The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response

Kenneth G.C.Smith1, Amanda Light, G.J.V.Nossal2 and David M.Tarlinton3

The Walter and Eliza Hall Institute of Medical Research, PO Royal Melbourne Hospital, Victoria 3050, Australia
1Present address: Department of Medicine, University of Cambridge School of Clinical Medicine, Addenbrooke’s Hospital, Hills Road, Cambridge, UK
2Present address: Department of Pathology, The University of Melbourne, Parkville, Victoria 3052, Australia
3Corresponding author
e-mail: tarlinton@wehi.edu.au

Immunization with protein-containing antigens results in two types of antigen-specific B cell: antibody forming cells (AFCs) producing antibody of progressively higher affinity and memory lymphocytes capable of producing high affinity antibody upon re-exposure to antigen. The issue of the inter-relationship between affinity maturation of memory B cells and AFCs was addressed through analysis of single, antigen-specific B cells from the memory and AFC compartments during the primary response to a model antigen. Only 65% of splenic memory B cells were found capable of producing high affinity antibody, meaning that low affinity cells persist into this compartment. In contrast, by 28 days after immunization all AFCs produced high affinity antibody. We identified a unique, persistent sub-population of bone marrow AFCs containing few somatic mutations, suggesting they arose early in the response, yet highly enriched for an identical affinity-enhancing amino acid exchange, suggesting strong selection. Our results imply that affinity maturation of a primary immune response occurs by the early selective differentiation of high affinity variants into AFCs which subsequently persist in the bone marrow. In contrast, the memory B-cell population contains few, if any, cells from the early response and is less stringently selected.

Keywords: affinity maturation/bone marrow/germinal center/(4-hydroxy-3-nitrophenyl)acetyl/somatic hypermutation

Introduction

A feature of T-cell-dependent antibody responses in higher vertebrates is an improvement in affinity of antibody for antigen with time—a process known as affinity maturation. Affinity maturation is apparent both in the higher average affinity of serum immunoglobulin for antigen late in the primary response (Eisen and Siskind, 1964) and also in the ability of memory B-cells upon challenge to produce a response of higher affinity than the early primary response (Steiner and Eisen, 1967; Siskind and Benacerraf, 1969). While substantial information has been gathered in relation to affinity maturation in the generation of B-cell memory (Gray, 1994; Rajewsky, 1996), comparatively little is known concerning the affinity maturation of serum antibodies which also occurs during the course of the primary response.

Antigen-specific antibody is initially produced by foci of antibody forming cells which develop during the first week of the response and which are associated with the T-cell areas along the periaorterolar lymphoid sheath (Van Rooijen et al., 1986; Jacob et al., 1991). The AFC of the foci first secrete IgM but subsequently switch in an almost synchronous manner to a downstream immunoglobulin isotype such as IgG1 (Nossal and Reidel, 1989; Jacob et al., 1991). Direct analysis of the AFCs comprising these foci reveals that they secrete low affinity antibody (Lalor et al. 1992) encoded by V gene segments that have not been altered by somatic hypermutation (Jacob et al., 1993; McHeyzer-Williams et al., 1993). During the second week of the response the foci involute (Jacob et al., 1991; McHeyzer-Williams et al., 1993), presumably due to death in situ as there is extensive apoptosis occurring in these areas during this time, a wave of AFC emigrants is not detected in other locations (Smith et al., 1996) and blocking apoptosis with transgenic BCL-2 prevents involution (Smith et al., 1994). Together these results indicate that the AFCs of the foci are not responsible for producing high affinity antibody as the primary response progresses.

The involution of the foci during the second week means that the continued production of high affinity antibody after this time must be due to AFCs in other locations. In some primary immune responses antigen-specific AFCs have been detected in bone marrow shortly after immunization (Zachau et al., 1989; Smith et al., 1996). The bone marrow has also been shown to be the location of long-term antibody production in certain responses (Bachmann et al., 1994; Hyland et al., 1994; Slifka et al., 1995) and to become a major site of IgG synthesis as animals age (Benner et al., 1981). These data suggest the bone marrow is an important location for the long-term production of high affinity antibody in the T-cell-dependent response to antigen. Identification, isolation and analysis of high affinity AFCs during the course of a primary immune response would add to our understanding of the mechanism underlying affinity maturation of serum immunoglobulin.

Generation of a high affinity memory B-cell population occurs in most instances within germinal centers (GCs). GCs are histologically discrete structures which develop in the B-cell follicles during the first week of the response and usually persist for a further three to four weeks. Within the GC, the processes of V gene somatic hypermutation, B-cell proliferation and selection are considered to act in concert to enrich efficiently for those cells bearing surface
immunoglobulin with increased affinity for the immunizing antigen (Nossal 1992; MacLennan, 1994; Kelsoe, 1996). During the first two weeks after immunization, the number of antigen-specific B cells in the GC increases substantially before entering a period of decline (Jacob et al., 1991; McHeyzer-Williams et al., 1993; Smith et al., 1994; Liu et al., 1996). During the period of expansion, the frequency of V gene mutation increases as does the clonal restriction of the GC B cells and the frequency of amino acid exchanges associated with enhanced affinity for the immunizing antigen (Weiss et al., 1992; Jacob et al., 1993). The decline in GC B-cell number after the second week leading to the establishment of the stable, recirculating memory B-cell pool is substantial; fewer than 5% of the peak GC B-cell number persist in the memory population that remains in the spleen more than 10 weeks after immunization (Smith et al., 1994). It is thus possible that the memory B-cell population is established by the selective recruitment from among GC cells of high affinity variants. One end result would be a memory B-cell population of uniformly high affinity. Whether or not this is the manner in which the memory B-cell population is actually formed is yet to be determined.

While these features of the immune response adequately explain the early production of low affinity antibodies and the improved affinity of memory B cells, the basis of the increasing affinity of serum antibody for antigen during the primary response and the mechanism for establishing the memory B-cell population remain to be clarified. In this report we examine affinity maturation of antigen-specific B cells during the primary response to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) in C57BL/6 mice. Individual NP-specific IgG1+ B cells of either a GC/memory B-cell phenotype or an AFC phenotype were purified by flow cytometry (McHeyzer-Williams et al., 1991; Smith et al., 1996) and examined for the extent of Vh gene somatic mutation, the presence of a particular affinity-enhancing amino acid exchange in the Vh gene, and the affinity of the antibody expressed by the cell. Our analyses show that as the primary response progresses, the cells of the GC produce antibody of increasing affinity, correlated with an increasing frequency of Vh gene somatic mutation. The GC B-cell population, however, does not mature to a point of being uniformly high affinity and, furthermore, the same distribution of low and high affinity B cells persists into the memory population. In contrast, a population of uniform high affinity AFCs is identified in the bone marrow and these cells appear to be responsible for long-term production of high affinity antibody after primary immunization. Analysis of the molecular basis of affinity maturation in these AFCs reveals that although they contain few somatic mutations, they are highly selected for the single affinity-enhancing amino acid exchange in the first complementarity determining region (CDR1) of the Vh segment characteristic of the anti-NP response in IgHb allotype mice (Cumano and Rajewsky, 1986). This enrichment is not seen to the same degree in the long-term memory B-cell population. In addition, such AFCs persist for several weeks in the bone marrow. Our results, which suggest that selection operates to different extents in the development of memory and effector B cells in the primary response, are discussed in the context of a model for B-cell selection in the T-cell-dependent humoral immunity.

Results

Somatic mutation and antibody affinity progressively increase in GC B cells during the primary response

The kinetics of the splenic GC/memory B-cell response to the hapten NP coupled to a protein carrier were established using a six parameter flow cytometry system. Antigen-specific GC B cells, defined as B220+IgM-IgD- IgG1+ and NP-binding (hereafter referred to as NP+), reached a maximum frequency of 0.3% of splenocytes at day 14 post immunization before declining such that by day 240 they represented 0.002% (Figure 1A). Single B cells of this phenotype were isolated at various times 6–56 days after immunization, cDNA was made and V<sub>186.2</sub>-Cy1 gene rearrangements amplified by PCR. The V<sub>186.2</sub> gene segment in association with Vλ1 dominates the response to NP in C57BL/6 mice (Bothwell et al., 1981; Cumano and Rajewsky, 1985). The PCR product was sequenced directly, meaning that the background frequency of mutation due to reverse transcriptase and/or Taq polymerase was essentially nil (Table I). In broad agreement with previous reports (Weiss et al., 1992; Jacob et al., 1993) few somatic mutations per Vh gene were seen at day 6, more substantial numbers by day 10, with the average number of mutations continuing to increase over time until reaching near maximum levels at day 17 (Table I and Figure 1B). The Trp to Leu exchange at amino acid position 33 of the V<sub>h</sub> chain, which alone confers a 10-fold increase in antibody affinity for NP (Allen et al., 1988), was absent at day 6 and rare at day 10 despite a substantial increase in the average frequency of mutations per Vh gene during this time. By day 14, however, this particular substitution was present in over half of the Vh genes analysed despite there being only a slight increase in the frequency of mutation, suggesting that most of this increase was due to selection on the basis of affinity. Beyond day 14 little change in the frequency of the position 33 mutation in the GC and memory populations was observed (Table I and Figure 1C). As the GC reaction proceeded the ratio of amino acid replacement (R) to silent (S) mutations in the CDRs and framework regions of the Vh genes of NP-specific B cells changed in a manner consistent with selection on the basis of improved antigen binding, namely increasing in the former and decreasing in the latter (Table I). These results confirm the progressive accumulation of somatic mutations and conserved amino acid exchanges in antigen-specific GC B cells thought to be consistent with the generation of a high affinity memory population.

To determine the extent of the correlation between the accumulation of mutations and increasing affinity of antibody, purified IgG1+ NP-specific GC and memory cells were used to establish clonal, in vitro cultures in a time course equivalent to that of the Vh gene sequencing described above. Cells were cultured on a feeder layer of fibroblasts transfected with CD40 ligand in the presence of IL-4 and IL-5. After 7 days supernatants were assayed by ELISA for the presence of NP-binding IgG1. Approxi-
was converted into arbitrary units by means of a high affinity standard IgM, IgD and GR-1 but positive for syndecan (Table III).

common to all assays. Each dot represents the measurement from a Within the syndecan were assayed for NP2-binding IgG1 by direct ELISA. Optical density AFCs making anti-NP IgG1 were negative for surface immunization. Serum samples from a cohort of five immunized mice secretion capabilities of these cells and showed that all properties of NP-specific hybridomas: 0–0.4 (low affinity), 0.5–0.8 for AFCs in the spleen (Lalor after in vitro culture. The ratios of NP2/NP15 binding of the antibodies spleen. A small population of IgG1 secretion were segregated into three groups on the basis of the binding (Figure 2) which had a phenotype similar to that described for further details. (C) Percentage of the V1.186.2 sequences shown in (B) containing the affinity enhancing Trp to Leu exchange at amino acid position 33. (D) Affinity distribution of NP-binding IgG1 produced by sorted splenic antigen-specific GC and memory B cells after in vitro culture. The ratios of NP2/NP15 binding of the antibodies have been segregated into three groups on the basis of the binding properties of NP-specific hybridomas: 0–0.4 (low affinity), 0.5–0.8 (intermediate affinity) and >0.8 (high affinity). See Table II for details. (E) Affinity maturation of NP-specific IgG1 in serum after primary immunization. Serum samples from a cohort of five immunized mice were assayed for NP2-binding IgG1 by direct ELISA. Optical density was converted into arbitrary units by means of a high affinity standard common to all assays. Each dot represents the measurement from a single mouse.

Fig. 1. Kinetics of the primary B-cell response to NP-KLH. (A) Frequency of antigen-specific GC/memory B cells in the spleen at various times after immunization. Each point is the average of three animals and is the percentage of splenocytes having the phenotype B220+IgM IgD IgG1^*NP-binding. (B) Average frequency of mutations in V1.186.2 genes PCR amplified from single antigen-specific GC/memory B cells at the indicated times. Cells were sorted from a pool of three spleens. See Table I and Materials and methods for further details. (C) Percentage of the V1.186.2 sequences shown in (B) containing the affinity enhancing Trp to Leu exchange at amino acid position 33. (D) Affinity distribution of NP-binding IgG1 produced by sorted splenic antigen-specific GC and memory B cells after in vitro culture. The ratios of NP2/NP15 binding of the antibodies have been segregated into three groups on the basis of the binding properties of NP-specific hybridomas: 0–0.4 (low affinity), 0.5–0.8 (intermediate affinity) and >0.8 (high affinity). See Table II for details. (E) Affinity maturation of NP-specific IgG1 in serum after primary immunization. Serum samples from a cohort of five immunized mice were assayed for NP2-binding IgG1 by direct ELISA. Optical density was converted into arbitrary units by means of a high affinity standard common to all assays. Each dot represents the measurement from a single mouse.

Affinity maturation of serum immunoglobulin
Having established the kinetics and extent of affinity maturation in the GC B-cell population, we next determined to what degree this was reflected in serum Ig. Serum was collected from a cohort of animals at regular intervals after primary immunization and the titre of total and high affinity NP-binding IgG1 determined. High affinity NP-specific IgG1 was first detected in the day 7 samples with the titre increasing thereafter to plateau at around day 21 (Figure 1E). The most rapid increase in high affinity IgG1 occurred between days 7 and 14, the same time period in which somatic hypermutation was first observed in GC cells and during which the frequency of position 33 mutations seen in the V1.1 genes of these GC cells increased substantially (Figure 1B and C). No further increase in the titre of high affinity NP-binding serum IgG1 was observed after day 21 indicating that affinity maturation was complete at this time for this dose of antigen.

Identification of high affinity AFCs in the bone marrow
The apparent concordance between affinity maturation of serum antibodies and GC B cells prompted us to isolate the cells responsible for antibody secretion after the decline of the splenic foci. A number of previous experiments suggested bone marrow as one location for high affinity AFCs. We therefore attempted to isolate primary response NP-specific IgG1^+ AFCs from the bone marrow with the flow cytometry system used to isolate such cells in the spleen. A small population of IgG1^+ AFCs was identified (Figure 2) which had a phenotype similar to that described for AFCs in the spleen (Lalor et al., 1992; Smith et al., 1996). ELIspot assays were used to measure the in situ secretion capabilities of these cells and showed that all AFCs making anti-NP IgG1 were negative for surface IgM, IgD and GR-1 but positive for syndecan (Table III). Within the syndecan^+ compartment, 90% of NP-specific AFCs could be further partitioned on the basis of cell surface binding of NP (in the form of an APC conjugate) and anti-IgG1 (Figure 2). Such NP^+IgG1^+ AFCs could be reliably sorted to 80–100% purity using these criteria, despite representing 1–2 per 100 000 nucleated BM cells (Table III). We were thus able to isolate antigen-specific AFCs from the bone marrow for further analysis.

At different times after immunization the relative affinity of antibody produced by individual sorted AFCs was while low affinity antibody will bind only at high haptenation ratios. The proportion of sorted B cells making antibody with improved affinity increased from 3% at day 6, to 40% at day 14, 50% at day 17 and 65% at day 21, thereby confirming the relationship between mutation and increasing affinity on a single cell level (Table II and Figure 1D). Surprisingly, of the day 50 antigen-specific memory B cells stimulated in vitro to produce antibody, 65% were found able to bind NP with enhanced affinity. Thus no increase in mutation frequency, affinity enhancing mutations or frequency of GC cells capable of producing high affinity antibody occurred after day 21 of the primary response. This resulted in a memory B-cell population of mixed rather than uniformly high affinity (Tables I and II and Figure 1D).

K.G.C.Smith et al.

2998
Table I. VH186.2 gene sequence summary of NP-binding IgG1+ B cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Day post immunization</th>
<th>Number of V genes sequenced</th>
<th>Sequences mutated (%)</th>
<th>Mutations per V gene average (range)</th>
<th>Position 33 Trp→Leu (%)</th>
<th>R/S ratio CDR 1 and 2</th>
<th>R/S ratio FWR 1–3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenic</td>
<td>6</td>
<td>21</td>
<td>25</td>
<td>0.4 (0–2)</td>
<td>0</td>
<td>0</td>
<td>2.3</td>
</tr>
<tr>
<td>Germinal center</td>
<td>10</td>
<td>15</td>
<td>100</td>
<td>4.5 (2–6)</td>
<td>6.7</td>
<td>4.4</td>
<td>3</td>
</tr>
<tr>
<td>Memory B cell</td>
<td>14</td>
<td>24</td>
<td>96</td>
<td>4.7 (0–11)</td>
<td>54</td>
<td>14</td>
<td>2.4</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
<td>15</td>
<td>100</td>
<td>5.1 (1–12)</td>
<td>50</td>
<td>5.5</td>
<td>3.2</td>
</tr>
<tr>
<td>42</td>
<td>19</td>
<td>95</td>
<td>95</td>
<td>8.7 (0–20)</td>
<td>58</td>
<td>7.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Splenic AFC</td>
<td>14</td>
<td>17</td>
<td>76</td>
<td>1.5 (0–4)</td>
<td>41</td>
<td>&gt;17</td>
<td>1.2</td>
</tr>
<tr>
<td>Bone marrow AFCs</td>
<td>14</td>
<td>31</td>
<td>74</td>
<td>1.9 (0–5)</td>
<td>71</td>
<td>&gt;38</td>
<td>2.2</td>
</tr>
<tr>
<td>35</td>
<td>21</td>
<td>95</td>
<td>0</td>
<td>4.8 (0–11)</td>
<td>62</td>
<td>9.8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

GSX2.1

aData from these experiments are shown in Figures 3 and 4.

bGSX2.1 is an IgG1b NP-specific hybridoma utilizing VH186.2 and made on day 7 of a primary response.

Table II. Clonal cultures of NP-specific germinal center and memory B cells

<table>
<thead>
<tr>
<th>Day post immunization</th>
<th>% Cultures secreting NP-binding IgG1</th>
<th>Numbers of NP+ cultures analyzed</th>
<th>% Cultures with NP2:NP15 &gt;0.8</th>
<th>IgG1 secreted per clone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>9.3</td>
<td>37</td>
<td>3</td>
<td>7.0 ± 4.6</td>
</tr>
<tr>
<td>10</td>
<td>11.0</td>
<td>25</td>
<td>12</td>
<td>6.7 ± 3.5</td>
</tr>
<tr>
<td>14</td>
<td>2.5</td>
<td>30</td>
<td>35</td>
<td>52.0 ± 4.3</td>
</tr>
<tr>
<td>17</td>
<td>8.0</td>
<td>43</td>
<td>43</td>
<td>12.5 ± 5.6</td>
</tr>
<tr>
<td>21</td>
<td>8.0</td>
<td>49</td>
<td>55</td>
<td>9.5 ± 3.2</td>
</tr>
<tr>
<td>56</td>
<td>9.0</td>
<td>34</td>
<td>58</td>
<td>9.1 ± 5.0</td>
</tr>
</tbody>
</table>

Supernatants from clonal cultures stimulated with CD40 ligand, IL-4 and IL-5 were titrated on NP2– and NP15–BSA plate coats in an ELISA. The NP2:NP15 ratio for IgG1 was determined from the optical density at a non-saturating point on the titration curve. Ratios are presented in full in Figure 1D.

assessed in both spleen and bone marrow (Table IV). This was done by comparing the frequency of AFCs producing anti-NP IgG1 of any affinity with that producing only high affinity antibody, again using differential binding to high and low haptenation plate coats. At early time points in the primary response all bone marrow and splenic AFCs produced antibody of low affinity. By day 28 splenic AFCs were present in very low numbers and, while those few cells seen were of high affinity, it was impractical to sort 50 at each time point so they are not shown in Table IV. In the bone marrow the proportion of AFCs secreting high affinity antibody increased progressively to reach 100% by day 28 and remained at this level thereafter (Table IV). This observation of affinity maturation of bone marrow AFCs at the cellular level recapitulates the phenomenon described for serum immunoglobulin (Figure 1E), and indicates bone marrow AFCs contribute to the long-term production of high affinity antibody during the primary response. The eventual uniform high affinity of the bone marrow AFCs is in contrast to the mixed affinity of GC/memory B cells at similar time points, indicating that the processes which select for increased antibody affinity operate to different extents in the memory and AFC populations.

VH genes of bone marrow AFCs show a distinct pattern of somatic mutation

To determine the basis of the improved affinity of the antibody secreted by a fraction of day 14 bone marrow AFCs we next examined these cells for the presence and distribution of VH gene somatic mutations. For comparison, splenic GC B cells from the same time point were similarly examined. The sequences of the VH186.2 genes recovered from the bone marrow AFCs and splenic GC B cells are shown in Figure 3 and summarized in Figure 4 and Table I. Single NP-specific IgG1 bone marrow AFCs were sorted 14 days after immunization, cDNA synthesized, VH186.2-Cγ1 rearrangements were amplified by PCR and sequenced as described above. The B220+NP+IgG1+ GC B cells sorted from the spleen at the same time constitute an independent experiment to that described in Figure 1. At day 14, all antigen-specific splenic GC B cells were mutated with an average of 5.1 mutations per VH gene (range 1–12) (Figure 3B). In contrast, the 74% of bone marrow AFCs which were mutated had an average of 2.5 mutations per VH gene (range 1–5), significantly fewer than seen in the GC (Student’s t-test P <0.001) and equivalent to the number of mutations seen in GC cells 8 days after immunization with NP-CGG (Weiss et al., 1992). This low number of mutations suggests that bone marrow AFCs are early products of the GC. The high affinity replacement mutation at position 33 was found in 50% of splenic day 14 GC B cells, in agreement with previous reports (Weiss et al., 1992). Remarkably, despite having fewer mutations, all but one of the mutated bone marrow AFCs carried the position 33 Trp to Leu exchange (Figure 3A) thereby explaining the improved affinity of a fraction of bone marrow AFCs at this time. The proportion of mutated VH genes with the position 33 exchange was higher among...
Persistence of early, affinity selected AFCs in the bone marrow

The unique distribution of somatic mutations in the $V_{H}$ genes of day 14 bone marrow AFCs suggested that if these cells persisted in that location, they should be identifiable by virtue of their mutational pattern. For this reason $V_{H}186.2$ genes were sequenced from single AFCs sorted from bone marrow at day 35 after primary immunization. Three main observations could be made by comparing sequences from day 14 and 35 AFCs (Figure 4A and C). First, the proportion of AFCs containing unmuted $V_{H}$ gene sequences declined from 26% at day 14 to 5% (a single sequence) at day 35, paralleling the loss of low affinity AFCs in the bone marrow between days 14 and 28 as measured by ELIspot (Table IV). Secondly, the unique population of somatically mutated AFCs seen at day 14 was still present at day 35 in at least similar absolute numbers [since the number of NP-specific AFCs in the bone marrow increases ~3-fold between days 14 and 35 (Smith et al., 1996) while the total cellularity remains constant]. This suggests that, once selected, high affinity bone marrow AFC clones are long-lived in a fashion analogous to memory B cells. These persistent mutated AFCs may correspond to the previously observed long-lived plasma cells in the bone marrow (Ho et al., 1986). Finally, additional GC-derived cells with more mutations but less dependence on the position 33 mutation pattern of mutations seen in the antigen-specific splenic memory cell population was again distinct from that seen in the bone marrow AFC population at day 35 (Table I and Figure 4C and D), comprising cells with a higher average number of mutations per $V_{H}$ gene and without the subpopulation of highly selected but sparsely mutated genes seen in the AFCs of the bone marrow.

Somatic mutation and selection in day 14 AFCs in the spleen

Our examination of bone marrow AFCs at days 14 and 35 post immunization had revealed a unique pattern of somatic hypermutation in the $V_{H}$ genes of a fraction of these cells. If these cells arose as a consequence of somatic mutation and selection in the GC, they should be detectable in organs containing GCs, such as the spleen. We therefore examined the distribution of somatic mutations in the $V_{H}$ genes of NP-specific IgG1 AFCs isolated from the spleen 14 days after immunization. Single AFCs were sorted and $V_{H}186.2$ containing rearrangements amplified from cDNA and sequenced as before. A summary of these sequences is presented in Figure 5 and Table I. For comparison, the distribution of mutations in the $V_{H}$ genes of day 14 NP-specific IgG1 GC B cells sorted from the spleen is

Table IV. Affinity maturation of NP-specific bone marrow AFCs

<table>
<thead>
<tr>
<th>Response</th>
<th>Day</th>
<th>Spleen</th>
<th>Bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>56</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>ND</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>ND</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

At each time point at least 50 NP$^{+}$IgG1$^{+}$syndecan$^{+}$ cells were sorted onto ELIspot plates coated with NP$_{15}$ and NP$_{2}$–BSA, which detect total and high affinity anti-NP antibody respectively. The ratio of anti-NP IgG1 ELIspots (NP$_{3}$/NP$_{15}$) allowed calculation of the percentages shown. ND, not determined; while AFCs could be detected in the spleen at this time, their scarcity made sorting and analysis of affinity impractical.

This result is representative of six other experiments. The cumulative results of these six experiments show that for 102 syndecan$^{+}$NP$^{+}$IgG1$^{+}$ cells plated, 97 anti-NP IgG1 ELIspots were observed (a sensitivity of 95%).

Table III. Isolation of NP-specific IgG1 bone marrow AFCs

<table>
<thead>
<tr>
<th>Phenotype of sorted cells</th>
<th>Number of cells plated</th>
<th>Anti-NP$^{+}$ AFCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>(IgM IgD Gr-1 PI)$^{+}$</td>
<td>$5 \times 10^{5}$</td>
<td>0</td>
</tr>
<tr>
<td>(IgM IgD Gr-1 PI) syndecan$^{−}$</td>
<td>$4 \times 10^{5}$</td>
<td>0</td>
</tr>
<tr>
<td>NP$^{+}$ syndecan$^{+}$NP$^{+}$IgG1$^{+}$</td>
<td>$3 \times 10^{5}$</td>
<td>2</td>
</tr>
</tbody>
</table>

30000
Affinity maturation in the primary B-cell response

Fig. 3. \(V_H\) gene nucleotide sequences from day 14 NP-specific B cells. (A) Single NP-binding IgG1\(^+\) antibody forming cells were sorted 14 days after immunization from bone marrow. \(V_H^{186.2}\) was the predominant \(V_H\) gene in these AFCs. (B) \(V_H^{186.2}\) gene sequences from single NP-specific IgG1 GC cells, sorted from spleen 14 days after immunization. Only those codons which differ from the germline sequence are shown and numbered according to Kabat et al. (1991). Amino acids are shown using the single letter code. \(D_H\) and \(J_H\) regions are compared with known germline sequences. Details of the amplification and sequencing are given in Materials and methods and Table I.

Interestingly, a sub-population of \(V_H\) genes with one or two nucleotide exchanges and containing the position 33 amino acid exchange is apparent amongst these splenic AFCs and is enriched in this compartment relative to the GC. That is, the proportion of splenic AFCs with 1–2 mutations and the position 33 exchange is 60% (6/10). The same fraction among GC B cells with 1 or 2 mutations per \(V_H\) gene is 33% (2/6). Taken together these results
of bone marrow AFCs compared with splenic GC/memory B cells strongly suggests that the AFCs are an early selected product of the GC reaction, while the memory population develops at a later time and under less stringent conditions. The finding of a population of AFCs in the spleen at day 14 with a distribution of somatic mutation similar to that of the bone marrow AFCs supports this notion. These observations provide the first detailed insight into the processes of affinity maturation in the effector B-cell compartment compared with that in the GC cells which give rise to the memory B-cell population. A number of interesting questions, however, remain to be addressed. How do low affinity B cells survive in what is presumed to be the intense selective environment of the GC? How do low affinity B cells enter the memory population and how is a memory population of heterogeneous affinity able to generate a uniformly high affinity secondary response? Finally, what is the mechanism by which a B cell within the GC differentiates into either an AFC or a memory B cell?

**Generation of the memory B-cell population**

The observation that the process of affinity maturation of GC B cells in response to NP was incomplete was unexpected, but affords some insight into how the memory B-cell population is established. We (Smith et al., 1994) and more recently others (Liu et al., 1996) have documented a substantial decline in the number of antigen-specific B cells in the spleen during the formation of the memory population. Indeed, <5% of the NP-specific GC B cells present at day 14 remain in the spleen by day 50 and beyond (Figure 1A). We proposed that this reduction was consistent with the dissolution of the GC and the concomitant selection of a fraction of GC B cells into the memory compartment on the basis of affinity (Smith et al., 1994). Our present results would suggest that this view was an over-simplification. It would now appear that in the anti-NP response, the composition of the memory population reflects the late GC and that no additional selection on the basis of affinity occurs at the transition from GC cell to memory cell. While we are aware of no previous study which has systematically analysed the affinity of antigen-specific memory B cells, some have
inferred that the memory population has the potential to give rise to AFCs of both high and low affinity upon re-exposure to antigen (Celada et al., 1969; Anderson, 1972; Elliot and Roszman, 1975). Since this work was done with an incomplete knowledge of the processes of somatic mutation, selection and isotype switching the basis for this potential was unclear. Most analyses of memory B cell affinity have supported the notion that the memory population is uniformly high affinity since the AFCs arising after secondary stimulation are all high affinity (e.g. Vitetta et al., 1991). Such an interpretation may, however, be biased by the methodology in that these experiments involved secondary stimulation with antigen after cell transfer and this may either selectively activate only the high affinity memory B cells or initiate a new round of affinity maturation. In terms of direct analysis of the memory population, Weiss and Rajewsky (1990) cloned two mutated V_{H}186.2 gene segments from NP-specific memory cells into an IgG1 expression vector and partially reconstructed the antibody by transfection into a myeloma expressing an unmutated λ1 light chain. While both reconstructed antibodies were high affinity, the number analyzed was too few to detect the degree of heterogeneity revealed by our in vitro cultures (Figure 1D). Thus our study provides a more comprehensive analysis of the affinity of purified memory B cells generated after primary immunization with a haptenic antigen. Our results contradict the commonly held assumption that all memory cells make antibody of high affinity.

The question of how such low affinity B cells are able to persist in what is considered to be the intensely competitive environment of the GC is difficult to answer. Three possible explanations come to mind. One is that in these experiments antigen was so abundant that high affinity cells had no selective growth advantage over low affinity cells in the GC, and consequently could not dominate representation of the memory population. Second is that each GC might operate in isolation and that only a fraction of the GCs generate high affinity B cells. If all GCs then contribute to the memory population it will be as heterogeneous in affinity as the GCs. Third is that somatic mutation continues to operate in the GC up until the point of generating the memory population and that the low affinity cells are ‘back’ mutations from high to low affinity which have yet to be deleted by selection (Brugeman et al., 1986). The plateau in V_{H}1 gene mutation frequency seen after day 17, however, would indicate that somatic mutation of GC B cells has substantially stopped by this time, which in turn would argue against this last possibility. Although there is currently little information on the inter-relationship of GCs, there is data on the effect of antigen dose on affinity maturation. These data indicate that large doses of antigen do delay affinity maturation, although whether this effect persists into the memory population is questionable (Goidl et al., 1968; Siskind et al., 1968). Clearly additional experimentation is required to resolve this point.

While not all memory B cells are high affinity, all secondary response AFCs are high affinity (Table IV; Cumano and Rajewsky, 1986; Ridderstad et al., 1996). We would suggest two possible explanations to account for this discrepancy. First, the secondary response, initiated by injection of small quantities of soluble antigen, may recruit a subset of memory B cells based on affinity for antigen. Previous analysis of the secondary anti-NP response in single animals has indicated that only a limited number of clones participate (Blier and Bothwell, 1987) and this number may be substantially less than the number of clones in the memory population. In the same vein, we have recently shown that the magnitude of the secondary response, as measured by the frequency of AFCs, is not necessarily proportional to the frequency of memory B cells present at the time of boosting, suggesting that the number of antigen-specific memory B cells available is greater than actually used (Ridderstad et al., 1996). Second, the generation of AFCs in GCs early in the secondary response (Dilosa et al., 1991; Van Rooijen, 1991) may operate under the same selective conditions that we have observed for the generation of high affinity AFCs early in the primary response. Together these results suggest that the generation of a high affinity secondary response from a memory compartment of mixed affinity is not an improbable event.

Affinity maturation of serum immunoglobulin is driven by highly selected bone marrow AFCs from the early GC

Improvements in the affinity of serum antibody for the immunizing antigen results from the activity of a population of high affinity AFCs. Amongst the antigen-specific AFCs detected in the bone marrow 14 days after primary immunization, half were found to be secreting high affinity antibody. These bone marrow AFCs had few V_{H}1 gene mutations when compared with GC cells at the same time, yet mutated cells had a far higher proportion of Trp to Leu exchanges at position 33 (e.g. Figure 4A and B). The fact that the position 33 mutation is not seen in early NP-specific GC cells and AFCs (Table I and Weiss et al., 1992; Jacob et al., 1993; McHeyzer-Williams et al., 1993) indicates it is not present in the pre-immune repertoire (see also Weiss and Rajewsky, 1990). In addition, as the mutation is not seen in >60% of the more highly mutated memory cells (Figure 1C; Weiss and Rajewsky, 1990), the 96% frequency in mutated bone marrow AFCs cannot be explained purely as the result of a mutational ‘hot spot’ (Betz et al., 1993). Rather, the frequent appearance of this single amino acid exchange indicates that the day 14 bone marrow AFC population has been selected on the basis of affinity. It is likely that possession of the position 33 mutation is the only way for an NP-specific B cell to gain high affinity if only few mutations are present. Thus if AFCs are to be affinity-selected and yet leave the GC early in the response when the mutational load is low, they must possess this mutation. Later in the response when more mutations are present, other mutational combinations may provide an equivalent increase in affinity (Allen et al., 1988) and so the requirement for the position 33 exchange will be less stringent. While we cannot determine whether the early bone marrow AFCs arise directly in the GC or first pass through a memory compartment, such a difference may be relatively unimportant. This is because effectively all of the cells with the genetic characteristics
of the early GC are seen in the bone marrow AFC compartment and none appear to enter the long-term memory population. Thus the AFCs responsible for secreting high affinity antibody early in the primary immune response arise as a distinct product of the GC reaction which is not seen in the long-term memory cell population.

A possible relationship between bone marrow AFCs and the maintenance of B cell memory

By 35 days post immunization, the mutational pattern of the NP-specific AFC population in the bone marrow is more diverse than at day 14. This is primarily due to the presence of NP-specific AFCs with a higher frequency of V gene mutations and less dependence on the position 33 Trp to Leu exchange. While the sparsely mutated, high affinity AFCs still present are presumed to have persisted since their generation early in the response, the origin of the more highly mutated AFCs is less clear. These cells may have been generated during the later stages of the GC reaction, prior to its dissolution and the formation of the memory cell population. It is also possible, however, that these AFCs are recruited from the memory B-cell population (Bachmann et al., 1994) in an antigen-dependent fashion with only limited proliferation. Interestingly when the proliferation of memory B cells was determined by BrdU incorporation, 10% of the antigen-specific cells incorporated BrdU during an 18 day labeling period which started 20 weeks post immunization (Schittek and Rajewsky, 1990), suggesting some degree of continual memory cell activation. If the memory cell encounter with antigen in the form of immune complexes was competitive with either serum immunoglobulin or other memory clones, then high affinity memory cells would be preferentially maintained. If the differentiation of memory B cells into AFCs constitutes one aspect of such a system for maintaining B-cell memory in an antigen-dependent fashion (Gray and Skarvall, 1988), then a consequence of this should be that the pattern of V gene mutations in the bone marrow AFC population will eventually come to resemble that of the memory population. Furthermore selective maintenance of high affinity memory cells would also be reflected in an increasing proportion of memory B cells being high affinity with time. The methods applied in the current study will allow the direct examination of this model by documenting changes in the pattern of V gene mutations in antigen-specific bone marrow AFCs and the affinity of memory cell antibody at later intervals after immunization.

A scheme for B-cell selection in the T-cell-dependent humoral immune response

The data presented here lead us to propose a scheme for the generation and selection of AFCs and memory B cells in the humoral immune response (Figure 6). A vital component of the primary response is its effector arm, the production of high affinity antibody. It would appear reasonable for the initial output of the GC to be high affinity AFCs, produced early and stringently selected on the basis of affinity. We find no evidence that a similar population of B cells from the early GC becomes memory cells. The increase in affinity seen in the GC population is both more gradual than in the bone marrow AFCs (perhaps in part due to the export of many high affinity cells to join the AFC population) and is incomplete. Despite the memory B-cell population containing only 65% high affinity cells, all AFCs of the secondary anti-NP response are high affinity. This suggests that selection is important in the secondary response, compensating for the incomplete affinity maturation of the memory population. It is tempting to speculate that formation of the memory B-cell population, in contrast to that of AFCs, coincides with the dissolution of the GCs, an event perhaps precipitated by the titre of high affinity antibody (Heyman, 1990; Van Rooijen, 1991).

Materials and methods

Mice and immunizations

C57BL/6 mice were immunized by intraperitoneal injection of 100 μg of alum-precipitated NP conjugated to keyhole limpet hemocyanin (NP:KLH conjugation ratio 13:1), prepared as described previously (Lalor et al., 1992). The secondary response was initiated by intravenous...
Affinity maturation in the primary B-cell response

injection of 10 μg of soluble NP2 KLH per mouse on day 49 of the primary response.

Cell staining
Bone marrow cells were prepared and stained to enable detection of cells bearing sydnean, surface IgG1, and binding the hapten NP essentially as described (Smith et al., 1994) except that granulocytes were excluded on the basis of reactivity with the monoclonal antibody 8C5. Cells were analyzed and sorted using a dual laser FACStar® (Becton Dickinson). When spleen cells were prepared for analysis a similar procedure was used except that anti-B220 (RA3-6B2) replaced anti-syndecan (281.2) in the antibody mixture. Single cells were sorted using the automatic cell deposition unit (ACDU) of the FACStar®.

ELIspots and ELISAs
ELIspot assays for IgG1 secreting cells were performed essentially as described (Lator et al., 1992). In brief, the indicated number of spleen or bone marrow cells were sorted with an ACDE into replicate wells of a cellulose ester based plate, coated with NP3-2 or NP2- bovine serum albumin (BSA) and then cultured for 20 h. After this time, the plates were washed and anti-NP IgG1 revealed with goat anti-mouse IgG1 conjugated to horseradish peroxidase (Southern Biotechnology Associates) and visualized by the subsequent addition of 3-aminomethyl carbazole. AFCs were counted using a dissecting microscope. Total and high affinity NP-specific AFCs were detected by ELIspot with NP1-BSA and NP2-BSA plate coats respectively, based on a published method (Herzenberg et al., 1980). NP-specific ELISAs were performed exactly as described (Smith et al., 1994).

Cell culture
Single cells of the phenotype IgM IgD B220 "NP" IgG1 + were deposited by ACDE into the wells of 96-well plates containing RPMI culture medium supplemented with 10% fetal calf serum, IL-4, IL-5 and 5000 3T3 fibroblasts stably transfected with mouse CD40 ligand (cDNA a gift from Dr. M. Spriggs, ImmuneX, Seattle, WA). Interleukins were added in the form of supernatants from a myeloma cell line transfected with the appropriate expression vectors (Karasuyama and Melchers, 1988), the concentration previously having been determined as optimal for B-cell cloning. The final volume of each well was 200 μl. After 7 days incubation, an aliquot of supernatant from each well was assayed for the presence of NP-binding IgG1 in a direct ELISA. Supernatant from positive wells was then harvested and the titre of NP1- and NP2-binding IgG1 determined in parallel ELISAs using an equal concentration of plate coat in both. The ratio of NP1 to NP2 binding IgG1 was used as a measure of the affinity of the anti-NP antibody at the time of the assay. The concentration of IgG1 in each well was also determined using a sheep anti-mouse IgG1 capture reagent and competition with a purified myeloma standard (Sigma). In each experiment the ability of the CD40 ligand-based culture system to clone IgM+ IgD+ splenic B cells was determined and used as a means of monitoring the reproducibility of the culture conditions.

VH gene sequencing
At various times after immunization, cell suspensions were prepared from the spleens and bone marrow of three mice, pooled, stained and analyzed by flow cytometry as described above. The sequences of VH186.2 genes from single IgG1 "NP" AFCs or GC/memory B cells were obtained essentially as described (Smith et al., 1995). Briefly, cells were sorted into tubes containing 11 μl of buffer, lysed and DNA was made using SuperScript II (Gibco BRL) and random hexamer primers (Pharmacia). Two and a half μl of this DNA solution was added to the first-round PCR. Two rounds of PCR using nested primers specific for VH186.2 and Cγ1 were performed as described (McHeyzer-Williams et al., 1991). Products with bands of the expected size were purified over a Quiaqquic column (Qiagen). Eight μl of the eluant from the column was used as a template for the PRISM fluorescent cycle sequencing system (Applied Biosystems) separated on an ABI model 373A automated DNA Sequence. For GC/memory phenotype B cells, 30% of the cDNAs made from single cells gave a PCR product, 75% of which were determined to be VH186.2 by sequence analysis. For AFCs, 66% of single cell cDNA's gave a PCR product with 75% of these being VH186.2. The entire PCR product was sequenced with the region between amino acids 10 and 96 being compared in detail with the germline sequence. No clonal repeats were found as indicated by unique CDR3 sequences.

Acknowledgements
We thank R. Muir, D. Constantinous and F. Battey for assistance with flow cytometry, and J. Scott and J. Merryfield for assistance with animal husbandry. We are grateful to T. Hewitson and G. Becker for their support, and to A. Strasser and A. Ritterheld for critical review of the manuscript. This work was supported by the NH & MR, Canberra, and by grant AI 03958 from the US National Institute of Allergy and Infectious Diseases, and by a grant from the Human Frontier Science Program, Principal Investigator Professor D. Mathis.

References