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Effect of Environmental Antigens on the Ig Diversification and the Selection of Productive V-J Joints in the Bursa

Hiroshi Arakawa, Kei-ichi Kuma, Masahiro Yasuda, Shigeo Ekino, Akira Shimizu, and Hideo Yamagishi

In chickens, a single set of unique functional segments of both Ig H and L chain genes is rearranged during early embryogenesis to generate a pool of B cell progenitors that will be diversified in the bursa by gene conversion, forming the preimmune repertoire. After hatching, bursal cells are exposed to environmental Ags in the bursal lumen. We prepared B cells from each single bursal follicle and used PCR-directed Ig L chain gene analysis to study the differentiation of B cells and the effect of antigenic stimulation from the bursal lumen on the neonatal chicken B cell repertoire formation. Selective amplification of B cell clones with a productive V-J joint was observed during the late embryonic stage, possibly by the interaction with ligands expressed on the bursal stroma and further accelerated in the neonatal chicken. Administration of the artificial Ags into the bursal lumen before the isolation of bursa by bursal duct ligation in the embryo caused a significant increase in lymphocytes with a productive V-J joint in the neonatal chicken bursa compared with the isolated bursa. Intra- and interclonal diversity of a complementarity-determining region measured by an evolutionary distance increased during bursal development. Clonal diversification did not require stimulation by artificial Ags from the bursal lumen. Thus, the preimmune repertoire in the bursa is generated by gene conversion during Ag-dependent B cell proliferation, and antigenic stimulation from the bursal epithelium to bursal B cells plays roles in the selection of clones with a productive V-J joint. The Journal of Immunology, 2002, 169: 818–828.

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Abbreviations used in this paper: BDL, bursal duct ligation; NP, 4-hydroxy-3-nitrophenylacetyl; CDR, complementarity-determining region; s, surface; NJ, neighbor joining.
environmental Ags play a significant role in the selective amplification of B cells with a productive V-J rearrangement but no critical role in the process to create the diversity required in the adult immune repertoire. Thus, the preimmune repertoire in the bursa is generated by the Ag-independent B cell proliferation, but antigenic stimulations to the bursa accelerate expansion of productive bursal cells driven by Ags.

**Materials and Methods**

**Isolation of single bursal follicles**

White Leghorn HB-15 chickens (15) were sacrificed on day 18 of incubation and day 7 of posthatching, and each single bursal follicle was isolated as described previously (2), with a few modifications. In brief, bursa of Fabricius was cut open and gently minced in HBSS in a 10-cm plastic dish. The fragments were teased with the backside of a curved tweezer. Individual follicles released into the medium were visible under a dissecting microscope. A single follicle was transferred into a microcentrifuge containing 30 μl of 1× LA Taq buffer (Takara, Kyoto, Japan) with 1 mg/ml proteinase K and 0.5% Tween 20. To avoid DNA degradation, the isolated follicles were immediately incubated for 45 min at 56°C for proteinase K-mediated proteolysis, followed by a 10-min incubation at 95°C to inactivate proteinase K. This crude lysate of a single follicle was stored at −20°C before use.

**Bursal duct ligation**

BDL was performed on day 18 of incubation as described previously (16). To prepare the NP-BDL chicken, 0.3 mg of sterile NP-BSA resuspended in 8 μl saline was administered into the bursal lumen via a vinyl tube immediately before BDL. Each single bursal follicle of BDL and NP-BDL chickens was isolated on day 7 of posthatching and stored as crude lysate prepared as described.

**PCR amplification**

A frozen lysate of a single bursal follicle was divided into three portions (10 μl each) and independently subjected to two rounds of PCR using pairs of nested primers. The primary PCR of 30 cycles was conducted with the nested L1/L4 primers in a 50-μl volume. The secondary PCR of 20 cycles was conducted with the L20/L21 primers internal to those used for primary PCR in separate reaction tubes using 2 μl of the first-round reaction mixture in a 50-μl volume. PCR products were monitored by the PCR product defined as a visible band with an expected 350-bp length on an ethidium bromide-stained 0.8% agarose gel. Because the PCR product reached its maximum after 30 cycles of amplification, PCR-introduced mutations are minimized. The primers were designed from the registered nucleotide sequence M24403 (European Molecular Biology Laboratory/GenBank/DNA Data Base in Japan). Primary PCR primers, L1 5′ of V4 in the leader intron and L4 3′ of J4 in the C-J intron, were described previously (17). The secondary PCR primers, L20 (TCCAAGCTTTCCTCTCCCTCTCCAGG) and L21 (GGCTCTAGACTCAGATGGGGGAAGAA) were flanked by HindIII and Xhol cloning sites, which facilitate the cloning of PCR products. Neither of the restriction sites HindIII and Xhol were found in the region including the φW4, V4, and J4 genes. PCR amplification was performed with LA Taq polymerase (Takara) as follows: an initial 4-min incubation at 94°C, 30 or 20 cycles consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s, with a final 5-min elongation step at 72°C.

**DNA cloning and sequencing**

Amplified DNA of expected length was purified by 0.8% agarose gel electrophoresis. Cloning was performed in pUC19 vector after digestion with HindIII and Xhol. Sequencing was done using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) and the autosequencer model 373 (PE Applied Biosystems). All sequences were confirmed by sequencing both strands using the universal and reverse oligonucleotide primers specific for M13. GenBank accession numbers for the sequences reported in this paper are AB061547-061652 and AB061654-061667.

**PCR-introduced mutations**

PCR amplification artifacts were measured by sequencing 14 cloned products of an analogous two rounds of amplification of a known L chain plasmid, p2115 (352 bp), equivalent to 5000 copies. We found only two substitutions in 4928 nucleotides (14 × 352 bp) during in vitro amplification. Therefore, these infrequent PCR artifacts are unlikely to account for the majority of the observed mutations.

**Gene conversion search and other statistical comparisons**

According to the Conversion Search computer program, as previously described (14), linked base modifications with counterparts in the pseudogene pool were assigned as templated gene conversions. An Ig L chain pseudogene mini-database was constructed from M12437, M13097–15099, M15137–15156, and AH002536 (European Molecular Biology Laboratory/GenBank/DNA Data Base in Japan). Other all single-base changes were assigned as point mutations. The average number of base modifications, point mutations, and gene conversion events among related sequences in genealogical trees were calculated as shown previously (17). The evolutionary trees were inferred by the neighbor joining (NJ) method (18) and calculations of the evolutionary distance were as described (14).

**Results**

**Selection of productive Ig L chain in the bursa**

We used a PCR to amplify all VA1-JA fusions of B cells isolated from individual single follicles of the bursa from the day-18 embryos and from the 7-day-old normal, BDL, and NP-BDL chickens. Amplified DNAs were cloned into plasmid vectors, and the V region inserts of individual PCR clones were sequenced. We analyzed a total of 215 L chain rearrangements obtained from 16 single bursal follicles (Table I). Despite the extensive sequence diversity by base modifications, each sequence can be related by unique V-J joint sequences. Because most follicles are populated by a very few prebursal stem cells that are committed to a particular Ig gene rearrangement at the very beginning of the development of the embryonic bursa (2–4), sequences related by a unique V-J joint have likely originated from the same precursor cell. N junctional diversification is achieved by some P base additions and moderate exonucleolytic nibbling of the coding ends (5–8).

When we examined the sequence of the V-J joints, about one-third of the L chains derived from the day-18 embryo and from the 7-day-old normal chickens were out-of-frame. This proportion of nonproductive V-J joints was significantly lower than the two-thirds observed at the earliest bursal stage (4, 5). Antigenic stimulation from the bursal lumen (NP-BDL) caused a more significant decrease in nonproductive joints to one-fifth (Table I). This indicates that a selection of B cells with a productively rearranged L chain locus within the bursa occurred during embryonic development and was further amplified by stimulation of artificial environmental Ags after hatching.

Selective amplification of B cells with productive gene rearrangements suggests that the productive V-J coding sequences may be amplified during extensive bursal clonal diversifications. We examined the proportion of abortive clones with out-of-frame joints or with the loss of a productive rearrangement by modifying the reading frame in all clones analyzed. Neonatal normal chickens stimulated possibly by the natural environmental Ags after hatching showed a significant decrease to one-fourth in the proportion of abortive clones (Table I). The neonatal NP-BDL chickens stimulated by the NP Ags showed a more pronounced decrease to less than one-tenth in the proportion of abortive clones (Table I).

**Clonal diversification of B cells in the bursa during development**

To reveal the clonal expansions present during late embryonic life and on day 7 after hatching, clonally related L chain sequences obtained from each single bursal follicle were aligned with germ-line sequence and their representative related clones from e2 and p2 are shown in Fig. 1. Germline precursor segments are identified in e2 clones (Fig. 1A). For the quantitative assignments of gene conversion, a computer program, Conversion Search, was used for
this study (14). Clonally related L chain sequences carried shared and unique nucleotide changes. The mutations in this collection of genes included both templated gene conversions and point mutations. Single-base changes were either templated or untemplated in the known pseudogene pool. The mutational patterns reflect an intraclonal generation of Ab mutants during the expansion of individual B cell clones. All the Ig L sequences derived from the single bursal follicles, e2 and p2, are represented in the form of genealogical trees (Fig. 2). The unrelated single clones of unique V-J joints may well be considered representative of a minor population of less diversification in the bursa.

As shown in Fig. 1A, gene conversion events with $\psi$V16 (e2204) and $\psi$V23 (e2103 and e2109) resulted in the out-of-frame sequences by shifting the reading frame of productive V-J joints. Repeated gene conversion events with $\psi$V23 (e2202) corrected the out-of-frame joints of e2103 or e2109. Repeated use of the gene conversion donors $\psi$V7 and $\psi$V10 was also observed in the CDR1 to CDR2 (Fig. 1B).

**Effect of Ags in the bursal lumen on the clonal diversification of B cells after hatching**

We aligned the clonally related L chain sequences from each single bursal follicle of BDL and NP-BDL chickens with the germline sequence. Representative related clones from the bursal follicles of b3 of BDL chickens and n1 of NP-BDL chickens are shown in Fig. 3. Putative precursor segments shared by BDL b3-I clones (Fig. 3A) show an out-of-frame V-J joining event, suggesting that nonproductive joining is not a lethal event by itself. The pseudogenes $\psi$V2, $\psi$V6, $\psi$V8, and $\psi$V23 are preferentially and repeatedly used as the gene conversion donor in the CDR1 and CDR3. The more complicated gene conversions were observed in the n1-I clones of NP-BDL chickens (Fig. 3B). The first common mutational event in this group was the point mutation A to T in the CDR3 flanking region. All the Ig L sequences derived from the single bursal follicles b3 and n1 are represented in the form of genealogical trees (Fig. 4).

Among several point mutations with no counterpart in the known L chain pseudogene pool, we found a clear indication of untemplated mutations in the JA and JA-CA intron of the rearranged L chain genes (Figs. 1A and 3B) as shown in the bursa of 3-wk-old chickens (7, 19).

**Mutation mechanism of B cells specified in the bursa**

In Table II, the number of mutation events was calculated for each pair-group of IgL sequences as shown in the genealogical trees (Figs. 2 and 4). The number of conversion events increased with time: two on day 18 of incubation and five on 7 days after hatching. The gene conversion events were not different between BDL and NP-BDL chickens and thus were unaffected by the environmental Ags. A significant number of point mutations were observed during the late embryonic stage. However, the increase in point mutations was very small during development and was not affected by the environmental Ags. Total base modifications during development were achieved mainly by a mechanism of gene conversion and were independent of the clonal selections by the environmental Ags.

The bar graph shown in Fig. 5 illustrates the frequency of pseudogene usage in the unique gene conversion events specified in the bursal follicles listed in Table I. Except for a striking increase in the usage of $\psi$V23 segments at the late embryonic stage and in the

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**Table I. Number of sequences characterized by the unique V-J junction and coding sequence**

<table>
<thead>
<tr>
<th>Bursal Follicle</th>
<th>No. of Sequences Analyzed</th>
<th>Abortive Clones (%)</th>
<th>Independent V-J Joints Total</th>
<th>Nonproductive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>e1</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>e2</td>
<td>30</td>
<td>13</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>e3</td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>e4</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total (day-18 embryo)</td>
<td>54</td>
<td>23 (42.6)</td>
<td>12</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>p1</td>
<td>7</td>
<td>1</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>p2</td>
<td>30</td>
<td>6</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>p3</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>p4</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Total (day-7 neonatal)</td>
<td>53</td>
<td>13 (24.5)</td>
<td>28</td>
<td>10 (35.7)</td>
</tr>
<tr>
<td>b1</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>b2</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>b3</td>
<td>30</td>
<td>13</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>b4</td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Total (BDL)</td>
<td>54</td>
<td>20 (37.0)</td>
<td>25</td>
<td>11 (44.0)</td>
</tr>
<tr>
<td>n1</td>
<td>30</td>
<td>1</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>n2</td>
<td>8</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>n3</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>n4</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Total (NP-BDL)</td>
<td>54</td>
<td>4 (7.4)</td>
<td>20</td>
<td>4 (20.0)</td>
</tr>
</tbody>
</table>

* Bursal follicles were isolated from day-18 embryos (e1–e4) or 7 days after hatching from normal (p1–p4), BDL (b1–b4) and NP-BDL (n1–n4) chickens. Each set of follicles (e1 and e2; e3 and e4; p1 and p2; p3 and p4; b1 and b2; b3 and b4; n1 and n2; n3 and n4) was isolated from the bursa of a single chicken.

* Each percentage is significantly different ($p < 0.05$).

* Each percentage is significantly different ($p < 0.002$). Data evaluation is based on the two-tailed Student $t$ test.
BDL chicken (Fig. 5, A and C), the preferential usage of pseudogenes located in the inverted orientation and more proximal to the rearranged V-J locus was generally confirmed as described previously for the random IgL clones (7, 20). The local follicle preference of the pseudogene donor as V9274/H9274/V22 may be an accidental event in a total of 11011 follicles per bursa. Only the pseudogene V9274/V22, carrying the shortest coding segment between CDR1 and CDR2, was not used for the gene conversion donors.

Evolutionary distance of Ig L chain protein from the germline

We translated the PCR sequences of the productive clone to the corresponding amino acid sequences. Then we searched amino acid sequence similarities between a pair of neighbor sequences and constructed a unique evolutionary tree under the principle of minimum evolution according to the NJ method (18) (Fig. 6). The NJ method provided not only the topology but also the horizontal branch lengths representing evolutionary distance of the final tree.
Figure 2. Genealogical relationship of IgL sequences derived from a single bursal follicle, e2 of a day-18 embryo (A) and p2 of a 7-day-old normal chicken (B). O, The mutated sequence clone; O, hypothetical intermediates. Crippling mutations as termination or frame-shift mutations are shown by notched circles. The two numbers alongside the branches refer to the additional numbers of gene conversion events/point mutations, respectively. The numbers in parentheses represent the additional base modifications. Clones e2101–e2113 and e2201–e2217 in A and clones p2101–p2115 and p2201–p2215 in B were derived from independent PCR, respectively.
The average evolutionary distance of IgL from the germline was 3.3% during late embryonic life and expanded to 15% 1 week after hatching. There were no significant differences in IgL in the evolutionary distance from germline between BDL and NP-BDL chickens.

Intra- and interclonal amino acid differences in CDR
Because the primary structure responsible for Ag binding is located in CDRs of each Ig chain, we examined the average evolutionary distances between clones of the same group and between clones of different groups in the region corresponding to the CDR germline of the Ig L chain (26 aa of CDR1, CDR2, and CDR3) as shown in Table III. Clonal diversity in CDRs was expanded during bursal development. No significant differences were observed between intra- and interclonal diversities or in the clonal diversities between BDL and NP-BDL chickens. The evolutionary distance from the germline was expanded 2-fold more in the CDRs than in the full length of IgL.

Discussion
Ag-independent bursal diversifications
In chickens, the Ig gene rearrangement is not the key event for Ig diversity, but the postrearrangement diversification by gene conversion in bursa generates the B cell repertoire. Therefore, the bursa of Fabricius is the primary site of B cell preimmune repertoire formation in the chicken. The evolutionary distance of the IgL sequence from the germline increases with time in the bursa: 3.3% at the intraembryonic phase and 15% at the posthatching period (Fig. 6). These bursal diversifications are expanded to the average evolutionary distance of 22% in the periphery, as shown by Ag-activated B cells migrating into germinal centers (14). Both bursal and germinal center diversifications contribute equally to the evolutionary distance at the periphery.

In the pseudogene sequences used for gene conversion, framework regions are well conserved but the CDRs are more diversified (1). Accordingly, the evolutionary distance from the germline is enlarged to >30% in neonatal bursal cells when compared with the CDR sequences (Table III). However, repertoire of bursal lymphocyte specificities shown by both intraclonal and interclonal evolutionary diversities in CDRs of the Ig L chain was not significantly affected by the NP antigenic administration. These results based on the sequence diversity are compatible with the functional evidence that the BDL treatment did not impede the rate of Ab diversification during embryonic development (21).

The postbursal Ig diversifications in the germinal center are equally induced by gene conversion and by point mutations in the early phase of Ag stimulation, but gene conversion events are strongly suppressed during the late stage (14). Although we iden-
FIGURE 4. Genealogical relationship of IgL sequences derived from a single bursal follicle, b3 of BDL chicken (A) and n1 of NP-BDL chicken (B). Clones b3101–b3115 and b3201–b3215 in A and clones n1101–n1114 and n1201–n1216 in B are derived from independent PCR, respectively. Other indications are as described in Fig. 2.
tified base modifications induced by gene conversion and point mutations separately, somatic point mutations remained suppressed to a low level during bursal development (Table II). Point mutations occurred at sites distant from gene conversion events (Figs. 1 and 3). The generation of the bursal preimmune repertoire is mostly brought out by gene conversion events.

### Table II. Average mutational events for each pair-group of IgL sequences in genealogical trees

<table>
<thead>
<tr>
<th>Bursal Follicle</th>
<th>Clonal Group (no. of sequences)</th>
<th>Gene Conversion Events</th>
<th>Point Mutations</th>
<th>Base Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>e2 (day-18 embryo)</td>
<td>I (26)</td>
<td>1.7</td>
<td>0.7</td>
<td>7.1</td>
</tr>
<tr>
<td>p2 (day-7 neonatal)</td>
<td>I (7)</td>
<td>4.1</td>
<td>0.9</td>
<td>22.6</td>
</tr>
<tr>
<td></td>
<td>II (7)</td>
<td>6.1</td>
<td>1.9</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>III (6)</td>
<td>4.7</td>
<td>0.7</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>IV (4)</td>
<td>7.5</td>
<td>2.3</td>
<td>32.5</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>5.4</td>
<td>1.3</td>
<td>24.0</td>
</tr>
<tr>
<td>b3 (BDL)</td>
<td>I (5)</td>
<td>4.4</td>
<td>3.4</td>
<td>18.4</td>
</tr>
<tr>
<td></td>
<td>II (5)</td>
<td>5.5</td>
<td>1.3</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td>III (5)</td>
<td>6.8</td>
<td>1.2</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>IV (4)</td>
<td>6.8</td>
<td>1.8</td>
<td>27.8</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>5.8</td>
<td>1.9</td>
<td>22.1</td>
</tr>
<tr>
<td>n1 (NP-BDL)</td>
<td>I (19)</td>
<td>5.1</td>
<td>2.8</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>II (5)</td>
<td>4.0</td>
<td>1.4</td>
<td>21.6</td>
</tr>
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<td></td>
<td>III (3)</td>
<td>4.0</td>
<td>1.3</td>
<td>19.7</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>4.7</td>
<td>2.4</td>
<td>21.7</td>
</tr>
</tbody>
</table>

**FIGURE 5.** Frequency of ψVL usage as gene conversion donors. The number of occurrences of each ψVL segment as possible conversion donors was tabulated in 31 gene conversion events specified in 28 IgL sequences from embryonic bursal follicles, e1–e4 (A); 118 conversion events in 30 IgL sequences from neonatal normal bursal follicles, p1–p4 (B); 119 conversion events in 30 IgL sequences from BDL bursal follicles, b1–b4 (C); and 108 conversion events in 30 IgL sequences from NP-BDL bursal follicles, n1–n4 (D). For events with more than two potential donors, each was counted as a fraction divided by the number of possible donors. ψVL segments are identified by number assignment, and orientation is distinguished by filled bars (antisense orientation) and open bars (sense orientation).
In both embryonic and posthatching stages, intraclonal CDR diversity shown by average evolutionary distances between clones of the same group is not markedly different from the interclonal CDR differences between clones of different groups (Table III). This is characteristic of the Ag-independent bursal diversifications generating the preimmune repertoire. In the Ag-dependent germinal center
reaction, in which Ag-activated B cells are diversified during the early phase and are subsequently subjected to selection for oligoclonality, intraclonal diversity was restricted to a narrow range in contrast with the expanded interclonal diversity (14).

Nonproductive clones are not lethal

Joining events were identified by unique V-J junctions. These numbers may be different from the actual number of independent joints, because many of the joints are clonally amplified and are counted as one independent event. Many nonproductive V-J joints and abortive clones were observed in sequences derived from the single bursal follicles at different developmental stages (Table I). Nonproductive rearrangements cloned at day 18 of incubation were 42% in total sequences and 33% in independent joints. This percentage is higher than the previous values, 6% of all V-J joints (5) and 4% of all V-D-J joints (22) cloned from pools of bursal cells. Minor nonproductive clones in each single follicle may not be counted in a large pool of bursal follicle cells.

Clonal expansion of nonproductively rearranged IgL alleles and the base modifications by gene conversion continued during bursal development without artificial Ag stimulation (Figs. 2B and 4A). We also characterized gene conversion events that result in either the loss of a productive rearrangement or correct out-of-frame joints by shifting the reading frame during late embryonic life (Fig. 1A). The nonproductive V-J joining event is not a lethal event by itself in bursa. These findings would account for the Ag-independent clonal diversification by gene conversion. Constitutive basal signaling may be sufficient to support B cell development in the bursa. In contrast, no abortive clones carrying crippling mutations in the Ig L chain have been diversified in the Ag-induced germinal centers (14).

Ag-dependent positive selection of B cells

The proportion (one-third) of nonproductive V-J joints at the late embryonic stage is lower than the two-thirds expected if V-J joining is random at the earliest bursal stage (5). Thus, selective amplification of cells with productive gene rearrangements could be

---

**Table III. Percentage of average amino acid difference in CDRs of IgL chain**

<table>
<thead>
<tr>
<th>Bursal Follicles Compared</th>
<th>No. of CDRs</th>
<th>Intraclonal Distance</th>
<th>Interclonal Distance</th>
<th>Distance from Germline</th>
</tr>
</thead>
<tbody>
<tr>
<td>e1, e2, e4</td>
<td>25</td>
<td>8.8 ± 7.6</td>
<td>11.6 ± 8.3</td>
<td>5.9 ± 5.8</td>
</tr>
<tr>
<td>p1, p2, p3</td>
<td>30</td>
<td>35.6 ± 9.0</td>
<td>47.0 ± 10.2</td>
<td>38.3 ± 7.8</td>
</tr>
<tr>
<td>b1, b2, b3, b4</td>
<td>29</td>
<td>36.6 ± 7.0</td>
<td>42.2 ± 10.6</td>
<td>35.5 ± 8.2</td>
</tr>
<tr>
<td>n1, n2, n3, n4</td>
<td>40</td>
<td>38.3 ± 11.4</td>
<td>48.3 ± 12.3</td>
<td>31.2 ± 10.6</td>
</tr>
</tbody>
</table>

*Percentage of amino acid difference is shown by a weight-average evolutionary distance, giving weights of a numerical factor of CDRs.

Clonal groups are shown with the number of CDR sequences (26 aa of CDR1, 2, and 3) in parentheses as follows: e1-II (4), e2-I (16), e4-I (5); p1-I (2), p1-II (2), p2-I (2), p2-II (7), p2-III (6), p3-I (2), p3-II (2), p3-III (2); b1-I (4), b2-I (2), b2-II (2), b3-II (5), b3-III (5), b3-IV (4), b3-V (2), b4-I (2), b4-III (3); n1-I (19), n1-II (5), n1-III (3), n2-I (3), n2-II (2), n3-I (3), n4-I (5).
induced by an interaction between the surface receptor and ligands in the bursal microenvironments.

After hatching, environmental Ags are transported along the bursal duct connecting the intestinal lumen to the bursal lumen across the follicle-associated epithelium into the medullary areas of the bursal follicle (23). Thus, bursal cells with the prediversified receptor are exposed to foreign Ags at a time and place in which the B cell repertoire is being generated. Although the proportion of productive joints was not changed during this time period, the selective expansion of B cell clones with in-frame V-J joints was observed (Table I). To clarify whether the selective amplification of bursal lymphocyte with productive rearrangements could result as a consequence of exposure to environmental Ags, we compared the IgL sequences of B cells from a single bursal follicle of BDL and NP-BDL chickens. Antigenic administration into the bursal lumen immediately before BDL caused a significant decrease in lymphocytes with a nonproductive V-J joint at the IgL locus (Table I) and a remarkable decrease in abortive IgL clones in the bursa (Table I) in comparison with nonimmunized BDL chickens. However, administration of bacterial Ag into the bursal lumen did not cause an increase in the proliferation rate of bursal cells in comparison with the bursa of BDL chickens (13). There is no evidence for Ag-specific B cell proliferation in bursa. Antigenic stimulation into the bursal lumen before BDL caused a significant increase in bursal cells expressing IgM+IgG+ double-positive immune complexes on their surface (12). These immune complexes were verified as the surface (sIgM-positive B cells bound to the complex of environmental Ag and maternal IgG trapped on the surface of follicular dendritic cells as illustrated in Fig. 7 (Ref. 24 and M. Yasuda, H. Arakawa, H. Yokoyama, Y. Yokomizo, and S. Ekino, unpublished results). Starting around the time of hatching, B cells in the epithelial buds segregate to form an outer cortex of cells surrounding the inner medulla, and most bursal cell proliferation occurs in the cortex (13, 24–26). The medulla contains largely nondividing cells. CD4+T cells were distributed not in the medulla but in the cortex, where some cytoplasmic IgG+ cells were found (24). The frequency of apoptotic cells in the normal bursa increases after hatching and is especially increased in B cells expressing a truncated IgA chain (27). Thus, we suggest that B cells stimulated by enormous environmental Ags are escaped from apoptotic cell death and potentiating to migrate to form the cortex of rapidly dividing cells. Bursal B cells emigrate directly from the cortex to the periphery via lymph vessels (26, 28). Introduction of Ag into the bursal lumen has been shown to induce the specific production of Ab-forming cells in the periphery following subsequent systemic challenge (11, 29).

Overall, these findings strongly suggest competition within the follicle of bursal cells expressing endogenous sIg receptors driven by environmental Ag. Thus, environmental Ag-induced amplification of bursal B cells with in-frame V-J joints may be accounted for by the Ag-induced B cell survival depending on the signaling through endogenous sIg receptors rather than the Ag-specific proliferation. Two distinct populations of peripheral B cells have been defined as major populations of short-lived B cells comprising a diverse repertoire which have not encountered the foreign Ag in the bursa, and minor populations of B cells exposed to environmental Ag within the bursa, acquiring a longer-lived state (30). B cell populations clonally expanded in the bursal microenvironment and positively selected by environmental Ags may acquire the longer-lived state and respond to the environmental Ags in the periphery after emigrating from the bursa.

Acknowledgments

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References