IN VITRO MODEL FOR NATURAL TOLERANCE TO
SELF-ANTIGENS

Inhibition of the Development of Surface-Immunoglobulin-Negative
Lymphocytes into T-dependent Responsive B Cells by Antigen*

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One explanation for natural tolerance to self-antigens is the clonal-deletion model
proposed by Burnet (1), or the elimination of self-reactive lymphocytes upon contact
with self-antigens. This was later expanded by Nossal (2) into the clonal abortion
theory that states that as a lymphocyte acquires immunological competence, there
is a particular differentiation stage at which contact with self-antigen results in elimi-
nation of potential self-reactive clones. The obvious prediction from this theory is that
if one starts with an immunologically incompetent population of B lymphocytes, i.e.,
those lacking surface-immunoglobulin (s-Ig) receptors, and allows them to mature in
the presence of specific antigen, then the developing antigen-specific lymphocytes
should be eliminated.

Experiments designed to study tolerance induction using the T-dependent splenic
focus assay, which examines tolerance at the level of the individual B cells without
the complication of T-cell tolerance, have indicated that the developing B cell is
highly susceptible to tolerance induction (3-5). This is consistent with the clonal
abortion theory but with one important stipulation: that the developing B cell is
tolerized by contact with antigen only if there are no helper T cells present for that
antigen (3). However, these experiments did not directly test the clonal abortion
hypothesis because the starting populations analyzed were not pure populations of s-
Ig-negative cells.

It has recently been suggested that the type of tolerance in which the T cell plays
a pivotal role affects B-cell precursors at an intermediate stage in ontogeny (6, 7).
That is, if the differentiating B cell could be examined for tolerance induction at the
earliest stage in which an antigen can be recognized, or during the initial development
of s-Ig expression, perhaps interaction with antigen would result in obligatory

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AI-0-3958 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.
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‡ Abbreviations used in this paper: BSS-FCS, Hepses-buffered, pH-indicator-free balanced salt solution
containing 5-10% fetal calf serum; DMEM, high glucose Dulbecco's modified Eagle's medium; DNP, the
haptenic determinant 2,4-dinitrophenol; DNP-BSA, DNP-bovine serum albumin; DNP-HGG, DNP-
human gamma globulin; DNP-KLH, DNP-keyhole limpet hemocyanin; FACS, fluorescence-activated cell
sorter; FITC, fluorescein isothiocyanate; HGG, human gamma globulin; KLH, keyhole limpet hemocyanin;
NIP, the haptenic determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid; NIP-HGG, NIP conjugated to
HGG; PBA, polyclonal B-cell activator; s-Ig, surface immunoglobulin; SPF, specific-pathogen free.

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tolerance regardless of the status of the T cell, i.e., clonal abortion sensu strictu.

To test this idea, neonatal and adult spleen-cell populations were separated into s-Ig-positive and s-Ig-negative subpopulations using the fluorescence-activated cell sorter (FACS). The subpopulations were then tested both for the frequency of B-cell precursors to the determinant 2,4-dinitrophenol (DNP), and also for their susceptibility to the tolerogen DNP-human gamma globulin (DNP-HGG) using the splenic focus assay. In addition, another paper from this laboratory has examined the clonal frequency and tolerance induction of adult bone-marrow s-Ig-positive and s-Ig-negative subsets using a T-independent system. The results strongly support the clonal abortion theory by indicating that the response from the s-Ig-negative subset is almost totally abrogated by the presence of tolerogen. However, the stipulation that helper T cells can intervene and prevent the tolerance signal is still maintained.

Materials and Methods

Mice, Immunizations. The mice used in these studies were inbred CBA/CaH/WEHI (Walter and Eliza Hall Institute of Medical Research) mice which were specific-pathogen free (SPF). Unprimed spleen-cell sources for FACS separation were obtained from 8-wk-old adult and 3-d-old neonatal mice. SPF female mice were injected intraperitoneally at 6–8 wk of age with 0.1 mg of keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant. These mice were used as recipients for transferred cells 6–8 wk later. All mice were bred and maintained at The Walter and Eliza Hall Institute of Medical Research.

Hapten-Protein Conjugates. The hapten DNP was conjugated to KLH, bovine serum albumin, or human gamma globulin (HGG) as previously described (8). DNP-keyhole limpet hemocyanin (DNP-KLH) contained ≈10 mol of DNP per 100,000 daltons of KLH. DNP-bovine serum albumin (DNP-BSA) and DNP-HGG contained ≈10 mol of DNP per mol of protein. The hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was conjugated to HGG (NIP-HGG) according to standard procedures (9) and contained 10 mol of NIP/mol of protein.

Fluorescent Antiserum. Polyclonal anti-mouse Ig was prepared from a sheep anti-MPC-86 (IgG2b,k) serum with high anti-light-chain activity. The antibody was purified on a mouse Ig-Sepharose (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) column and conjugated with fluorescein isothiocyanate (FITC) as described previously (10).

Preparation of Cell Suspensions and Fluorescent Labeling. Spleen-cell suspensions from adult and neonatal mice were prepared using previously described procedures for removal of erythrocytes (11) and damaged cells (12). Cell suspensions were adjusted to 50 × 10⁶ viable cells per ml in a Hepes-buffered, pH-indicator-free balanced salt solution (11) containing 5–10% fetal calf serum (BSS-FCS) and labeled with a polyvalent FITC-anti-Ig at 4°C for 30 min. The antiserum was used at concentrations previously determined to give optimal labeling (≈40 μg/ml). Cells were washed and resuspended in BSS-FCS at 5 × 10⁶/ml for the FACS.

FACS Separation and Analysis. A FACS II (Becton, Dickinson FACS Systems, Mountain View, Calif.) was used to analyze and sort labeled cell suspensions. The light-scatter profile was determined and the scatter gates set to exclude the lowest light-scatter peak that contained a high proportion of dead cells and no Ig-positive cells. The fluorescence profile of the remaining cells was then determined. The threshold for separating bright from dull cells was set slightly lower than the threshold used for counting fluorescent cells under the microscope to minimize contamination of the dull population with bright cells. Only one or two channels were omitted between bright and dull populations. Cells were sorted at a rate of 3,500–4,000 per s and collected into siliconized glass tubes containing a small amount of fetal calf serum. Recoveries were ≈50% of the FACS count.

Fluorescence Microscopy. Samples of unfractionated and sorted spleen cells were usually fixed in 1% paraformaldehyde in phosphate-buffered saline and counted the next morning. Cells were mounted in 1% paraformaldehyde and overlaid with a coverslip sealed with nail polish.

FACS Separation of S-Ig-Positive and S-Ig-Negative Cells. To separate s-Ig-positive and s-Ig-negative subpopulations, spleen-cell suspensions were labeled with FITC-anti-Ig and separated by the FACS as described in Materials and Methods. In adult populations, the fluorescence profile showed a clear distinction between positive and negative cells (Fig. 1a), and it was relatively straightforward to determine the
table I
S-Ig Characteristics of Spleen Cells Separated by FACS

<table>
<thead>
<tr>
<th>Cell population*</th>
<th>Percentage of cells bearing s-Ig‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Adult (7–8 wk)</td>
<td></td>
</tr>
<tr>
<td>Unfractionated</td>
<td>46.5 ± 1.3</td>
</tr>
<tr>
<td>S-Ig-positive fraction</td>
<td>94.0 ± 1.5</td>
</tr>
<tr>
<td>S-Ig-negative fraction</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>Neonatal (3 d)</td>
<td></td>
</tr>
<tr>
<td>Unfractionated</td>
<td>15.0 ± 2.5</td>
</tr>
<tr>
<td>S-Ig-positive fraction</td>
<td>69.3 ± 11.9</td>
</tr>
<tr>
<td>S-Ig-negative fraction</td>
<td>1.1 ± 1.1</td>
</tr>
</tbody>
</table>

* Adult and neonatal spleen cells were stained with FITC-anti-Ig and separated into S-Ig-positive and S-Ig-negative fractions by the FACS. Fractions were subsequently analyzed for the percentage of S-Ig-positive cells by fluorescence microscopy.

‡ Data expressed as mean ± SE of three experiments.

thresholds for sorting. In contrast, the thresholds for sorting S-Ig-positive from S-Ig-negative cells in neonatal spleen-cell suspensions were more difficult to determine because the distribution of cells versus fluorescence intensity was continuous (Fig. 1 b). However, if the number of cells were plotted on a log scale, there was usually a point of inflection in the fluorescence distribution which corresponded to a point giving <1% positive cells in the unlabeled sample. Consequently, this was used as a reasonable cut-off point.

S-Ig Characteristics of Spleen Cells Separated by FACS. Both adult and neonatal spleen cells were separated into S-Ig-positive and S-Ig-negative subsets using the FACS. The subsets were then tested for the percentage of S-Ig-positive cells by fluorescence microscopy. The results of this analysis are presented in Table I. It was apparent that approximately one-half of the cells in adult spleen were S-Ig positive and that there was little contamination of the S-Ig-negative fraction with S-Ig-positive cells. The 3-d neonatal spleen-cell population contained 15% S-Ig-positive cells, and again the S-Ig-negative fraction was only slightly contaminated with S-Ig-positive cells. However, there was somewhat more contamination of the S-Ig-positive fractions with S-Ig-negative cells, particularly in the neonatal subset. (Only one or two channels were discarded between positive and negative cells because these fractions contained lymphocytes). Apparently, the contaminating cells in the S-Ig-positive fractions have not bound enough FITC-anti-Ig to be detectable by fluorescence microscopy. Moreover, for these studies it was more important to ensure that the S-Ig-negative fractions were pure.

T-Dependent Responses of Neonatal Spleen Cells Separated by FACS. In previous communications, it was shown that the splenic focus assay developed by Klinman and Press (14) is capable of measuring less-mature subpopulations of B lymphocytes (5, 16). It was of great interest therefore, to determine if the splenic focus assay could support the maturation and subsequent cloning of S-Ig-negative B-cell precursors. To this end, neonatal spleen cells, separated by the FACS, were analyzed for the frequency of DNP-specific precursors. The results are shown in Table II. To determine
Table II

Clone Frequency and Tolerance Induction of S-Ig-Positive and S-Ig-Negative Fractionated Neonatal Spleen Cells

<table>
<thead>
<tr>
<th>Cell population*</th>
<th>Tolerogen (3 × 10⁻⁷ M hapten)</th>
<th>Number of DNP precursors†</th>
<th>Statistical significance of difference§</th>
<th>Percent tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>per 10⁸ cells transferred</td>
<td>P</td>
<td>%</td>
</tr>
<tr>
<td>Unfractionated unlabeled</td>
<td>-</td>
<td>0.80 ± 0.20</td>
<td>&lt;0.05</td>
<td>68.7</td>
</tr>
<tr>
<td></td>
<td>DNP-HGG</td>
<td>0.25 ± 0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NIP-HGG</td>
<td>0.78 ± 0.15</td>
<td>NS†</td>
<td></td>
</tr>
<tr>
<td>Unfractionated labeled</td>
<td>-</td>
<td>0.62 ± 0.08</td>
<td>&lt;0.05</td>
<td>66.2</td>
</tr>
<tr>
<td></td>
<td>DNP-HGG</td>
<td>0.21 ± 0.08</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>S-Ig-positive</td>
<td>-</td>
<td>2.02 ± 0.17</td>
<td>&lt;0.01</td>
<td>64.4</td>
</tr>
<tr>
<td></td>
<td>DNP-HGG</td>
<td>0.72 ± 0.09</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>S-Ig-negative</td>
<td>-</td>
<td>0.41 ± 0.09</td>
<td>&lt;0.05</td>
<td>87.8</td>
</tr>
<tr>
<td></td>
<td>DNP-HGG</td>
<td>0.05 ± 0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

* Spleen cells from the sources indicated were transferred to carrier-primed recipients. 2-5 × 10⁸ donor cells were injected per recipient, 3-4 mice per experimental group. Fragment cultures were incubated in the presence or absence of DNP-HGG for 24 h followed by 3-d stimulation with DNP-KLH (3 × 10⁻⁷ M). Supernates were collected at day 10 and DNP clones were detected in radioimmunoassay by ¹²⁵I-labeled anti-Fab.
† Results of three experiments ± SEM.
§ Calculated by the t statistic for two means.
|| NS, not significant.

if labeling with FITC-anti-Ig affected the response, the DNP-precursor frequency for both unlabeled and labeled unfractionated spleen cells was determined. Although the frequency in the labeled unfractionated population was slightly lower, it was not significantly different from the unlabeled control. When neonatal spleen cells were fractionated on the basis of s-Ig, over a three-fold increase in DNP-precursor frequency was found in the s-Ig-positive fraction compared with unfractionated controls. This frequency was marginally lower than expected because there was over a four-fold increase in s-Ig-positive B cells in this fraction compared with unfractionated controls. Most noteworthy however, was the small but significant precursor activity found in the s-Ig-negative subset.

Tolerance Induction of Neonatal Spleen Cells Separated by FACS. Because it was found that s-Ig-negative spleen cells were detectable in the assay system, it was of great interest to study the effect of tolerogen. The assay system provided a direct test of the clonal abortion theory because s-Ig-negative cells would have been acquiring Ig receptors in the presence of tolerogen. Neonatal spleen cells were therefore sorted on the basis of s-Ig and analyzed for tolerance susceptibility by a modified splenic focus assay as described by Metcalf and Klinman (3). The results of prior exposure of spleen fragments to the tolerogen DNP-HGG are summarized in Table II.

It has been reported previously that ≈60-70% of unfractionated 3-d-old neonatal spleen cells are susceptible to tolerance induction (3, 5), and it appears that labeling with FITC-anti-Ig does not affect the extent of tolerance susceptibility. In addition, the tolerance observed was specific because incubation with NIP-HGG did not alter...
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### Table III

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Tolerogen</th>
<th>Isotype distribution: *</th>
<th>percentage of clones expressing‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>IgM plus IgA</td>
</tr>
<tr>
<td>Unfractionated unlabeled</td>
<td>−</td>
<td>35.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Unfractionated labeled</td>
<td>−</td>
<td>42.8</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>26.3</td>
<td>15.8</td>
</tr>
<tr>
<td>S-Ig-positive</td>
<td>−</td>
<td>29.4</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20.0</td>
<td>0</td>
</tr>
<tr>
<td>S-Ig-negative</td>
<td>−</td>
<td>50.0</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>40.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

* DNP-specific clones detected in radioimmunoassay by 125I-labeled anti-Fab were reanalyzed for isotype distribution using the following 125I-labeled, purified anti-heavy-chain antibodies: anti-IgM, anti-IgG1, anti-IgG2, anti-IgG2b, anti-IgG3, and anti-IgA.

‡ Positive clones from each group were analyzed from 3 separate experiments, 10-30 total clones were analyzed per group except the tolerogen-treated s-Ig-negative group in which only 5 were assayed. Results expressed as percentage of clones containing the isotypes indicated. Due to the complexity of the data, IgG1, IgG2a, IgG2b, and IgG3 were grouped as IgG.

§ This grouping includes those clones positive for IgA plus IgM plus IgG.

|| This grouping includes those clones positive for IgA plus IgG.

the response. Similar to the unfractionated neonatal control, the s-Ig-positive subset was highly sensitive to tolerogen, resulting in an ~65% reduction in precursor frequency. Most interesting though was the almost complete abrogation of DNP-response precursors in the tolerogen-treated s-Ig-negative subset.

Isotypes Secreted by Neonatal Spleen Cells Separated by FACS. Those clones found to be positive for anti-DNP antibody were reanalyzed to determine the isotypes of anti-DNP that were secreted. The isotype analysis is summarized in Table III. All positive foci were screened for the presence of six isotypes (IgM, IgG1, IgA, IgG2a, IgG2b, and IgG3) but the data were grouped as indicated in Table III due to the complexity involved.

Approximately 35–45% of the clones from unfractionated controls were secreting IgM only, 15–20% IgM plus IgA or IgA only, and 40–45% IgM plus IgG or IgG only. When neonatal spleen cells were fractionated, the isotypes secreted by resulting clones from the s-Ig-positive fraction were not substantially different from unfractionated controls. In the s-Ig-negative subset however, there was a striking depletion of clones secreting IgM plus IgG or IgG with an increased incidence of clones secreting IgA. When both the unfractionated neonatal population and the s-Ig-positive and s-Ig-negative subpopulations were exposed to tolerogen, the resistant precursors remaining resulted in a lower incidence of IgM-only clones and a higher incidence of IgM-plus-IgG- or IgG-secreting clones compared with the corresponding, untreated controls.

T-Dependent Responses of Adult Spleen Cells Separated by FACS. A large, immature, rapidly sedimenting, tolerance-susceptible splenic lymphocyte that results in IgM-only-secreting clones has been previously described (16, 17). Even though the cell was found in adult spleens, it was suggested that this large lymphocyte could be a pre-B-
Table IV
Clone Frequency and Tolerance Induction of S-Ig-Positive and S-Ig-Negative Fractionated Adult Spleen Cells

<table>
<thead>
<tr>
<th>Cell population*</th>
<th>Tolerogen (DNP-HGG, 5 × 10⁻⁷ M)</th>
<th>Number of DNP precursors‡</th>
<th>Statistical significance of difference§</th>
<th>Percent tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>per 10⁶ cells transferred</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Unfractionated unlabeled</td>
<td>-</td>
<td>1.14 ± 0.30</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.40 ± 0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfractionated labeled</td>
<td>-</td>
<td>0.85 ± 0.14</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.90 ± 0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Ig-positive</td>
<td>-</td>
<td>2.10 ± 0.30</td>
<td>NS</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.70 ± 0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-Ig-negative</td>
<td>-</td>
<td>0.45 ± 0.10</td>
<td>&lt;0.05</td>
<td>82.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.08 ± 0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Spleen cells from the sources indicated were transferred to carrier-primed recipients. 2-5 × 10⁶ donor cells were injected per recipient, three-four mice per experimental group. Fragment cultures were incubated in the presence or absence of DNP-HGG for 24 h followed by 3-d stimulation with DNP-KLH (5 × 10⁻⁷ M). Supernates were collected at day 10 and DNP clones were detected in radioimmunoassay by ¹²⁵I-labeled anti-Fab.
‡ Results of three experiments ± SEM.
§ Calculated by the t statistic for two means.
‖ NS, not significant.

Cell (16), the putative cytoplasmic-Ig-positive, functional-Ig-receptor-negative B-cell precursor (18, 19). It was of interest, therefore, to see if the s-Ig-negative subpopulation from adult spleens contained any DNP-specific B-cell precursors. The results are shown in Table IV. It was apparent that there was a small but significant DNP-precursor activity found in the s-Ig-negative subset of adult spleen.

The effect of labeling with FITC-anti-Ig on the DNP-responsive B-cell precursors was determined by comparing the frequency of DNP precursors in labeled and unlabeled unfractionated spleen-cell populations. Similar to results found with neonatal spleen, the frequency obtained in the labeled unfractionated population was slightly lower but not significantly different from the unlabeled control. When fractionated on the basis of s-Ig, there was about a twofold enrichment of DNP precursors in the s-Ig-positive subset. This was to be expected because there was about a twofold enrichment of s-Ig-positive B lymphocytes in this subset.

Tolerance Induction of Adult Spleen Cells Separated by FACS. Sensitivity to tolerogen was also determined for adult spleen cells sorted by s-Ig positivity; the results are summarized in Table IV. The unfractionated adult spleen-cell population as well as the adult s-Ig-positive subpopulation were not susceptible to tolerance induction, which is in marked contrast to the tolerance susceptibility of the corresponding neonatal populations. The adult s-Ig-negative subset, on the other hand, was extremely susceptible, with exposure to tolerogen resulting in nearly total abrogation of the DNP-specific clonable precursors.

Isotypes Secreted by Adult Spleen Cells Separated by FACS. Adult anti-DNP clones were also analyzed for the class of antibody produced and the results are presented in Table...
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Table V
Isotypes Secreted by Adult Spleen Cells Separated by FACS

| Cell population          | Tolerogen | IgM | IgM plus IgA | IgA | IgM plus IgG§ | IgG|| |
|-------------------------|-----------|-----|--------------|-----|--------------|-----|
|                         |           | %   |              |     |              |     |
| Unfractionated unlabeled| −         | 25.6| 12.5         | 5.6 | 32.2         | 24.1|
|                         | +         | 22.1| 11.0         | 2.5 | 44.6         | 19.8|
| Unfractionated labeled  | −         | 16.1| 9.7          | 0   | 35.4         | 38.8|
|                         | +         | 19.4| 2.8          | 11.1| 38.8         | 27.8|
| S-Ig-positive           | −         | 28.5| 14.3         | 9.5 | 23.8         | 23.8|
|                         | +         | 16.1| 6.5          | 9.7 | 35.4         | 32.3|
| S-Ig-negative           | −         | 60.0| 10.0         | 10.0| 20.0         | 0   |
|                         | +         | 40.0| 20.0         | 40.0| 0            | 0   |

* DNP-specific clones detected in radioimmunoassay by 125I-labeled anti-Fab were reanalyzed for isotype distribution using the following 125I-labeled purified, anti-heavy-chain antibodies: anti-IgM, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, and anti-IgA.

§ Positive clones from each group were analyzed from 3 separate experiments, 10-40 total clones were analyzed per group except the tolerogen-treated s-Ig-negative group in which only 5 were assayed.

Results expressed as percentage of clones containing the isotypes indicated. Due to the complexity of the data, IgG1, IgG2a, IgG2b, and IgG3 were grouped as IgG.

§ This grouping includes those clones positive for IgA plus IgM plus IgG.

II This grouping includes those clones positive for IgA plus IgG.

It appeared that ≈15–25% of the clones in the unfractionated groups secreted IgM only, 10–20% secreted IgM plus IgA or IgA only, and 55–75% secreted IgM plus IgG or IgG. A comparison with the neonatal isotype analysis of unfractionated controls indicated that the adult spleen cells tended to produce a smaller percentage of IgM-only-secreting clones with a subsequently larger percentage of clones secreting the IgG classes. The isotypes secreted by clones from the s-Ig-positive subset were not substantially different from the unfractionated controls. However, the s-Ig-negative subset was highly enriched for clones secreting IgM only, with a subsequent paucity of clones secreting IgM plus IgG or IgG.

The unfractionated populations and the s-Ig-positive subpopulation which showed no decrease in precursor frequency due to exposure to tolerogen also showed no differences in the isotypes secreted when exposed to tolerogen. In contrast, the few resistant clones from the s-Ig-negative subset more frequently secreted IgA than the untreated control.

Discussion

The main purpose of these studies was to test the clonal abortion theory directly by examining tolerance induction in a starting population of lymphocytes lacking an s-Ig receptor. The s-Ig-negative lymphocytes, separated by the FACS, were tested from both neonatal and adult spleens. The data indicate that the presence of the tolerogen DNP-HGG during the acquisition of s-Ig receptors results in the elimination of potential anti-DNP clones. Similar results were obtained in another study from this laboratory in which the maturation of s-Ig-negative adult bone-marrow cells was
examined in the presence of tolerogen using a T-independent system. The results provide very persuasive evidence for clonal abortion.

The T-dependent splenic focus assay developed by Klinman and Press (14) was used for these studies because it had been previously shown that this assay system could measure a range of splenic-B-cell subpopulations including immature, tolerance-susceptible subpopulations (5, 16, 17). In addition, tolerance as well as immune induction could be assessed at the level of the individual B cell without complications involving T-cell tolerance or T-cell suppression (3, 4). Finally, the characteristics of tolerance induction in this system correlate well with the prerequisites one would expect for a relevant physiological mechanism designed to eliminate potential self-reactive clones (20). These characteristics include: (a) the target cell for tolerance induction is an immature B-cell precursor rather than the mature adult cell; (b) tolerance is inducible with a wide variety of antigenic structures; (c) tolerance is inducible over a wide range of antigenic concentrations including relatively low concentrations that are normally immunogenic with mature B cells; and (d) an antigen-specific T cell is not required to induce tolerance (20). Thus, the splenic focus assay seemed an ideal system to analyze the development of s-Ig-negative lymphocytes in terms of both immune induction and tolerance induction.

In light of several reports indicating the suppressive effect of anti-Ig on B cells, particularly immature B cells, (21, 22), it was important to determine if labeling lymphocytes with FITC-anti-Ig affected immune function. It was found that neither the degree of immune induction nor the degree of tolerance induction was significantly altered by the labeling of adult or neonatal cells with FITC-anti-Ig. This probably relates to the low concentrations of anti-Ig used as well as to the short exposure time, because it has been reported that 24 h is necessary to irreversibly suppress immature B cells (21).

When both neonatal and adult spleen cells were sorted into s-Ig-positive subsets, there was an enrichment in the frequency of DNP precursors which corresponded well with the enrichment of s-Ig-positive cells. The isotypes secreted by the s-Ig-positive subsets were not significantly different from unfractionated controls, but the neonatal unfractionated control and s-Ig-positive subset resulted in a greater incidence of clones secreting IgM only, with a subsequent decrease in IgG compared with the corresponding adult fractions.

Interestingly, both neonatal and adult spleen cells sorted into s-Ig-negative subsets gave rise to a low but substantial frequency of DNP-specific clones. The fact that these clones are secreting a large percentage of IgM with little or no IgG indicates not only the relative immaturity of these cells compared to the s-Ig-positive cells, but also the effectiveness of the sorting procedure. It is interesting that there is also a considerable percentage of clones secreting IgA. Perhaps lymphocytes capable of secreting IgA upon appropriate triggering appear earlier in ontogeny than has generally been recognized (23). This point is a subject of further study.

Presumably, the DNP-precursor frequency from the receptor-negative subsets reflects the acquisition of functional s-Ig receptors on lymphocytes during the antigenic stimulation phase of the culture, with subsequent proliferation and differentiation into anti-DNP-secreting clones. This is particularly plausible in light of recent findings of Ryser and Vassalli (24) and Fairchild and Cohen (25) as well as results from this laboratory that indicate that the s-Ig-negative small lymphocytes isolated from adult bone marrow progressively acquire s-Ig in tissue culture with up to 50–60% of the
small lymphocytes bearing s-Ig after periods varying from 2-4 d.

Numerous reports have documented the extreme sensitivity of neonatal B cells to multivalent antigens compared with adult B cells (5, 20, 26-30). Likewise, in this study it was found that neonatal unfractionated spleen cells and s-Ig-positive B cells were sensitive to tolerogen with an $\approx 60-70\%$ reduction in responsive B cells, although adult unfractionated spleen cells and adult s-Ig-positive B cells remained refractory to tolerogen. A comparison of the isotype distribution in control versus tolerogen-treated cultures indicates that the precursors for IgM-only-secreting clones are the most susceptible to tolerance induction which is consistent with past results (3).

The presence of the tolerogen DNP-HGG during the culturing of s-Ig-negative cells resulted in a drastic reduction of clonable precursors, a finding certainly consistent with clonal abortion. However, it should be noted that when the same s-Ig-negative cells were cultured with the antigen DNP-KLH for which there are KLH-specific helper T cells present, a response occurs. Thus, it would appear that the helper T cell overrides the tolerance signal even at the earliest phase during which it is possible to affect the cell functionally (i.e., that point at which surface-Ig receptors appear). One possible objection to this reasoning is that the clonal activity observed in the s-Ig-negative subset is due to the small proportion of s-Ig-negative cells that have acquired s-Ig during the 14-h adoptive transfer part of the splenic focus assay (a period in which there is no antigen present). However, it is unlikely that such cells could account for the observed activity in view of the relatively high clone frequency in the s-Ig-negative fractions (Tables II and IV). In addition, clonable B-cell precursors have been detected in 14-d fetal liver using the splenic focus assay (manuscript in preparation). Because s-Ig receptors do not appear in ontogeny until day 16 or 17 of gestation (31), it is unlikely that the clonal activity is due to maturation of s-Ig-positive lymphocytes during the adoptive transfer part of the assay. Furthermore, Rosenberg and Cunningham (32) could find no evidence for B-cell tolerance of fetal lymphocytes in an adoptive transfer system in which specific helper T cells were present. Consequently, the evidence indicates that the T cell overrides the tolerant signal regardless of the developmental stage of the B-cell precursor.

However, it is worth noting that the development of B cells, both in the embryo and in the neonate, more than likely takes place in the absence of self-reactive helper T cells. In fact, if anything, there seems to be an overabundance of nonspecific suppressor T cells during this time (33). Thus, the ability either to immunize or to tolerize the same cell may be very important in terms of molecular mechanisms involved in triggering, but may not be so important in terms of eliminating, self-reactive lymphocytes in the developing animal.

The ability to measure DNP precursors from the s-Ig-negative subpopulation of spleen cells is particularly interesting because the response is T-dependent. Recent work by Cambier et al. (34) suggests that the precursor for a primary T-dependent response is a more mature B cell bearing both IgM and IgD receptors. That such an immature B cell just acquiring surface receptors can be triggered in a T-dependent manner tends to argue against the requirement for the more mature precursor. Indeed, the ability to clone out T-dependent responsive precursors from a s-IgD-negative subset in the presence of anti-IgD further substantiates this point.3

It is noteworthy that the adult spleen contains a subpopulation of immature, tolerance-susceptible B-cell precursors, albeit to a lesser extent than the neonatal spleen, a result which is consistent with earlier studies from this laboratory (5, 16). This reveals the heterogeneity of B cells with respect to differentiation states in adult spleen, a tissue often used as a control source of mature B cells. This could in part explain recent results by Hamarström et al. (35) and Primi et al. (36) in which the inducibility of self-reactive antibodies in mature adults by polyclonal B-cell activators (PBA) was cited as evidence against the physiological role of a central B-cell tolerance. However, it is by no means excluded that the source of autoreactive antibodies is from stimulation of immature B cells that have been immunized instead of tolerized due to the intervention of a PBA in much the same way as helper T cells can intervene. Evidence for the intervention of a PBA during tolerogenesis has been reported (6, 37). In the final analysis, it may be that clonal abortion is most important during the development of the immune system, although in the adult animal where there are increased chances of polyclonal B-cell activation, a central B-cell tolerance for self-renewing lymphocytes exists but is less crucial because of the ancillary regulatory mechanisms available such as T-cell suppression (38, 39), idiotype networks (40), and receptor blockade (41).

In this regard it is interesting that the onset of autoimmune diseases in both man and animals appears after the development of the immune system (42) and often in early adult life or later (43). Moreover, the causes of these diseases have often been attributed to a breakdown in ancillary regulatory mechanisms (44).

**Summary**

Neonatal and adult splenic cell suspensions were labeled with fluorescein isothiocyanate-anti-Ig and fractionated into surface-immunoglobulin- (s-Ig) positive and s-Ig-negative subpopulations by the fluorescence-activated cell sorter. The subpopulations were then tested by splenic focus assay for both frequency and tolerance susceptibility of clonable 2,4-dinitrophenol (DNP) precursors. It was shown that both adult, and neonatal, s-Ig-negative subsets contained clonable DNP-specific B-cell precursors. However, because these precursors result in fewer clones secreting IgG, they appeared to be less mature than the s-Ig-positive precursors.

In the absence of helper T cells, it was found that exposure of s-Ig-negative lymphocytes to tolerogen during the process in which they were acquiring surface receptors resulted in nearly total abrogation of potential DNP clones. This finding provides compelling evidence for clonal abortion.

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