The Immunoglobulin Allotype Contributed by Peritoneal Cavity B Cells Dominates in SCID Mice Reconstituted with Allotype-disparate Mixtures of Splenic and Peritoneal Cavity B Cells

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Summary

We have studied potential regulatory interactions between mature B lymphocyte populations by analysis of C.B-17 severe combined immunodeficient (SCID) mice reconstituted simultaneously with immunoglobulin allotype-congenic mixtures of spleen (SP) and peritoneal cavity (PerC) B cells. We have previously shown that the independent transfer of B cells from these sources leads to the long-term survival of donor B cells and reconstitution of immunoglobulin levels in SCID mice (Riggs, J.E., D.L. Robertson, R.S. Stowers, and D.E. Mosier, manuscript submitted for publication). SP and PerC B cells differ in numerous respects, with the PerC having higher proportions of large, activated B cells that express the IgM>IgD phenotype and greater numbers of CD5 B cells. The injection of equal numbers of B cells from SP and PerC into SCID recipients (e.g., BALB/c SP + C.B-17 PerC → SCID) has led to the following observations: (a) serum IgM allotypes in B cell chimeras revealed strict dominance by the allotype contributed by the PerC B cells; (b) this dominance was not due to regulatory T cells; (c) B cells of the unexpressed (i.e., SP) allotype were present in the chimera in the spleen but not the peritoneal cavity; and (d) immunization with TI and TD antigens failed to elicit the SP IgM allotype, whereas secondary TD antigen immunization elicited low levels of the SP IgG2a allotype. Additional experiments demonstrated concurrent expression of IgM allotypes derived from both SP and PerC B cells in recipients that: (a) received a 10-fold excess of SP B cells; (b) received SP B cells before PerC B cell transfer; or (c) received SP B cells intravenously and PerC B cells intraperitoneally. We conclude that the establishment of IgM synthesis by PerC B cells leads to a feedback inhibition of subsequent IgM synthesis by SP B cells, and that the frequency of B cells that can lead to this effect is substantially higher in peritoneal cavity than in spleen. These data provide further confirmation of regulatory interactions between B cells in the absence of T lymphocytes (18), but confound the interpretation of experiments supporting the existence of a separate CD5+ B cell lineage (9).

B lymphocytes isolated from adult mice show evidence of heterogeneity in a large number of functional and phenotypic assays (1-11). These observations give rise to three questions: (a) are distinct functions associated with distinct B cell subsets; (b) do distinct lineages of B cell development exist (e.g., in a sense analogous to T cells expressing α/β or γ/δ receptors); and (c) do different B cell subsets (or lineages) influence each other? Historically, these questions first focused on B cells responsive to thymus-dependent (TD) versus thymus-independent (TI) antigens (1), then on B cells expressing or not expressing Lyb-5 (12), and the current candidates of conventional B cells and CD5 (Ly-1)-positive B cells (8). Characterization of peritoneal cavity (PerC) CD5+ B cells has provided the following evidence in support of their being a distinct B cell subpopulation/lineage. These cells exhibit a specific array of cell surface antigens [CD5+/−, Mac-1+, IgM>IgD, B220low (7, 8)], express unique Vμ genes (13), have a distinct slg+ progenitor (9), and display unique functional properties such as spontaneous Ig secretion (8), responsiveness to “environmental” antigens (8, 14), unresponsiveness to TD antigens (15), and autoantibody production (7, 15). However, controversy exists as to the stability of the CD5+ phenotype, as evidence accumulates that this marker is also an activation antigen (7, 8, 16, 17). Precursors of CD5+ B cells in adult animals appear to exist in the PerC but not the bone marrow (9), which is the clearest argument...
for their being a separate lineage. Our previous work (Riggs, J.E., D.L. Robertson, R.S. Stowers, and D.E. Mosier, manuscript submitted for publication) described the survival and function of mature B cells from different lymphoid organs when transferred to C.B-17 scid (SCID) mice, and documented engraftment of both conventional B cells and large, high sIgM, Mac-1+ *B cells that are identical to or closely related to the CD5+ (or their CD5- “sisters”) population. The engraftment of the latter population was seen with transfer of all B cell sources studied, including bone marrow from aged animals. This paper focuses on the interaction of allotype-marked B cells derived from spleen (SP) and from PerC when both are cotransferred to SCID recipients.

Our results extend recent experiments (18) suggesting feedback inhibition of Ig synthesis by CD5-like B cells derived from the PerC. We find that the Ig allotype derived from PerC B cells dominates over the allotype associated with transferred SP B cells. This allotype dominance is not dependent upon T cells, and appears to interfere with Ig synthesis rather than B cell survival. B cells capable of mediating feedback suppression exist both in the spleen and PerC; they are simply more frequent in the latter site. These studies have implications for the issue of separate B cell lineages as well as for regulatory interactions among B cells.

Materials and Methods

Mice. BALB/c (H-2b, Igh-1), BALB-xid (XID), C.B-17 (H-2b, Igh-1), and C.B-17-xid (hereafter, SCID) mice, bred and maintained at the Medical Biology Institute (MBI), were used between 8 and 12 wk of age. All donors were age- and sex-matched for establishing chimeras. All mice were handled in accord with National Institutes of Health (NIH) guidelines. The MBI SCID colony was derived from breeding pairs kindly provided by Ken Dorschkind, UC Riverside. As 10% of SCID mice “break through” and produce Ig, all mice were bled at 8 wk of age and only those mice with < 5 µg/ml of serum IgM were used in these studies.

Cell Preparations and Adoptive Transfer. SP suspensions were prepared by gently mincing spleens between two sterile glass slides in HBSS. PerC cells were harvested by flushing the peritoneum with 10 ml HBSS. T cells were depleted by anti-Thy-1.2 plus complement treatment. Unless specified otherwise, a total of 10 × 10^6 cells in a volume of 0.2 ml of HBSS (5 × 10^6 of each allotype) were injected intravenously into the lateral tail vein or intraperitoneally into SCID recipients.

Antigens and Immunizations. 0.3 ml (~ 10^8 organisms) of a heat-killed Streptococcus pneumoniae (strain R36a, obtained from American Type Culture Collection [ATCC], Rockville, MD) preparation and 100 µg of crl (1 →3) dextran (Dextran, B1355S) in saline, generously provided by the laboratory of Dr. Roy Riblet (MBI), were simultaneously injected intraperitoneally with 100 µg KLH. (Calbiochem-Behring Corp., La Jolla, CA) in saline mixed with CFA (H37Ra; Difco Laboratories, Detroit, MI), or 50 µg phosphorylcholine (PC)-substituted KLH, prepared as described (19) and mixed with IFN (Sigma Chemical Co., St. Louis, MO), were injected intraperitoneally.

Allotype- and Antigen-specific ELISA. Allotype-specific serum IgM levels were determined by ELISA employing flexible polyvinylchloride (PVC) plates (Dynatech Laboratories, Alexandria, VA) coated with an appropriate dilution of affinity-purified rabbit anti-mouse IgM antibody. Serial twofold dilutions of test sera and the standard HPCM2 (IgMr, k) for IgMr or total serum IgM detection, or C.B-17 sera for IgMr detection were applied in duplicate to the plates. Total IgM or Ig levels were determined using an appropriate dilution of rabbit anti-mouse Ig F(ab')2-specific horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA). IgMr and IgM levels were determined, respectively, with biotinylated (20), protein A-purified (Pierce ImmunoPure IgG purification kit; Pierce Chemical Co., Rockford, IL) DS-1 (mouse IgG; anti-IgMr [21]) generously provided by Dr. Donna Sieckmann (NMRI, Bethesda, MD), and AFS-78.25 (mouse IgG, anti-IgM [22]) mAbs at appropriate dilutions. Biotinylated mouse anti-mouse IgG2a (clone 8.3; [23]), purchased from PharMingen, La Jolla, CA, was used to detect IgG2a anti-KLH antibodies. Streptavidin-HRPO (Fisher Biotech) was used to detect binding of the biotinylated mAbs. Antigen-specific ELISAs were conducted in a similar manner. PVC plates were coated with KLH, or PC-substituted BSA, prepared as described (19), or dextran-substituted BSA, the latter generously provided by Dr. Gary Gilmore (MBI). HPCM2 and MOPC-104E (IgMr, λ) served as IgM anti-PC and IgM anti-Dextran standards. PC-specific antibodies were detected with the rabbit anti-mouse Ig F(ab')2 specific HRP conjugate described above. Dextran-specific antibodies were detected with an appropriate dilution of goat anti-mouse λ alkaline-phosphatase conjugate (Fisher Biotech), with phenolphthalein monophosphate serving as the substrate. Calculations of all Ig levels were done by comparison to standard curves using a minimum of 5 data points with correlation coefficients > 0.95.

Flow Cytometric Analyses. SP or PerC cells were cultured with tris-buffered NH4Cl to lyse red blood cells. 106 cells were then incubated with various airflowed (100,000 g for 10 min) antibody preparations. T cells were detected with fluoresceinated anti-Thy-1.2 obtained from Becton Dickinson Immunocytometry Systems, Mountain View, CA. Total IgMr cells were detected with fluoresceinated goat anti-mouse IgM antibody (Fisher Biotech). Sequential incubations, first with biotinylated, protein A-purified DS-1 or AFS-78.25 followed by avidin-FITC (Fisher Biotech) were used to detect IgMr or IgM bearing cells. An appropriate dilution of biotinylated APF-122 (mouse IgG, anti-IgD [22], Pharmingen) followed by streptavidin-FITC (Fisher), or a saturating amount of AMS 9.1.1.1 (mouse IgG2b anti-IgD [22]) tissue culture supernatant followed by a predetermined amount of fluoresceinated goat anti-mouse IgG2b (Fisher Biotech) were used to detect IgD alleles. Each step was conducted on ice for 30 min with extensive washing between steps. All samples included propidium iodide to allow exclusion of dead cells from the analyses. A minimum of 3 × 106 cells were then analyzed on a Coulter EPICS 753 flow cytometer.

Results

IgM Produced by PerC B Cells Dominates after Co-transfer of Allotype-Congenic SP and PerC to SCID Mice. We have previously shown that PerC B cells restore serum Ig levels in SCID recipients more rapidly than do SP B cells (Riggs, J.E., et al., manuscript submitted for publication). Given this result, it is of interest to determine if the simultaneous intravenous transfer of mixtures of SP and PerC cells leads to partial or total dominance of Ig produced by the PerC cells. We used transfers of Ig-alloallotype marked cell populations to perform these co-transfer experiments. All combinations of SP
and PerC cells from C.B-17 (Ighb) and BALB/c (Igha) donors were transferred to SCID mice. The recipients were bled weekly after intravenous transfer of cells, and their sera were assayed by ELISA for levels of total IgM, IgMa, and IgMb. The results of a representative experiment are presented in Table 1. The co-transfer of cells from the same tissue source (e.g., Sp + Spb) led to almost equal representation of each IgM allotype in the serum of the SCID chimera. However, the co-transfer of SP and PerC cells led to dominance by IgM expressing the allotype of the PerC donor. This dominance was stable as it persisted in mice for at least 60 wk after cell transfer (not shown).

Table 1. Co-Transfer of PerC SP B Cells Demonstrates the Dominance of PerC B Cells

| SCID recipients† of: | Total IgM | IgM* | IgMb |
|----------------------|-----------|------|------|
|                      | µg/ml     |      |      |
| SP + SPb             | 353 ± 135 | 122 ± 14 | 140 ± 12 |
| PerC + PerCb         | 1,082 ± 239 | 565 ± 33 | 750 ± 20 |
| PerC + SPb           | 1,351 ± 178 | 584 ± 90 | <5    |
| SP + PerC            | 1,080 ± 201 | <5    | 1,131 ± 27 |
| BALB/c control       | 1,000     | 600   | <5    |
| C.B-17 control       | 1,534     | <5    | STD   |

* Serum IgM levels determined 2 wk after transfer by ELISA as outlined in Materials and Methods. Analogous results are noted 40 wk after transfer.
† Three recipients per group.

T Cells Are Not Responsible for the Dominance of the PerC Allotype. Dominance of the PerC-contributed allotype in PerC + SP chimeras could be the result of regulatory T cell influences (24). To test this possibility, SP and PerC preparations were treated with anti-Thy-1.2 plus complement to deplete T cells before co-transfer to SCID recipients. Table 2 illustrates that T cell depletion did not alter the dominance of the IgM allotype contributed by the PerC B cells. The completeness of T cell depletion was confirmed by experiments in which SCID recipients of T cell-depleted mixtures of PerC and SP were killed at weekly intervals and tested for the presence of T cells by PHA responsiveness and flow cytometric analyses. In no instance was there any evidence of T cells by both criteria for at least 6 wk after transfer (data not shown). As a further test of the hypothesis that the dominance exerted by PerC cells is attributable to B cells and not T cells, we used BALB.xid mice as PerC B cell donors. xid mice have an intrinsic B cell defect manifest at several levels, including reduced serum IgM levels (6), but their T cells are normal in most respects (25). Equal numbers (5 × 106) of B cells from BALB.xid PerC and C.B-17 SP were injected intravenously into SCID recipients. The results shown in Table 3 demonstrate that the IgMb allotype contributed by the C.B-17 SP B cells dominated, in contrast to the result when PerC cells from normal donors were used. These data strongly imply that the allotype dominance exerted by normal PerC cells is due to B cells and not T cells.

Table 2. T Cell Depletion of Donor Populations Does Not Alter PerC B Cell Dominance

| SCID recipients† of: | Total IgM | IgM* | IgMb |
|----------------------|-----------|------|------|
|                      | µg/ml     |      |      |
| Total                |           |      |      |
| SP + PerCb           | 871 ± 97  | <5   | 762 ± 71 |
| PerC* + SPb          | 1,052 ± 285 | 514 ± 164 | <5    |
| T cell-depleted§     |           |      |      |
| SP + PerCb           | 625 ± 149 | <5   | 270 ± 75 |
| PerC* + SPb          | 996 ± 228 | 464 ± 82 | <5    |
| BALB/c control       | 928       | 524   | <5    |
| C.B-17 control       | 918       | <5    | STD   |

* Serum IgM levels determined 2 wk after transfer by ELISA as outlined in Materials and Methods.
† Three recipients per group.
§ All donor populations consisted of <2% Thy-1.2+ cells after T cell depletion.

Table 3. Failure of BALB.xid PerC to Dominate in Co-Transfer with C.B-17 SP

| SCID recipients† of: | Total IgM | IgM* | IgMb |
|----------------------|-----------|------|------|
|                      | µg/ml     |      |      |
| PerC*+SPb            | 266 ± 38  | 26 ± 17 | 193 ± 80 |
| PerC*+SP§            | 54 ± 8    | ND   | ND   |
| BALB.xid control     | 290       | 114   | <5    |
| BALB/c control       | 882       | 895   | <5    |
| C.B-17 control       | 1,084     | <5    | STD   |

* Serum IgM levels determined by ELISA 2 wk after transfer.
† Three recipients/group; equal numbers of B cells (5 × 106), determined by flow cytometry, were transferred.
Figure 1. Flow cytometric analyses of IgM<sup>+</sup> (A, E, I, K), IgM<sup>−</sup> (B, F, J, L), IgD<sup>+</sup> (C, G) and IgD<sup>−</sup> (H) expression for the spleen (A-H) and peritoneal cells (I-L) of SCID mice reconstituted 5 wk previously with 5 × 10<sup>6</sup> PerC<sup>+</sup> + 5 × 10<sup>6</sup> SP<sup>+</sup> i.v. Each profile compares the cells of chimeric (PerC<sup>+</sup> + SP<sup>+</sup>) recipients (dark solid line) and control PerC<sup>+</sup> or SP<sup>+</sup> (light dotted line) cells.
allotypes. Table 4 lists the number of spleen and PerC B cells expressing membrane IgM, IgD, IgMb, or IgDb, in PerC + SP recipients, and Fig. 1 illustrates representative staining profiles from which the data in Table 4 were derived. Splenic B cells from PerC + SP allotype chimeras predominately expressed mIgM and mIgD of the SP donor allotype (Table 4; Fig. 1, A, E, C, G), although some B cells expressed IgMb and thus were derived from the PerC donor. In contrast, PerC B cells from the same mice had a relative predominance of IgMb cells derived from the PerC donor (Table 4; Fig. 1, I and K vs. J and L). B cells recovered from the PerC expressed too little mIgD to be reliably analyzed with the allotype-specific anti-IgD reagents. Analogous results were obtained in 10 separate experiments using this as well as the reciprocal (PerC + SP) combination of B cells and by analyzing recipients 3-40 wk after transfer. These data lead to two conclusions: (a) that B cells derived from the SP donor persist even though IgM derived from them is not detected in the serum; and (b) that B cells bearing the donor SP allotype predominate in the spleen of SCID recipients and those bearing the donor PerC allotype predominate in the peritoneal cavity.

Thymus-independent Immunization Fails to Rescue the Suppressed SP Allotype. Having demonstrated the presence of donor SP B cells in PerC + SP → SCID mice, we attempted to promote the secretion of antibody of the SP allotype by deliberate immunization. SCID recipients of PerC + SP B cells were immunized 10 wk after transfer with R36a (S. pneumoniae) to elicit anti-PC antibodies and with dextran B1355S to elicit anti-α (1 → 3) dextran antibodies. IgMb mice respond to dextran, while IgMb mice do not (26). Use of allotype-specific reagents to develop antigen-specific ELISAs permits assessment of the contribution of each donor allotype in the response to PC. The results in Table 5 show that SP + SPb and PerC + PerCb recipients had relatively equal representation of each allotype in their IgM anti-PC response. In contrast, both the PerC + SP and the PerCb + SP recipients showed relative dominance of PC antibodies contributed by the PerC B cell donor. Table 5 also illustrates that PerC + SP mice failed to respond to dextran immunization with antibody levels greater than the C.B-17 control, while all other combinations of B cells in the SCID chimeras produce good antidextran responses. Thus, immunization with TI antigens either fails to elicit antibody production by the “suppressed” B cells of splenic origin, as is the case with dextran immunization, or generates only a meager response from such cells, as seen with PC immunization.

Primary TD Immunization Fails to Rescue SP IgM Allotype Expression. It is possible that the use of TI antigens leads to the selective activation of B cell subpopulations (8, 14). Accordingly, we also tested the ability of TD antigens to rescue the “suppressed” SP allotype. PerC + SP (not T-depleted) chimeras, BALB/c, C.B-17, and unreconstituted SCID controls were primed with 100 μg KLH 4 wk after transfer and boosted with 50 μg PC-KLH 4 wk later. The augmented primary antibody response to PC and the secondary antibody response to KLH were assayed 7 d after immunization. The primary IgM response to PC is restricted to the IgM bearing the PerC allotype (Table 6). Similar results were obtained for primary immune sera after KLH and SRBC immunization (data not shown). As these mice were carrier-primed to enhance T cell help for the primary anti-PC response, we also tested their secondary IgM and IgMb anti-KLH response. While the secondary response to KLH was quite low in these animals, the IgM allotype contributed by PerC cells appear to predominate although a small amount of the SP IgM allotype was now detectable. Furthermore, when the IgG2a response to KLH was measured, a better antibody response was seen and the SP allotype was readily detectable. Comparison between groups suggests that the major part of this response came from B cells derived from the SP donor. These data suggest that the PerC IgM allotype is predominant in both primary and secondary responses to TD antigens, yet the SP IgG2a allotype is elicited in a secondary TD response. This result suggests that the “suppressed” B cell is present and indicates that it can be activated by antigen under appropriate circumstances.

Secondary Transfer to SCID Recipients Fails to Rescue the “Suppressed” SP Allotype. We performed secondary transfer experiments to address two issues: (a) how stable is the suppression of SP B mediated by PerC B; and (b) do all the cells capable of maintaining suppression exist in the spleen of the primary SCID recipient, or might transfer of cells from the

Table 4. Staining of Spleen and Peritoneal Cells from SP/PerC SCID Chimeras

| Group                  | IgM | IgMb | IgMb | IgD | IgDb |
|------------------------|-----|------|------|-----|------|
| Spleen cells:          |     |      |      |     |      |
| BALB/c control         | 62.4| 38.5 | 1.8  | 52.5| 23.4 |
| C.B-17 control         | 84.2| 9.5  | 29.1 | 3.1 | 69.2 |
| C.B-17 scid control    | 1.8 | 3.9  | 1.5  | 2.1 | 2.7  |
| PerC + SP → SCID†      | 47.3| 39.2 | 11.5 | 24.7| 13.9 |
| PerC + SP → SCID‡      | 46.3| 40.1 | 13.4 | 24.3| 13.0 |
| Peritoneal cells from: |     |      |      |     |      |
| BALB/c control         | 7.6 | 5.1  | 0.2  | NT  | NT   |
| C.B-17 control         | 5.4 | 0.1  | 4.2  | NT  | NT   |
| PerC + SP → SCID       | 3.2 | 0.5  | 1.4  | NT  | NT   |

* Number of cells expressing specific markers determined by multiplying percent positive cells (determined by subtraction from appropriate staining controls) by the number of viable cells (determined by Trypan blue exclusion).
† SCID recipient of 5 × 10⁶ BALB/c spleen cells (SP) + 5 × 10⁶ C.B-17 peritoneal cells (PerC) i.v. 5 wk before being killed.
§ Not tested; too few cells were recovered for analyses; No cells recovered from C.B-17-scid control peritoneal cavity.
Table 5. Thymus-independent Immunization Fails to Rescue the SP Allotype

| SCID recipients of: | Total IgM | IgM<sup>a</sup> | IgM<sup>b</sup> | Dextran µg/ml |
|---------------------|-----------|-----------------|-----------------|--------------|
| SP<sup>a</sup> + SP<sup>b</sup> | 433 ± 76  | 283 ± 17        | 238 ± 85        | 84 ± 4       |
| PerC<sup>a</sup> + PerC<sup>b</sup> | 852 ± 195 | 655 ± 216       | 1,102 ± 498     | 61 ± 3       |
| PerC<sup>a</sup> + SP<sup>b</sup> | 239 ± 56  | 184 ± 48        | 61 ± 6          | 170 ± 70     |
| SP<sup>a</sup> + PerC<sup>b</sup> | 256 ± 55  | 34 ± 6          | 248 ± 49        | 11 ± 3       |
| BALB/c control | 361 ± 82  | 500 ± 16        | <5              | 156 ± 10     |
| C.B-17 control | 324 ± 16  | 47 ± 6          | 252 ± 68        | 15 ± 2       |

* Antibody levels determined 6 d post-immunization.
† SCID recipients (n = 3) of 5 × 10<sup>6</sup> cells of each type were immunized 10 wk after transfer.

peritoneal cavity be required to maintain suppression. We prepared SP cells from primary recipients of allotype-disparate PerC and SP B cells and injected these cells into secondary SCID recipients. The dominance of the original PerC donor allotype was maintained following secondary transfer (Table 7). PerC allotype dominance in secondary recipients was stable as similar results were observed at 30 wk after transfer. Secondary transfers conducted 40 wk after establishing primary recipients also failed to rescue the SP allotype (data not shown). These data indicate that B cell–dependent allotype suppression is long-lasting and that the spleen contains all the cells necessary to maintain the PerC B cell dominance established in the primary SCID recipient.

A 9:1 SP to PerC B Cell Ratio Results in Dominance of the Allotype of the SP Donor. All of the above experiments were performed with equal numbers of PerC and SP B cells injected into SCID recipients. To ask if there were B cells in the SP that could exert the allotype dominance effect, T cell–depleted PerC and SP were prepared from allotype-congenic donors, combined in various ratios, and transferred to SCID mice. The results (Table 8) demonstrate that a 9:1 SP–PerC B cell ratio at the time of transfer resulted in dominance of the IgM allotype of the SP donor. Both 3:1,
and as shown previously, 1:1 ratios of B cells resulted in dominance of the PerC allotype, although the 3:1 recipients have detectable levels of the SP allotype. SP B cell dominance is maintained in these recipients 40 wk after transfer (data not shown). These data indicate that some resident splenic B cells are capable of mediating allotype dominance over PerC B cells, yet the cells necessary for this dominance must be present in the spleen at a lower frequency than in the peritoneal cavity.

**Lack of Allotype Dominance in SCID Recipients of Large SP and Large PerC B Cells.** Previous experiments demonstrated that PerC B cells most rapidly restored B cell function in SCID mice (Riggs, J.E., et al., manuscript submitted for publication). However, transfer of large (Percoll gradient selected) SP B cells resulted in reconstitution kinetics identical to large PerC B cell recipients. Thus, co-transfer of equal numbers of density-fractionated, large PerC and SP B cells may lead to expression of both allotypes. The results in Table 9 show that this is the case; equal representation of both allotypes is detected in recipients of equal numbers of large PerC and SP B cells. These data confirm that the spleen harbors B cells capable of competing with PerC B cells and suggest that the SP B cells that mediate this dominance are enriched in a large B cell fraction.

**Sequential Transfer of PerC B Cells to SCID Mice Previously Reconstituted with SP B Cells Results in Co-expression of Both Allotypes.** The results presented above indicate that differences in rates of B cell growth or Ig secretion may explain PerC B cell dominance in PerC + SP chimeras. To test this hypothesis, sequential transfers were conducted in which SCID recipients of SP B cells received PerC B cells 5 wk later. This sequential transfer protocol should result in the establishment of B cells from the initial source, effectively negating growth rate differences, which permits an assessment of the ability of the second B cell source to compete with the established primary graft. Table 10 illustrates results for experiments in which SP and PerC B cells are compared for their ability to establish IgM synthesis in SCID mice previously engrafted with SP B cells. Prior transfer of SP B cells precluded subsequent reconstitution of IgM<sup>b</sup> production by SP<sup>b</sup> B cells, indicating again that B cells from SP can mediate allotype suppression. However, those same animals allowed establishment of IgM<sup>b</sup> synthesis by PerC<sup>b</sup> B cells. Equal representation of both allotypes was evident in such chimeras, suggesting that differences in B cell growth rates alone are not sufficient to explain PerC B cell dominance. The data in Table 10 also show that prior injection of PerC B cells of one allotype prevents expression of the IgM allotype of PerC B cells injected subsequently. These results suggest that the allotype dominance established by transfer of PerC B cells is more effective than that established by SP B cell transfer.

**Injection Route Influences B Cell-mediated Allotype Dominance Early after Transfer.** Our previous studies have shown that intraperitoneal transfer reduced the marked differences in IgM and B cell reconstitution rates noted after intravenous transfer of SP and PerC B cells (Riggs, J.E., et al., manuscript submitted for publication). In addition, the above results (e.g., Table 7) led to the question of whether the majority of the B cells mediating allotype dominance ended up in the spleen versus the PerC of SCID recipients. To address these issues, PerC<sup>b</sup> and SP<sup>b</sup> cells were co-transferred in all possible combinations of intravenous and intraperitoneal routes. The data in Table 11 demonstrate that transient allotype dominance mediated by the SP donor was evident in recipients of SP<sup>b</sup> intravenously and PerC<sup>b</sup> intraperitoneally at 2 wk after transfer, but not at 10 wk after transfer. All other combinations of injection routes led to allotype dominance by the PerC donor. These results suggest that the PerC of the recipient is not the only or primary site of residence of the B cells responsible for allotype suppression, and further suggest that the mechanism of suppression may require relatively close proximity of the B cells mediating suppression and those being suppressed.

| Table 8. Increasing the SP/PerC B Cell Ratio Results in SP Dominance |
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| SCID recipients<sup>1</sup> of SP<sup>b</sup> + PerC<sup>c</sup> at a ratio (SP/PerC)<sup>1</sup> of: | Serum IgM levels<sup>*</sup> |
| | Total IgM | IgM<sup>a</sup> | IgM<sup>b</sup> | µg/ml |
| 9:1<sup>1</sup> | 366 ± 22 | <5 | 185 ± 64 |
| 3:1 | 571 ± 7 | 573 ± 63 | 49 ± 9 |
| 1:1 | 735 ± 98 | 781 ± 150 | <5 |
| BALB/c control | 859 | 464 | <5 |
| C.B-17 control | 569 | <5 | STD |

<sup>*</sup> Serum IgM levels determined 2 wk after transfer by ELISA as outlined in Materials and Methods.
<sup>1</sup> Two recipients per group.
<sup>2</sup> A total of 10<sup>6</sup> cells were transferred to each recipient.
<sup>3</sup> Transfer of 10<sup>6</sup> Per C alone results in >100 µg/ml serum IgM by 2 wk.

| Table 9. Lack of Dominance in SCID Recipients of Large SP and Large PerC B Cells |
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| SCID recipients<sup>1</sup> of: | Serum IgM levels<sup>*</sup> |
| | Total IgM | IgM<sup>a</sup> | IgM<sup>b</sup> | µg/ml |
| Large SP<sup>b</sup> + Large PerC<sup>c</sup> | 946 ± 90 | 132 ± 7 | 198 ± 12 |
| BALB/c control | 528 | 339 | <5 |
| C.B-17 control | 986 | <5 | STD |

<sup>*</sup> Serum IgM levels determined 10 wk after transfer by ELISA.
<sup>1</sup> Three recipients/group.
<sup>2</sup> Large B cells isolated on Percoll gradients as previously described (11); 2 × 10<sup>6</sup> cells of each type were injected intravenously.
Table 10. *PerC or SP B Cell Engraftment in SCID Mice Previously Reconstituted with SP or PerC B Cells*

| Group                                      | Serum levels* | IG Levels |
|--------------------------------------------|---------------|-----------|
|                                            |               | Total IgM | IgM<sup>a</sup> | IgM<sup>b</sup> |
|                                            |               | µg/ml     | µg/ml          | µg/ml          |
| Primary SP<sup>a</sup> recipients<sup>1</sup> that receive: |               |           |               |               |
| 5 x 10<sup>6</sup> SP<sup>b</sup>            | 831           | 723       | <5             |               |
| 5 x 10<sup>5</sup> PerC<sup>b</sup>         | 489 ± 39      | 238 ± 55  | 140 ± 18       |               |
| Primary SP<sup>b</sup> recipients that receive: |               |           |               |               |
| 5 x 10<sup>6</sup> SP<sup>b</sup>            | 639           | <5        | 645            |               |
| 5 x 10<sup>5</sup> Per C<sup>b</sup>        | 736 ± 174     | 317 ± 157 | 326 ± 126      |               |
| Primary Per C<sup>b</sup> recipients<sup>1</sup> that receive: |               |           |               |               |
| 5 x 10<sup>6</sup> PerC<sup>b</sup>        | 1,117 ± 141   | 882 ± 276 | <5             |               |
| Primary PerC<sup>b</sup> recipients that receive: |               |           |               |               |
| 5 x 10<sup>6</sup> PerC<sup>b</sup>        | 977 ± 32      | <5        | 780 ± 10       |               |
| BALB/c control                             | 1,013         | 820       | <5             |               |
| C.B-17 control                             | 849           | <5        | STD            |               |

* Serum IgM levels determined as described in Materials and Methods.
† Primary recipients reconstituted 5 wk previously with 5 x 10<sup>6</sup> PerC; all primary recipients expressed only the donor allotype when tested the day of the second transfer.

**Discussion**

The transfer of mature B lymphocyte populations to SCID recipients allows a detailed analysis of their survival, function, and interaction in the absence of endogenous T and B lymphocytes. This is particularly true if B cells or their products influence the subsequent development or function of other B cells, as has recently been observed in transfers of CD5<sup>+</sup> B cells to neonatal animals (18), and is well documented by the data presented in this paper. The transfer of cells to SCID mice has some potential pitfalls as well; e.g.,

Table 11. *Injection Route Determines B Cell Dominance Early After Transfer*

| SCID recipients<sup>1</sup> of: | Serum IgM Levels* |
|--------------------------------|-------------------|
|                               | Time<sup>4</sup> | Total IgM | IgM<sup>a</sup> | IgM<sup>b</sup> |
|                               | µg/ml             | µg/ml     | µg/ml          | µg/ml          |
| Per C<sup>+</sup>(i.v.) + SP<sup>b</sup>(i.p.) | 2                 | 1,257 ± 201 | 1,277 ± 277 | <5             |
|                               | 10                | 926 ± 177  | 951 ± 285     | <5             |
| Per C<sup>+</sup>(i.p.) + SP<sup>b</sup>(i.v.) | 2                 | 422 ± 7    | 37 ± 3        | 215 ± 9        |
|                               | 10                | 642 ± 114  | 321 ± 125     | 48 ± 16        |
| Per C<sup>+</sup>(i.v.) + Sp<sup>b</sup>(i.v.) | 2                 | 1,171 ± 112| 956 ± 16      | 28 ± 1         |
|                               | 10                | 993 ± 403  | 1,015 ± 484   | <5             |
| Per C<sup>+</sup>(i.p.) + Sp<sup>b</sup>(i.p.) | 2                 | 272 ± 21   | 154 ± 25      | 22 ± 2         |
|                               | 10                | 586 ± 169  | 444 ± 210     | <5             |
| BALB/c control                | 908               | 830       | <5             |               |
| C.B-17 control                | 724               | <5        | STD            |               |

* Serum IgM levels determined by ELISA as outlined in Materials and Methods.
† Three recipients per group; each recipient receiving 5 x 10<sup>6</sup> each of SP<sup>b</sup> and Per C<sup>b</sup>.
§ Weeks after transfer.
selective survival of certain long-lived lymphoid subsets, and potential contributions by endogenous SCID NK cells (27) to the survival or function of donor lymphocytes. Despite these considerations, the results presented in this paper further our understanding of B lymphocyte function and longevity, and document potentially important regulatory interactions between B cells.

The main findings presented in this paper are that feedback inhibition of spontaneous and antigen-induced IgM secretion can be mediated by B cells that exist in higher frequency in the PerC as compared with the SP. In co-transfer of PerC and SP B cells from allogeneic donors, this feedback inhibition is manifest as a failure of the IgM allotype of the SP donor to be detected in the serum of SCID recipients. B cells from PerC can also exert feedback inhibition on allotype-mismatched PerC B cells transferred subsequently to the same SCID recipients. Feedback inhibition or B cell-mediated allotype dominance does not appear to require T lymphocytes. B cells capable of mediating allotype dominance are found both in the PerC and the spleen of normal BALB/c or C.B-17 donors (albeit at different frequencies), but are absent or substantially reduced in BALB.xid donors. Feedback inhibition probably affects primarily Ig synthesis or secretion, since significant numbers of B cells expressing the "suppressed" allotype can be detected in the spleens, but not in the peritoneal cavity, of allotype chimeric SCID mice. However, the possibility that a small but important fraction of B cells of the "suppressed" allotype are missing in the PerC + SP B cell chimeras cannot be excluded. Independent but simultaneous transfer of PerC cells to the peritoneal cavity (intraperitoneal injection) and SP cells to the spleen (intravenous injection) leads to a delayed onset of allotype dominance by the PerC cells, suggesting that feedback inhibition is not mediated by a secreted product such as Ig, and instead may require either cell contact or close proximity. Sera from SCID recipients showing allotype dominance do not transfer dominance to secondary recipients (data not shown), whereas secondary transfer of B cells does maintain allotype dominance (Table 7).

The B cells responsible for allotype dominance in our experiments share several characteristics with the CD5 + (and their CD5 - "sisters") B cell population (7, 8): (a) they are present in the spleen at a lower frequency than in the peritoneal cavity; (b) they are enriched in "large" B cell fractions; (c) they preferentially repopulate the peritoneal cavity; (d) they are long-lived; and (e) they appear to account for the majority of spontaneous IgM secretion, as has been noted previously (8). As noted in previous studies (Riggs, J.E., et al., manuscript submitted for publication), CD5 expression is not a reliable marker of this population following transfer to SCID recipients, but the general concept that a CD5-like subset with high representation in PerC is the primary mediator of feedback inhibition is attractive. Feedback inhibition mediated by CD5 + B cells has recently been reported by Lalor et al. (18) under different experimental conditions. An antibody IgM mAb was used to temporally block development of B cells in C.B-17 neonatal mice; subsequent injection of sorted Ly-1 + BALB/c B cells led to a long-term decrease in both endogenous Ly-1 + B cells and IgM of host origin. In contrast to our results, however, the reduction in cell numbers was more impressive than the reduction in IgM levels. Raveche et al. (28) also have reported that hyperdiploid CD5 + B cells from old NZB mice downregulate both endogenous B cell numbers and IgM secretion in unirradiated young (NZB × DBA/2) F1 recipients. A similar type of feedback inhibition has been postulated to be involved in multiple myeloma patients, although the mechanism of this inhibition remains to be elucidated (29). The finding that mice with the xid mutation have reduced numbers of CD5 B cells and accept long-term grafts of normal B cells without prior irradiation (8, 30) is also consistent with a role for CD5 B cells in feedback inhibition.

The allotype dominance seen in the co-transfer of PerC and SP B cells could have alternative explanations that do not involve feedback inhibition. The simultaneous transfer of two B cell populations to a SCID recipient creates a competition in many ways analogous to the competition of two species for a restricted habitat. The more rapid expansion of one population, even if their advantage is small, may lead to the extinction of the other population. Many observations in our experiments are consistent with a growth advantage of PerC B cells, but one experiment suggests that this explanation may be insufficient. The sequential transfer of SP cells followed by PerC cells led to a replacement of the originally engrafted SP B cells by PerC B cells, with a transition phase when both allotypes are expressed. In contrast to this result, the simultaneous transfer of a 9:1 excess of SP to PerC B cells led to a long-lived dominance of the SP B cell allotype. These apparently conflicting results could be explained if the act of B cell isolation and transfer lead to cell activation, such that delayed transfer of PerC B cells led to a competition between activated PerC B cells and less activated (established) B cells of SP origin. In contrast, both populations would be subject to activation in the simultaneous 9:1 SP/PerC transfer experiment. While the simplest explanation for our results is the competition of two populations for a limited B cell microenvironment, these experiments do raise the question of whether B cell allotype dominance reflects a direct competition, competition in addition to feedback inhibition, or both. Direct competition could reflect limitations in the availability of suitable microenvironments for B cell engraftment (e.g., the marginal zone of spleen where CD5 B cells appear to reside (8)) or competition for limited amounts of cytokines necessary for B cell differentiation. Feedback inhibition would appear from our experiments to involve either direct cell-to-cell contact or close proximity, and cannot be mediated by passive transfer of serum, so it is unlikely that Ig mediates the inhibition.

Our results do not allow resolution of the issue of independent conventional and CD5 + B cell lineages as first proposed by Hayakawa et al. (9), but they do call into question the interpretation of those experiments. If transfer of one population enriched for CD5 B cells can block development of Ig synthesis by another, then an experiment in which allotype-marked Ly-1 B cells are transferred to an irradiated recipient and result in dominant expression of that allotype (9) does
not necessarily coincide with the interpretation that Ly-1 B cells represent a separate lineage. Our data do not eliminate this possibility; we simply point out that it remains an open issue.

The important and unresolved question is the extent to which competition between B cell subsets influences the normal pattern of B cell development in intact mice. CD5 B cells are prominent in neonatal life (8), yet they obviously do not maintain that dominance in any site other than the PerC. Nonetheless, our results imply that the first wave of B cell differentiation could have profound effects on any B cells that develop subsequently, and that such effects are not necessarily mediated by idiotypic networks (31). If these effects occur at close range, then the order in which newly emerging B cells emigrate to distinct microenvironments (e.g., splenic marginal zone, follicular B cell zone, gut-associated lymphoid tissue, and PerC) could impact the adult B cell representation in those sites. Anatomical segregation of B cell subpopulations may be required for normal immune function. Finally, if feedback inhibition is operative between B cell subsets, what is the nature of the mediator of inhibition? The results reported in this paper raise many questions, but they also confirm that PerC B cells are an important focus for further study independent of the unresolved issues of how best to describe their phenotype and lineage relationship with conventional B cells.

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