**Summary**

The normal migration route of B cells into follicular areas of spleen and lymph nodes is altered in the case of autoreactive cells that have bound self-antigen. To begin characterizing the molecular requirements for B cell migration into follicles, cells were treated with pertussis toxin (PTX), an inhibitor of signaling by many G protein-coupled chemokine receptors. Lymphocyte accumulation in the spleen is not inhibited by PTX and, therefore, the distribution of transferred cells was examined in this tissue. In contrast to untreated cells that localized predominantly in follicular areas within white pulp cords, PTX-treated B cells failed to enter white pulp areas altogether and accumulated in the splenic red pulp. T cells were also excluded from white pulp cords and in the case of both cell types, the adenosine diphosphate-ribosylating subunit of the toxin was required to block white pulp entry. These findings implicate a G protein-coupled receptor in lymphocyte migration into splenic white pulp cords. Exclusion of PTX-treated cells from all organized areas of secondary lymphoid tissues raises the possibility that the association observed between PTX treatment and predisposition to autoimmune disease results from inhibition of tolerance mechanisms that normally operate within secondary lymphoid tissues.

**Materials and Methods**

Preparation and Treatment of Lymphocytes. Splenocytes from anti-HEL Ig transgenic (MD4 line [21]) or Ly5-congenic C57BL/6 mice were isolated as previously described [21] and resuspended in RPMI, 2% calf serum, 10 mM Hepes, 2 mM glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. After washing, the cells were incubated in six-well plates (Costar Corp., Cambridge, MA) at $3 \times 10^7$ cells per ml with 100 ng/ml pertussis toxin, 100 ng/ml B oligomer (both from List Biological Laboratories, Campbell, CA)
or an equivalent volume of the carrier (PBS) for 2 h at 37°C in 5% CO₂. 5 × 10⁷ washed cells were injected into the tail vein of 6-12-wk-old C57BL/6 mice that were sex-matched with the donors.

Flow Cytometry: Three color FACS® analysis (Becton Dickinson & Co., Mountain View, CA) was performed on a FACSscan® with FACS desk software (Beckman Center Shared FACS Facility, Stanford, CA). Surface marker staining was as previously described (9, 21, 22) using the mAbs B220, RA3-6B2-PE (Caltag Laboratories, San Francisco, CA); IgD⁺, AMS9.1-fluorescein (FITC); Ly5⁺, A20-1.7-FITC; Thyl.2-biotin (Caltag) followed by streptavidin-cychrome (PharMingen, San Diego, CA). Hen egg lysozyme (HEL) binding was measured by incubating the cells with 200 ng/ml HEL (Sigma Immunochemicals, St. Louis, MO) followed by anti-HEL mAb HyHEL9-biotin and streptavidin-cychrome.

Immunohistochemistry and Immunofluorescence. 6- or 7-μm cryo-stat sections were fixed and stained for immunohistochemistry as previously described (9, 22). HEL was detected with HyHEL9 biotin, and Ly5⁺ was detected with AS20-biotin, in each case followed by avidin-conjugated alkaline phosphatase (Sigma). The marginal zone was outlined with MOMA-1 (kindly provided by G. Kraal, Free University of Amsterdam, The Netherlands) an mAb specific for metallophilic macrophages (3), followed by goat anti-rat-conjugated horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL). For fluorescence microscopy, cryostat sections were air dried and stained with HEL/HyHEL9-biotin/streptavidin-PE (Caltag); MOMA-1/goat anti-rat IgG-FITC (Caltag); anti-CD79a rabbit antisem/Donkey anti-rabbit IgG-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA). The rabbit anti-CD79a serum was raised against a glutathione-S-transferase fusion protein incorporating the cytoplasmic tail (Asp 181-Glu228) of CD79a. Two-color fluorescence was visualized and photographed through a dual filter system: excitation 480–500 nm and emission 517–532 nm (FITC) and excitation 545–578 nm and emission 585–620 nm (PE) (Chroma, Brattleboro, VT). Cells were enumerated in immunohistochemically stained sections using a grid in one eyepiece containing 56 × 56-μm squares. Cells falling within a minimum of 200 squares of red pulp and 200 squares of white pulp were counted per section, and the number of cells counted was divided by the number of squares to give a value per unit area.

Results

To determine whether or not πt B cell migration into follicular areas within the spleen was altered by PTX, Ig-transgenic mice expressing Ig specific for HEL were used as a source of B cells with a uniform high level of antigen receptor expression and a resting phenotype (9, 21). Furthermore, the high affinity HEL-binding Ig expressed on the transgenic B cells allowed them to be tracked specifically without an in vitro labeling step (9). Splenocytes from Ig-transgenic donor animals were incubated with or without PTX in vitro for 2 h before injection into the tail vein of nontransgenic, syngeneic recipients. The sham-treated (control) B cells were detected in the spleen and lymph nodes within 6 h of transfer and were present in similar numbers 19 h after transfer (Fig. 1). By contrast, PTX-treated B cells failed to accumulate in cervical and mesenteric lymph nodes 6 and 19 h after transfer (Fig. 1), whereas their frequency in the spleen was similar to controls. The frequency of PTX-treated B cells in the blood 19 h after transfer was fourfold greater than that of sham-treated cells. This lymphocytosis effect, first observed in Bordetella pertussis–infected patients (reviewed in reference 23), most likely reflects the reduced exit of B cells from blood into lymph nodes, Peyer’s patches, and gut (17, 20).

To examine the location of transferred B cells within recipient spleens, sections were stained to detect HEL-binding B cells and their distribution was compared with all B cells revealed by a polyclonal antibody specific for CD79a/Igβ (Fig. 2 A–C). Sham-treated HEL-binding B cells in control recipients were distributed evenly among the recipients’ endogenous B cells in the follicular areas of the spleen (Fig. 2 B) and lymph nodes (data not shown). In the spleen, small numbers of transferred cells were also detected in the marginal zone and red pulp as previously observed (24). By contrast, PTX-treated, HEL-binding B cells were not only excluded from lymph nodes, but they also could not be detected in follicles in the splenic white pulp cords (Fig. 2 C). Unlike autoreactive B cells that accumulate in the outer T cell zone (9), PTX-treated B cells appeared to be completely excluded from the white pulp. This was confirmed by double staining to detect metallophilic macrophages lining the marginal zone and HEL-binding cells (Fig. 2 D–F). The marginal zone staining outlined the white pulp cords and few or no PTX-treated cells were detected within the cords, whereas large

Figure 1. Pertussis toxin treatment does not inhibit B cell accumulation in the spleen. (A) Flow cytometry of spleen cells from mice 19 h after intravenous injection of sham-treated (control) or PTX-treated Ig-transgenic cells, or a control that did not receive transferred cells (no cells), stained to detect HEL binding and IgD⁻positive cells. The percentage of spleen cells in the HEL-positive, IgD⁻positive window are shown. (B) Frequency of transferred cells detected in spleen, lymph nodes (a pool of mesenteric and cervical), and blood 6 and 19 h after transfer, determined by flow cytometry as in A. Values from individual mice are shown by dots and means are shown by columns.

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numbers of cells were visible in the red pulp (Fig. 2F). Similar distributions of HEL-binding cells were observed in spleens taken from recipients 6 or 19 h after cell transfer (data not shown).

To exclude the possibility that the PTX-sensitive step of B cell entry into the splenic white pulp was restricted to transgenic B cells, the fate of nontransgenic B cells and of T cells was followed using cells from Ly5a-congenic C57BL/6 donors. In recipients of sham-treated cells, Ly5a-positive cells detected by immunohistochemical staining of spleen cryosections were concentrated within white pulp cords (Fig. 2G), and the presence of both transferred B and T cells was confirmed by double staining with antibodies specific for either B220 or CD4 and CD8 versus Ly5a (data not shown). By contrast, as observed for Ig-transgenic B cells, pretreatment with PTX completely blocked appearance of Ly5a-positive lymphocytes in splenic white pulp cords (Fig. 2I).

Failure to detect PTX-treated B or T cells in the white pulp did not reflect surface marker downregulation because sham-treated and PTX-treated B and T cells in the recipient spleens expressed equivalent levels Ly5a, as assessed by flow cytometry (Fig. 3A). Furthermore, the number of Ly5a-positive cells detectable by immunohistochemistry per unit area of red pulp was elevated 2.5-fold in the spleen of PTX-treated groups (Fig. 3C). Since the red pulp volume/white pulp volume ratio is ~2:1 (25-27) and, conversely, the ratio of sham-treated lymphocytes in these zones is 1:2.6 (Fig. 3C), a 2.5-fold increase in the number of PTX-treated cells in the red pulp, combined with a complete absence of cells in the white pulp, leads to the prediction that similar numbers of cells should be present in the spleen as a whole. This prediction was borne out by the flow cytometry results where the frequencies of sham-treated and PTX-treated Ly5a-positive cells in the spleen were equivalent (Fig. 3B).

PTX is composed of a membrane-permeabilizing complex, the B oligomer, and an ADP-ribosylating G protein-inactivating subunit (14). The B oligomer acts as a mitogen for B and T cells under some in vitro culture conditions (30) and could therefore influence lymphocyte migratory behavior in vivo. To exclude this possibility cells were treated with purified B oligomer and tested in the same manner as cells incubated with the intact holoenzyme. B oligomer treatment
Our results demonstrate that lymphocyte entry into the splenic white pulp is a PTX-sensitive step. This finding implicates G protein-coupled receptors in this process, and it reveals similarities between migration into splenic white pulp and into lymph nodes. Lymphocyte entry into lymph nodes and Peyer's patches involves transient rolling along the endothelial surface followed by switching to a strongly adherent state in which the cells bind and begin migrating between the endothelial cells. The transition from rolling to sticking involves signaling through a PTX-sensitive G protein (20).

Lymphocyte entry into the splenic parenchyma has been less well characterized than entry into lymph nodes. Blood flow through the mouse spleen is mostly of the open type since the arterioles do not connect directly to venules but terminate within the marginal zone or in the red pulp (4, 5). Open circulation is not observed in lymph nodes and this difference may account for the insensitivity to PTX treatment of lymphocyte accumulation in the spleen as a whole because entry into the red pulp probably does not require active migration across an endothelial layer. The increased frequency of PTX-treated lymphocytes in the red pulp is expected given both the failure of cells to move into the white pulp and their elevated frequency in the blood itself.

The route of lymphocyte traffic into splenic white pulp cords is unclear. From the observations of several early studies showing an initial accumulation of transferred cells in the marginal zone with a later appearance in the PALS, it was argued that cells migrated from the marginal zone directly into the PALS (6, 25, 29). Although this route may operate, recent studies support a more prominent role for lymphocyte trafficking into the white pulp from distal PALS present around arterioles that terminate in the red pulp. Lymphocytes were suggested to gain access to the distal PALS by actively crossing the endothelium immediately upstream of the arteriole terminus (30, 31). Since most forms of lymphocyte migration across endothelium are PTX sensitive, the failure of PTX-treated lymphocytes to enter white pulp cords supports the hypothesis that the major site of entry into the white pulp is by first crossing endothelium in the distal PALS. It is equally possible, however, that lymphocyte migration into white pulp cords from the marginal zone or after release from terminal arterioles into the red pulp is along a chemotactic gradient. By studying the distribution of PTX-treated lymphocytes in the spleen at early time points after transfer, it should be possible to determine whether entry into marginal zones, distal PALS, or both are reduced and, therefore, to define the main routes of lymphocyte trafficking into the white pulp.

Pertussis toxin promotes the development of autoimmune disease in animal models such as experimental allergic encephalomyelitis (EAE) and experimental allergic uveoretinitis (EAU) (reviewed in reference 25). Although the basis for this
effect of PTX on EAE and EAU is unknown, it is thought that its capacity to increase vascular permeability may be an explanation \((23, 32)\). The findings described here raise an alternative possibility, namely that PTX treatment may impede peripheral B and T cell tolerance mechanisms normally operating within lymph nodes or white pulp cords of the spleen. Elimination of autoreactive B cells by follicular exclusion \((9)\), for example, may require the B cells to enter white pulp cords or lymph nodes and be held up in the T cell zone.

Similarly, B cell presentation of autoantigens to T cells, which may be tolerogenic for the latter cells in certain circumstances, would be inefficient without concentration of B and T cells in white pulp cords and T zones. Interference with peripheral tolerance mechanisms in combination with increased vascular permeability in some peripheral sites may allow autoreactive T or B cells to become activated and enter nonlymphoid tissues.

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