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Induction of Tolerance in B-1 Cells for Bromelain-Treated Mouse Red Blood Cells by a Transient Presence of Anti-Idiotype Antibodies in Neonatal and Adult Mice

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Murine B-1 cells are thought to develop from Ig^+^-precursors early in ontogeny and to expand by self-renewal. To examine the early development of Ig^+^-precursors of B-1 cells for bromelain-treated mouse RBC, the transient presence of RidA, a rat anti-Id mAb for V<sub>H</sub>11/V<sub>k</sub>9-type anti-bromelain-treated mouse Abs, was produced in neonatal mice. The presence of RidA during days 0 to 10 of age resulted in an 80% reduction in peritoneal RidA-Id<sup>-</sup> B cells and B cells secreting RidA-Id<sup>+</sup> Ig after LPS stimulation in 8-wk-old mice. This suggests that most Ig^+^-precursors for adult RidA-Id<sup>+</sup> B cells already exist in 10-d-old mice. However, RidA injected into mice on day 10 had to persist for >4 days to result in a significant reduction in adult B cells. Similarly, although RidA injected into adult mice bound immediately to membrane Ig (mIg) of the peritoneal RidA-Id<sup>-</sup> B cells, a RidA persistence for >4 days was required to suppress LPS reactivity of peritoneal and splenic B cells. The binding of RidA to mIg preexisting on B cells has no apparent effect on the ability of neonatal B cells to expand clonally or on the ability of adult B cells to secrete RidA-Id<sup>+</sup> Ig after LPS stimulation. Both abilities evidently are suppressed by the accumulation of reaction between freshly expressed mIg and RidA. The Journal of Immunology, 1998, 160: 4796–4800.

In this study, RidA, a rat mAb specific for V<sub>H</sub>11/V<sub>k</sub>9-type Abs, was prepared and injected into neonatal mice. Injected RidA could be eliminated by neutralization with injection of a mouse RidA-Id<sup>-</sup> mAb; thus, a transient RidA presence could be produced. The presence of RidA during days 0 to 10 of age remarkably reduced adult peritoneal RidA-Id<sup>-</sup> B cells and B cells that secrete RidA-Id<sup>-</sup> Ig after LPS stimulation. The development of RidA-Id<sup>-</sup> B cells from Ig^+^-precursors evidently occurs mainly by day 10 of age. As to signals through mIg on neonatal B cells, we observed that the binding of RidA to the B cells could not immediately cause their inactivation. We also injected RidA into adult mice and assessed its effects on mature RidA-reactive B cells. RidA persistence for >4 days was required both for neonatal B cells to be able to expand clonally and for suppression of adult B cell ability to secrete RidA-Id<sup>-</sup> Ig after LPS stimulation. We suggest that the suppressive signals are delivered by the binding of RidA to freshly expressed mIg on RidA-Id<sup>-</sup> B cells.

Materials and Methods

Mice

Female F<sub>1</sub> mice (bred in our animal facility from female CBA/N and male BALB/c mice) were used.

Monoclonal antibodies

A clone secreting a V<sub>H</sub>11/V<sub>k</sub>9-type mAb (IgMs) designated MBMa1 and a clone secreting a V<sub>H</sub>12/V<sub>k</sub>4-type mAb (IgMs) designated MBMb1 were selected from hybridomas of LPS-activated BALB/c peritoneal cells and Sp2/0 cells. To select hybridomas, two previously prepared rabbit anti-Id Abs were used: RAI-7-7a-4a and RAI-9-9a-1a (15). F344/N rats were immunized with MBMa1 and MBMb1 in their foot pads, and popliteal lymph node cells from the immunized rats were fused with Sp2/0 cells. Two mAbs (both IgG2a/k isotype) were obtained: RidA, specific for V<sub>H</sub>11/V<sub>k</sub>9-type Ab; and RidB, specific for V<sub>H</sub>12/V<sub>k</sub>4-type Ab.

Production of a transient presence of RidA in neonatal and adult mice

In neonatal mice, 10 μg of RidA were injected i.p., and on various later days 100 μg of MBMa1 were injected i.p. to remove the RidA. In adult
mice, 0.2 mg of RidA was injected i.v., and on various later days 1 mg of MBMa1 was injected i.v. for RidA removal.

Flow cytometric analysis

Peritoneal cells were prepared in PBS containing 0.3% BSA, 1 mM EDTA, and 0.1% sodium azide. To detect Id+ B cells, cells were incubated with biotin-RidA or biotin-RidB for 30 min at 0°C. After washing, they were stained with phycoerythrin (PE)-streptavidin (Sigma, St. Louis, MO) and FITC-anti-mouse IgM (Caltag, San Francisco, CA). To detect B220+ B cells, cells were stained with PE-anti-B220 (Caltag) and FITC-anti-IgM. Stained cells were analyzed by FACStar (Becton Dickinson, Sunnyvale, CA).

Estimation of the number of RidA-Id+ B cells per peritoneal cavity

The percentage of RidA-Id+ B cells in peritoneal whole nucleated cells was estimated by a FACS profile of peritoneal cells stained with biotin-RidA (+PE-streptavidin) and FITC-anti-IgM. The number of RidA-Id+ B cells per peritoneal cavity was calculated as \[\text{no. of whole nucleated cells} \times \% \text{ of RidA-Id+ B cells}] \div 100.

Culture medium

GIT medium (Wako, Osaka, Japan) supplemented with FCS (1%) and 2-ME (0.05 mM) was used for culturing cells.

Estimation of the amount of LPS-induced RidA-Id+ Ig per peritoneal cavity and spleen

 Appropriately diluted peritoneal and splenic cells were cultured with feeder thymocytes (8 \times 10^5 cells/ml) from 3-wk-old CBA/N mice and LPS (50 \mu g/ml), as described previously (18). On day 3 of culture, the concentration of RidA+ Id+ Ig in the culture supernatant was estimated by ELISA, as described below. The amount of LPS-induced RidA-Id+ Ig per peritoneal cavity or spleen was calculated as \[\text{concentration of cultured peritoneal or splenic nucleated cells} \times \text{no. of peritoneal or splenic whole nucleated cells}\].

ELISA for RidA-Id+ Ig

The diluted culture supernatant was added to the wells of ELISA plates coated with RidA and incubated at room temperature for 2 h. Plates were washed and incubated with biotin-RidA for 1 h. Following washing, peroxidase-streptavidin (Sigma) was added, and the plates were incubated again for 1 h. Then, they were washed, and 3,3',5,5'-tetramethylbenzidine (TMBZ) (Wako) was added. One hour later, the reaction was stopped by adding 2 N H_2SO_4 solution and OD 450 nm was measured by an ELISA reader.

Results

Tolerance induced by a transient presence of RidA in neonatal mice

Peritoneal anti-BrMRBC B cells was detected previously using fluorescent phospholipid Ags. Mercolino et al. (19) reported that 5 to 15% of peritoneal lymphocytes bound fluorescent liposomes of phosphatidylcholines, and I (20) observed that 5 to 15% of peritoneal cells bound fluorescent low density lipoproteins from chicken egg yolk. Two rat anti-Id mAbs for anti-BrMRBC Abs, RidA and RidB, also bound to a substantial portion of peritoneal cells. PE fluorescence intensity of Id+ cells correlated well with their FITC fluorescence intensity, indicating that, in the present staining procedures, the binding of anti-Id mAb to membrane IgM (mIgM) rarely inhibited the binding of FITC-labeled anti-IgM Abs. Splenic RidA-Id+ and RidB-Id+ cells were below a significantly detectable level (0.2% of spleen cells). Eight-week-old mice that had received RidA at birth had few peritoneal RidA-Id+ cells (Fig. 1C), but they had as many peritoneal RidB-Id+ cells (Fig. 1D) as did normal mice. RidA injection into newborn mice caused a specific tolerance for RidA-Id+ B cells.

To examine whether the induction of tolerance requires the persistence of RidA in mice, RidA was injected at birth, and on various later days MBMa1 was injected to remove the RidA. When the mice were 8 wk old, peritoneal cells were collected, and the number of RidA-Id+ B cells and the amount of RidA-Id+ Ig produced by LPS-stimulated cells was determined (Fig. 2). The presence of RidA during days 0 to 6 of age hardly affected the number of adult RidA-Id+ B cells. However, a significant reduction of RidA-Id+ B cells was observed in mice exposed to RidA for 8 days. MBMa1 injection into RidA-untreated mice on day 8 (Fig. 2) or day 0 (data not shown) had no effect on RidA-Id+ B cells in 8-wk-old mice. The number of RidA-Id+ B cells correlated well with the amount of LPS-induced RidA-Id+ Ig, indicating that...
RidA-Id+ B cells detectable in RidA-treated mice were not functionally anergic cells.

The above results suggest that the differentiation of most Ig+ precursors for adult RidA-Id+ B cells from Ig- precursors occurs by day 10 of age. To assess whether persistent RidA is necessary for inactivation of newly differentiated RidA-reactive B cells, RidA was injected on days 2 to 8 of age and removed by MBMa1 on day 10. As shown in Table I, the presence of RidA during days 4 to 10 of age reduced adult RidA-Id+ B cells by 71%. However, the presence of RidA only during days 6 to 10 reduced the B cells by only 20%, and the presence of RidA only during days 8 to 10 caused little reduction. Probably, the RidA binding to newly differentiated RidA-reactive B cells does not immediately lead to inactivation of the B cells, and a persistent reaction between them for >4 days is necessary for the inactivation.

The results shown in Figure 2 also suggest that RidA-sensitive Ig+ precursors for most peritoneal RidA-Id+ B cells in 8-wk-old mice are already present at day 10 of age. To assess the sensitivity of those precursors to RidA, RidA was injected into 10-d-old mice, and on various later days MBMa1 was injected. As shown in Table 1, a 2-day contact with RidA hardly affected the number of peritoneal RidA-Id+ B cells at 8 wk of age, but a 4-day contact reduced them by >60%. This indicates that neonatal RidA-reactive B cells with the ability to expand clonally are not immediately inactivated by the binding of RidA. The inactivation requires the persistent reaction of RidA for >4 days.

Change of RidA-Id+ B cells by RidA injection into young adult mice

To investigate further the effects of RidA binding to mIgM on RidA-reactive B cells, RidA was injected into adult mice. Injected RidA bound to peritoneal RidA-Id+ B cells; the FACS profiles (Fig. 3) show that RidA-IdlowIgMlowB220+ cells in normal mice turned to RidA-IdhighIgMhighB220+ cells in mice given RidA 14 days before assay. Because RidA-Idlow cells were also IgMlow, RidA-bound mlgM was eliminated, probably by internalization. Those RidA-IdlowIgMlowB220+ cells were still detectable 3 h after RidA injection (data not shown). Peritoneal B-1 cells are reported to be long-lived cells with a low turnover rate (21, 22). The RidA-IdlowIgMlowB220+ cells appeared to survive for >2 wk.

The peritoneal RidA-IdlowIgMlow cells could reexpress their mIgM in the absence of RidA in vitro and in vivo. In a 24-h culture of peritoneal cells collected from mice given RidA 14 days before culturing, RidA-IdlowIgMlow cells disappeared, and RidA-IdhighIgMhigh cells appeared (Fig. 4A). In the peritoneal cavity of mice given MBMa1 14 days after RidA injection, RidA-IdlowIgMlow cells disappeared, and RidA-IdhighIgMhigh cells appeared 2 days after MBMa1 injection (Fig. 4, B and C). It also took 2 days after

<table>
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<th>Age (Day) of Mice Treated with</th>
<th>Peritoneal RidA-Id+ B Cells (% of Control)</th>
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<tr>
<td>MBMa1 (100 μg)</td>
<td>22 ± 3</td>
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<tr>
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<td>12</td>
<td>18 ± 2</td>
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<tr>
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<td>&lt;5</td>
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*At 8 wk of age, the number of RidA-Id+ B cells per peritoneal cavity was estimated. Percentage of control was calculated as [cell no. in treated mice + av. cell no. in normal mouse] × 100. In five normal 8-wk-old mice, the average number of RidA-Id+ B cells per peritoneal cavity was 26.9 × 106. Results are expressed as mean ± SD of five mice.*

FIGURE 3. Appearance of peritoneal RidA-IdlowIgMlowB220+ cells after RidA injection. Eight-week-old mice received i.v. injection of RidA (0.2 mg), and 14 days later their peritoneal cells were collected and stained with biotin-RidA (+PE-streptavidin) or PE-anti-B220 and FITC-anti-IgM. FACS profiles represent cells falling within the lymphocyte gate determined by forward and 90-degree light scatter.

FIGURE 4. Reexpression of mIgM on peritoneal B cells in RidA-treated mice in the absence of RidA in vitro and in vivo. Eight-week-old mice received i.v. injection of RidA (0.2 mg). To examine the reexpression in vitro, peritoneal cells were collected 14 days after RidA injection and cultured for 1 day in the absence of RidA. The cultured cells were stained with biotin-RidA (+PE-streptavidin) and FITC-anti-IgM (A). To examine the reexpression in vivo, MBMa1 (1 mg) was administered i.v. 14 days after RidA injection. At 1 day (B) and 2 days (C) after MBMa1 injection, peritoneal cells were collected and stained. The FACS profile shows cells falling with the lymphocyte gate determined by forward and 90-degree light scatter.
MBMa1 injection to reexpress mIgM on RidA-Id<sup>low</sup>IgM<sup>low</sup> cells in the peritoneal cavity of mice given MBMa1 1 day after RidA injection (data not shown). The ability of RidA-reacted cells to reexpress mIgM suggests that RidA binding to mIgM delivers no signal inhibiting mIgM synthesis and that the low mIgM expression on RidA-Id<sup>low</sup>IgM<sup>low</sup> B cells in the presence of RidA is due to the elimination of mIgM by its persistent reaction with RidA.

Several reports have shown that the ability of adult B cells to synthesize mIgM does not necessarily correlate with their ability to synthesize secretory IgM after LPS activation (18, 23–26). RidA was injected into adult mice, and at various later days LPS-induced secretion of RidA-Id<sup>+</sup> Ig by their peritoneal and splenic cells was assessed (Fig. 5). Two days after RidA injection, LPS reactivity of both peritoneal and splenic cells had changed only slightly. By 14 days, it had decreased to an undetectable level. These results indicate that 2 days after RidA injection, almost all peritoneal RidA-Id<sup>low</sup>IgM<sup>low</sup> cells have an LPS reactivity equal to that of normal cells, but by 14 days most lack LPS reactivity, although the B cells in both populations have the ability to synthesize mIgM. The excellent correlation between peritoneal and splenic cells suggests that the injected RidA equally affects peritoneal and splenic cells and probably all RidA-reactive B cells in mice.

Figure 5 shows that a 2-day contact of RidA-reactive B cells with RidA hardly changed their LPS reactivity, but an 8-day contact decreased it remarkably. To examine whether the presence of RidA for 6 days after the 2-day contact was necessary to induce the inactivation, MBMa1 was injected into adult mice 2 days after they received RidA, and 6 days later LPS reactivity of their peritoneal cells was assessed. The cells from the treated mice were able to secrete LPS-induced RidA-Id<sup>+</sup> Ig as much as those from normal mice (Table II), indicating that the cells exposed to RidA for 2 days did not progress toward the hyporesponsive state.

Peritoneal cells exposed to RidA for 8 days lost much of their LPS reactivity (Fig. 5); yet they could reexpress mIgM in the absence of RidA (Fig. 4). To examine whether these cells could also restore LPS reactivity in the absence of RidA, MBMa1 was injected into mice 8 days after administration of RidA, and 6 days later LPS reactivity of their peritoneal cells was assessed. The cells showed a severely suppressed LPS reactivity (Table II), suggesting that the hyporesponsive state is irreversible and that the RidA-Id<sup>high</sup>IgM<sup>high</sup> cells shown in Figure 4C were functionally anergic cells.

**Discussion**

The unusual age-related increase of anti-BrMRBC B cells has been noticed since Cunningham’s 1974 report (6). This increase is especially observable in the peritoneal cavity of normal mice. The number of peritoneal RidA-Id<sup>+</sup> B cells of an F<sub>1</sub> female mouse used in this current study was <10<sup>3</sup> at Day 10 of age and about 3 × 10<sup>5</sup> at 8 wk of age. Hayakawa et al. (2) suggest that an increase of B-1 cells in adult mice depends mainly on self-replenishment. Lalor et al. (4, 5) find that redundant injection of anti-IgM mAb into neonatal mice causes the deletion of B-1 cells, with the absence continuing after the disappearance of the Ab. They suggest that the development of B-1 cells from Ig<sup>−</sup> progenitors stops before weaning or the onset of puberty. As to the early development of anti-BrMRBC B cells, Arnold et al. (27) observe that liposome-binding cells are detectable in the spleen of VH<sub>12</sub>-transgenic mice at birth and increase 20-fold by day 6 of age. They assert that the increase is mainly due to clonal expansion. In this study, the presence of RidA during days 0 to 10 of age reduced the peritoneal RidA-Id<sup>+</sup> cells in 8-wk-old mice by ~80% (Fig. 2). I believe the Ig<sup>+</sup> precursors present in 10-d-old mice expand clonally to occupy the major part of peritoneal RidA-Id<sup>+</sup> B cells in 8-wk-old mice; i.e., the development of anti-BrMRBC B cells, and probably other B-1 cells, from Ig<sup>+</sup> progenitors probably occurs mainly by day 10 of age.

Some studies have shown that B-2 cells have an immature IgM<sup>+</sup> IgD<sup>−</sup> B cell stage during the development from Ig<sup>−</sup> progenitors to mature IgM<sup>+</sup> IgD<sup>−</sup> B cells and that immature B cells are inactivated much more easily in vitro by anti-IgM Abs or Ags than mature B cells are (28, 29). In this study, a transient contact with RidA during days 0 to 6 of age reduced the peritoneal RidA-Id<sup>+</sup> B cells in 8-wk-old mice by ~80% (Fig. 2). I believe the Ig<sup>+</sup> precursors present in 10-d-old mice expand clonally to occupy the major part of peritoneal RidA-Id<sup>+</sup> B cells in 8-wk-old mice; i.e., the development of anti-BrMRBC B cells, and probably other B-1 cells, from Ig<sup>−</sup> progenitors probably occurs mainly by day 10 of age.

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the binding of anti-Id Abs to preexisting mlg has no effect on LPS reactivity and that the binding to mlg freshly reexpressed on the B cells delivers the suppressive signal. This view is supported by the present in vivo finding. RidA-reactive B cells can reexpress mlgM (Fig. 4) so that a persistent binding of RidA to newly synthesized mlgM should occur in the presence of RidA in mice. The binding seems to deliver the negative signals. To accumulate enough negative signals to significantly suppress the ability of neonatal RidA-reactive B cells to expand clonally and on the ability of adult B cells seems to deliver the negative signals. To accumulate enough negative signals it is necessary to observe a persistent reaction between RidA and the B cells is necessary for >4 days.

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References