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Role of the Polymeric Ig Receptor in Mucosal B Cell Homeostasis

Tania K. Uren,* Finn-Eirik Johansen,† Odilia L. C. Wijburg,* Frank Koentgen,‡ Per Brandtzaeg,† and Richard A. Strugnell2*

Secretory IgA (SIgA) is the most characteristic component of the mucosal immune system and has long been considered the major protective factor that prevents pathogens from invading hosts through the mucosae. Recent studies, however, have suggested that complete immunity against a range of mucosal bacterial and viral pathogens can be achieved in the absence of IgA. Therefore, to further dissect the role of SIgA, we generated mice deficient in the polymeric Ig receptor (pIgR−/− mice). As a result of an inability to transport dimeric IgA to the secretions, pIgR−/− mice are deficient in SIgA and accumulate circulating dimeric IgA, with serum levels 100-fold greater than those observed in normal mice. Examination of lamina propria mononuclear cells showed that pIgR−/− mice had ~3 times as many IgA-secreting cells as C57BL/6 mice. Further analysis showed that these cells displayed the differentiated IgA+ B220− phenotype and accounted for a 2-fold increase in the number of lamina propria blast cells in the pIgR−/− mice. Subsequent experiments showed that OVA-specific CD4+ T cell expansion following OVA feeding was not elevated in pIgR−/− mice. Furthermore, no differences in CD8+ T cell tolerance or induction of influenza virus-specific CD8+ T cells were detected in pIgR−/− mice compared with controls. Therefore, while SIgA is clearly involved in maintaining some parameters of mucosal homeostasis in the intestine, the mechanisms associated with its barrier function and the clinical consequences of its deficiency are yet to be identified. The Journal of Immunology, 2003, 170: 2531–2539.

The mucosal immune system is widely held to be responsible for the defense of the large expanse of mucous membranes that form a barrier between the external environment and the body’s interior. In particular, the gastrointestinal and respiratory tracts are exposed to a multitude of ingested or inhaled microorganisms and environmental and food Ags. Priming of T and B cells that preferentially home to the various mucosal effector sites is thought to occur mainly in organized mucosa-associated lymphoid tissue, such as Peyer’s patches (PP)† in the distal ileum. While a primary role of the mucosa-associated lymphoid tissue is considered to be the provision of protection against colonization and invasion by microorganisms, it must also function to prevent the induction of a vigorous and harmful response against innocuous environmental and food Ags. This functional dichotomy clearly delineates the effector arm of the mucosal immune system from the systemic counterpart. The many mechanisms likely to be involved in this regulation of productive local immunity and mucosally induced tolerance remain, to a large extent, unknown.

Secretory IgA (SIgA) is the most characteristic component of productive mucosal immunity. This is particularly true for the gastrointestinal tract, where the large numbers of Ig-producing immunocytes in the lamina propria (LP), which constitute ~80% of all Ig-producing immunocytes in the body, produce dimeric IgA (dIgA) in association with the J chain (1). After binding to the polymeric Ig receptor (pIgR), a 100-kDa glycoprotein expressed on the basolateral surface of secretory epithelial cells (2), dIgA Abs are actively transcytosed to the apical cell surface following receptor-mediated endocytosis of the entire ligand-receptor complex. The pIgR is then cleaved at the junction between the extracellular domain and the membrane-spanning region to release into the secretions covalently stabilized SIgA, which contains the disulfide-linked extracellular portion of the pIgR (known as bound secretory component) (3). This process results in the production of ~40 mg SIgA/kg of body weight in the human gut each day (4). Larger IgA polymers as well as pentameric IgM can be similarly transported by the pIgR to the secretions (5).

The importance of SIgA in immune exclusion (that is, the prevention of adherence or invasion of the mucosal epithelium by microorganisms and other foreign Ags) as a means of protecting the host against disease has generally been inferred from correlations between the induction and secretion of SIgA Abs, and resistance to infection. However, there is only limited experimental evidence that directly attributes mucosal protection to specific SIgA activity. Recently, three different gene-targeted mouse strains have been used to study the role of SIgA in immunity, namely IgA−/− mice (lacking IgA in serum and secretions) (6), J chain−/− mice (producing only monomeric IgA, which is not transported by the pIgR) (7), and pIgR−/− mice (which accumulate polymeric IgA in serum and cannot transport IgA to the secretions) (8). Results from studies using these gene-targeted mice have shown that mice producing only monomeric IgA (i.e., J...
or a systemic immune response. Thus, while we see the increase in IgA ASC and serum dIgA and the lack of SIgA did not indicate a mucosal immune response against luminally administered soluble Ags. In addition, a selective mechanism(s) responsible for this dysregulation was not identified as the role of pIgR in mucosal B cell homeostasis.

**Materials and Methods**

**Mice**

Generation of the targeting vector used to generate pIgR−/− mice has previously been described in detail (6). The internal exons 3 of the pIgR locus was targeted for disruption to limit any effects on adjacent loci. Briefly, the targeting vector contained a 1.9-kb upstream fragment, a neoβ cassette inserted at the PmlI site in exon 3, a 7.5-kb downstream fragment, and a herpes simplex virus thymidine kinase gene to enable negative selection of nonhomologous recombinants. To analyze the targeting vector or genomic DNA of pIgR−/− mice, Southern blots were conducted with embryonic stem cell DNA or tail biopsy DNA. The DNA was digested with HindIII, separated by agarose gel electrophoresis, and probed with a 1.4-kb genomic NcoI fragment adjacent to the targeting vector. Heterozygote pIgR+/− mice were generated in a pure C57BL/6 genetic background at the Walter and Eliza Hall Institute (WEHI, Parkville, Australia) gene targeting facility using C57BL/6 embryonic stem cells. The mice were then bred to the homozygous pIgR−/− state at University of Melbourne, Department of Microbiology and Immunology animal facility (Parkville, Australia). C57BL/6 mice were obtained from University of Melbourne, Department of Microbiology and Immunology animal facility. OT-II transgenic mice (in which all T cells recognize an MHC class II-restricted determinant of OVA) (22) and green fluorescent protein (GFP) transgenic mice (23) were obtained from Dr. F. Carbone (University of Melbourne, Parkville, Australia) and Dr. F. Alderuccio (Monash University, Prahran, Australia), respectively. These mice were mated to obtain offspring that were heterozygous for the OT-II and GFP transgenes. Consequently, all OVA–specific CD4+ T cells express GFP in these double-transgenic mice, enabling the use of flow cytometry to follow adoptively transferred cells in recipient mice. All mice were kept under conventional housing, with free access to gamma-irradiated mouse food and sterilized tap water. When necessary, mice were euthanized by either cervical dislocation or carbon dioxide asphyxiation. All mice were age- and sex-matched for each experiment. Animal experiments were approved by the University of Melbourne animal ethics committee.

**Immunizations**

Alum-precipitated (4-hydroxy-3-nitrophenyl) acetyl (NP) conjugated to keyhole limpet hemocyanin (KLH; NP-KLH conjugation ratio, 17:1) was prepared as described previously (24) and was supplied by Dr. D. Tarlinton (WEHI). To generate systemic Ab responses to NP, mice were immunized with 100 μg of NP1–KLH in 100 μl of PBS by i.p. injection. Mice were fed 5 mg of OVA in 200 μl PBS on days 0, 2, 4, and 7 by gastric lavage to induce oral tolerance to OVA (Sigma-Aldrich, St. Louis, MO). Before each feeding, 100 μl of 10% sodium bicarbonate was orally administered. Mice were systemically challenged by i.v. injection of irradiated C57BL/6 spleenocytes coated with 10 mg/ml OVA 7 days after the final OVA feeding as previously described (25). Influenza virus infection models were established by infecting mice with Men71, a reassorted influenza A virus bearing the hemagglutinin of A/Memphis/171 (H3) and neuraminidase of A/Bellamy/42 (N1; supplied by Dr. L. Brown, University of Melbourne). Mice were anesthetized with Penthrane (Abbott Laboratories, North Chicago, IL), and a dose of 10^4.5 PFU in 50 μl PBS was administered i.n.

**Preparation of fecal extracts**

Fecal extracts were prepared by a method modified from one previously described (26). Three to five fresh fecal pellets were collected, to which 1 ml of PBS containing 0.1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich) was added for every 0.1 g of feces. The tubes were vortexed vigorously and centrifuged at 13,000 x g for 15 min at 4°C. PMSF was added to the supernatant to a final concentration of 1 mM before storage at −20°C.

**ELISAs**

Mice were bled from the orbital sinus with heparinized capillary tubes, and the serum was separated from blood cells by centrifugation at 7000 x g for 5 min. Sera were stored at −20°C until use. Ag-specific Ab end-point titers and total Ab levels in serum and fecal samples were measured by a standard ELISA. Microtiter plates were coated overnight at 4°C with 5 μg/ml of unconjugated anti-mouse IgA, unconjugated anti-mouse IgG, or unconjugated anti-mouse IgM (Sigma-Aldrich) to determine the total amount of IgM that is constitutively and constantly transported to the lamina propria, abrogating tolerance and triggering an inflammatory immune response to what is usually a well-tolerated Ag. This suggests that intolerance to intestinal flora may trigger UC, and also that a deficiency in IgA, which is a predisposing factor in UC, might be one of the causes of this intolerance. This could arise in a manner similar to that described above for CD, whereby the lack of the intestinal SIgA results in bacterial proteins accessing the LP in a deregulated manner, triggering an abnormal and potentially harmful immune response. Other studies have shown that the enteric bacterial environment has a major impact on the severity of inflammatory bowel disease (IBD), such that experimental IBD is abrogated in germ-free animals (20, 21). Furthermore, the high levels of mucosal IgA Abs in UC are largely directed against intestinal bacteria (18). This suggests that intolerance to intestinal flora may trigger UC, and also that a deficiency in IgA, which is a predisposing factor in UC, might be one of the causes of this intolerance. This could arise in a manner similar to that described above for CD, whereby the lack of the intestinal SIgA results in bacterial proteins accessing the LP in a deregulated manner, triggering an abnormal and inflammatory immune response to what is usually a well-tolerated Ag.

The aim of this study was to generate mice deficient in SIgA by knocking out the pIgR, to then assess their ability to maintain normal mucosal homeostasis, and to evaluate their potential to induce mucosal and systemic immune responses against both pathogenic and nonpathogenic stimuli, with particular emphasis on intestinal induction and effector functions. As described previously in pIgR−/− mice which were generated on a different genetic background, loss of pIgR-mediated transport of dIgA in pIgR−/− mice resulted in intestinal SIgA deficiency, paralleled by increased levels of dIgA in serum. In contrast to other studies in pIgR−/− mice, we further analyzed the mucosal B cell compartment in these mice. Interestingly, there was a significant increase in the number of blast cells, in particular IgA Ab-secreting cells (ASC), in the intestinal LP of these mice compared with C57BL/6 mice. However, this could not be attributed to a significantly heightened immune response against luminally administered soluble Abs. In addition, the increase in IgA ASC and serum dIgA and the lack of SIgA did not have any effect on the induction of a respiratory mucosal immune response or a systemic immune response. Thus, while we could clearly demonstrate that a lack of SIgA secretion had a profound effect on some parameters of mucosal homeostasis, the precise mechanism(s) responsible for this dysregulation was not immediately apparent. Additional investigations are therefore required to determine not only the factors involved in this SIgA-dependent regulation of the mucosae, but also the clinical consequences, if any, of its disruption.
each respective isotype. Serial dilutions of sera or fecal extract were added to the plates in addition to serial dilutions of a known concentration of the appropriate purified IgA, IgG, or IgM hybridoma protein (ICN Biomedicals, Costa Mesa, CA) to generate a standard curve. Bound Ab was detected with HRP-conjugated anti-mouse IgA, IgG, or IgM (Southern Biotechnology Associates, Birmingham, AL) and was visualized using Immunopure o-phenylenediamine (Pierce, Rockford, IL) with H2O2 as a substrate.

For detection of NP-specific high and low affinity Abs, microtiter plates were coated with 20 μg/ml NP2-BSA (NP conjugated to BSA at a molar ratio of 2:1) or 20 μg/ml NP2-BSA (NP conjugated to BSA at a molar ratio of 17:1), respectively (supplied by Dr. D. Tarlinton, WEHI). Serial dilutions of sera were incubated on the microtiter plates, and bound IgG1 was detected with biotinylated rat anti-mouse IgG1 and HRP-conjugated streptavidin (both from Silenus, Boronia, Australia). Serum end-point titters were designated as the reciprocal of the dilution of specific Ab that gave an OD492 3 times above that obtained for preimmune serum.

Chromatographic analysis of mouse sera
Serum proteins were fractionated by size using a Sepharose 12 column (Amersham Pharmacia Biotech, Castle Hill, Australia). Mouse serum was first delipidated by incubation with 6 mg/ml of Aerosil 380 (Degussa, Teterboro, NJ) for 30 min at 4°C with gentle shaking. After centrifugation at 13,000 × g for 15 min, the supernatant was filtered to remove any remaining Aerosil. The delipidated serum (500 μl) was separated on a 125-μl column at a flow rate of 0.5 ml/min in sterile, filtered PBS, and 0.5-ml fractions were collected every minute. The fractions were stored at −20°C until further analyses.

Preparation of murine cell populations
Single-cell suspensions of spleens and lymph nodes were prepared by gentle sieving through a 40-gauge wire mesh, and erythrocytes were depleted with Tris/ammonium chloride. Lungs and PP were minced with a scalpel blade before incubation at 37°C with 200 μg/ml collagenase D (Roche, Mannheim, Germany) and 40 μg/ml DNase I (Roche). The enzyme-digested lungs and PP were sieved through a 40-gauge wire mesh to obtain single-cell suspensions and were depleted of erythrocytes with Tris/ammonium chloride. LP mononuclear cells (LPMC) were isolated from the combined small intestines of three mice for optimal cell recovery by a method adapted from one previously described (27). Briefly, fecal matter was flushed out and PP and mesentery were removed. The intestine was cut longitudinally before being further cut into 2- to 3-cm lengths and incubated in 5 mM EDTA (pH 7.2) to remove the epithelium. After washing, any remaining mesentery or PP were removed with the aid of a dissecting microscope and fine forceps. The intestinal segments were incubated with 100 μg/ml collagenase D, 1.5 μg/ml DNase I, and 1.5 μg/ml soybean trypsin inhibitor for 15 min, and the supernatant (containing mainly intraepithelial cells) was removed and discarded. The remaining intestinal segments were digested fully with three successive 1-h incubations with the collagenase mix. LPMC were separated from the remaining cell populations and cellular debris by an initial gradient separation with Lympholyte-M (Cedarlane Laboratories, Ontario, Canada), followed by a 40/70% discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient separation.

ELISPOT assay
The number of ASC present in tissues was determined with the ELISPOT technique (28). Microtiter plates were coated overnight at 4°C with 5 μg/ml of unconjugated anti-mouse IgA, unconjugated anti-mouse IgG, or unconjugated anti-mouse IgM (Sigma-Aldrich). Serial dilutions of the relevant single-cell preparations were added to the plates and incubated overnight. Isotype-specific ASC were detected with HRP-conjugated anti-mouse IgA, IgG, or IgM (Southern Biotechnology Associates) and visualized with aminoethylcarbazole (Sigma-Aldrich). The results of the ELISPOT assays are expressed as the number of ASC per 106 cells.

OVA-specific CD4+ lymphocyte activation
Mesenteric, mediastinal, axillary, caudal, popliteal, and inguinal lymph nodes were removed from OT-II × GFP transgenic mice, and single-cell suspensions were prepared. One day after i.v. injection of 2 × 106 OT-II × GFP transgenic cells into C57BL/6 and plgR−/− mice, the mice were fed either 20 mg of OVA in 200 μl of PBS or PBS alone. The mesenteric lymph nodes (MLN), PP, and spleens were removed from all mice 3 days later, and single-cell suspensions were prepared and stained with a CyChrome-conjugated anti-CD4 mAb (clone H129.19; BD PharMingen, San Diego, CA). The samples were then analyzed by flow cytometry to determine the number of CD4+ GFP+ cells present in the cell suspensions, which represented the number of OVA-specific CD4+ cells in the respective organs.

OVA-specific in vivo CTL assay
Seven days after priming with OVA, in vivo elimination of target cells was measured in mice using CFSE-labeled and peptide-pulsed spleen cells as target cells (modified from a method previously described (29)). Briefly, spleen cells were resuspended at 1 × 106 cells/ml and incubated with 1 μg/ml OVA2533-2540 peptide for 1 h at 37°C. The cells were washed, resuspended at 1 × 106 cells/ml, and incubated for 15 min at 37°C following the addition of 2 μl of 5 mM CFSE (CFSEnew). Control target cells that had not been pulsed with peptide were labeled with a lower concentration of CFSE by the addition of 2 μl of 0.5 mM CFSE (CFSEOLD). The two populations of target cells were thoroughly washed, resuspended and mixed together. Mice were i.v. injected with 200 μl of cells (2 × 107) and bled 24 h later to isolate PBMCs. The differential elimination of the target cell populations was analyzed by flow cytometry. The percent specific lysis was defined as (1 − [r (primed mice/r (naive mice)]) × 100], where r is the number of CFSEnew cells per number of CFSEOLD cells.

Influenza virus-specific in vitro CTL assay
Influenza A virus-specific cytolytic activity in freshly prepared lung cell suspensions and in vitro restimulated spleen and mediastinal lymph node cell cultures, was detected with a standard 51Cr release assay 7 days after mice were infected with Mem71. Cells from the spleen and mediastinal lymph nodes (3 × 106 cells) were cultured in 10 ml in 25-cm2 tissue culture flasks with 3 × 106 Mem71-infected stimulator cells for 5 days at 37°C in 5% CO2. Influenza A virus-infected stimulator cells were prepared by incubating 107 spleen cells with 100 μg PFU of infectious influenza Mem71 virus for 30 min at 37°C in serum-free RPMI culture medium.

After 5 days viable cells were harvested, counted, and used as effector cells in a cytotoxicity assay, in which 51Cr-labeled EL4 cells were used as target cells. In other experiments freshly prepared lung cells obtained from Mem71-infected mice were used as effector cells. Before labeling with Na251CrO4 (Amersham International, Sydney, Australia; 100 μCi/106 cells), EL4 cells were infected with 100 μCi/106 PFU Mem71 by incubation for 1 h at 37°C. Noninfected control cells were also incubated at 1 h at 37°C. Serial dilutions of effector cells were incubated with either 107 noninfected or Mem71-infected EL4 cells at 37°C in 5% CO2, and the supernatant was assayed for 51Cr after 5 h. Results are presented as the percent specific lysis defined as (experimental lysis − spontaneous lysis)/(total detergent lysis − spontaneous lysis), in which spontaneous lysis represents 51Cr present in supernatants from target cells alone, and detergent lysis represent 51Cr in supernatants from target cells incubated with 100 μl of 1% Triton X-100. Maximum spontaneous release values were always <10% of total lysis.

Flow cytometry
Single-cell suspensions were surface-stained according to standard protocols with the following Ab reagents: PE-conjugated anti-CD45R/B220 (RA3-6B2) and CyChrome-conjugated anti-CD4 (H129.19). After permeabilization with 0.5% saponin (Sigma-Aldrich) for 10 min at 37°C, LPMC were intracellularly stained for 40 min on ice with FITC-conjugated anti-IgA (C10-3) in 0.5% saponin. All Ab reagents were obtained from BD PharMingen. All samples were acquired and analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA) using the CellQuest software program. Collected events were gated for total lymphocytes defined by forward and side scatter characteristics.

Statistical analysis
Two-tailed Student’s t test was used for statistical analysis of the results. Differences were considered significant when p < 0.05.

Results
plgR−/− mice are deficient in intestinal IgA, but have elevated serum dIgA levels
The plgR is the sole known receptor for active transcytosis of polymeric Abs into the intestinal lumen. Previous reports of independently generated plgR−/− mice demonstrated that most dIgA produced by plasma cells (PC) in the LP of these mice is not transported to the intestinal lumen (8, 30). The total amount of IgA in fecal extracts was measured by ELISA to confirm that this was also true for the plgR−/− mice derived on a pure C57BL/6 background. The intestinal IgA levels in plgR−/− mice were markedly

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FIGURE 1. Comparison of mucosal and systemic Ig levels in C57BL/6 and plgR⁻/⁻ mice. Fecal extracts (A and B) and serum samples (C–E) were analyzed by ELISA for total concentrations of IgA (A and C), IgG (B and D) and IgM (E). The data presented were obtained from groups of five C57BL/6 (●) and plgR⁻/⁻ (○) mice. The mean value for each group is represented by a solid line. Statistical analysis was performed using Student’s t test (*, p < 0.05; **, p < 0.001).

The number of IgA ASC in the intestinal LP of plgR⁻/⁻ mice is elevated compared with that in C57BL/6 mice

The grossly elevated levels of dIgA in the serum of plgR⁻/⁻ mice could be solely attributable to the accumulation of locally produced IgA in the LP that was not transported to the intestinal lumen. If this were true, the total amount of IgA generated by ASC or the absolute number of IgA ASC in plgR⁻/⁻ mice would be the same as that in C57BL/6 mice. To investigate the validity of this hypothesis, IgA ELISPOT assays were performed with LPMC from C57BL/6 and plgR⁻/⁻ mice. The number of cells secreting IgA in plgR⁻/⁻ mice was consistently higher than the number of IgA ASC in C57BL/6 mice (Fig. 3A). The results of these experiments showed that between 7.1 × 10⁴ and 1.1 × 10⁵ IgA ASC/10⁶ cells were detected in the intestinal LP of plgR⁻/⁻ mice, a 3-fold increase over the number of IgA ASC in C57BL/6 mice (2.5 × 10⁴ and 3.5 × 10⁴ IgA ASC/10⁶ cells).

The numbers of IgA ASC in PP, MLN, and spleen were examined to determine whether this disparity extended beyond the intestinal LP. Five separate experiments were performed in which cells from these organs were isolated from C57BL/6 and plgR⁻/⁻ mice. ELISPOT assays were performed on pooled cells in two of the five experiments and on individual cell populations in the other experiments. The results are expressed as the ratio of plgR⁻/⁻ IgA ASC to C57BL/6 IgA ASC for each experiment (Fig. 3B). In contrast to the LP, similar numbers of IgA ASC were found in PP of C57BL/6 and plgR⁻/⁻ mice. Although one experiment indicated that plgR⁻/⁻ mice had 14 times more IgA ASC in MLN than C57BL/6, the other four experiments demonstrated an ~2-fold increase. Interestingly, there were elevated numbers of IgA ASC in the spleens of plgR⁻/⁻ mice compared with C57BL/6 mice (between 2.5- and 7-fold).

Blast cell populations are more abundant in the intestinal LP of plgR⁻/⁻ mice

Intracellular IgA staining and flow cytometric analysis were used to confirm the observed increase in LP IgA ASC in plgR⁻/⁻ mice (Fig. 4). The light scatter profiles revealed a significant difference in the ratio of small lymphocytes (designated R1) to blast cells (designated R2) when comparing C57BL/6/ (●:1) with plgR⁻/⁻ mice (●:2). Surface B220-PE staining and intracellular IgA-FITC staining indicated that while the blast population of both groups of mice contained ≥95% of the total number of LP IgA⁺ cells (data not shown), there were ~3.5-fold as many IgA⁺ B220⁻...
ppIgR/H11002 Mononuclear cells isolated from the LP of three C57BL/6 (f) mice were pooled, and the number of IgA ASC was determined by ELISPOT assay. The results presented show data obtained from three independent experiments. A, Lymphocytes were isolated from LP, PP, MLN, and spleen of C57BL/6 and ppIgR/H11002 mice, and the number of IgA ASC was determined by ELISPOT assay. The results are expressed as the ratio of the number of IgA ASC detected in ppIgR/H11002 mice to C57BL/6 mice. The bars in the histogram presented here represent the mean ratio detected in three to five independent experiments; the individual points represent individual experiments.

ASC in the blast population of ppIgR /− mice (15.1%) compared with C57BL/6 mice (4.3%). When expressed as a percentage of the total number of events, the percentage of IgA-containing cells in ppIgR /− mice was 2.7%, compared with 0.4% in C57BL/6 mice. Interestingly, the percentage of IgA− B220+ cells in the blast population of C57BL/6 mice was 3 times that in ppIgR /− mice. However, when expressed as a percentage of the total number of events, the number of IgA− B220+ cells was approximately the same in both groups of mice.

**Activation of OVA-specific cells following OVA feeding is equivalent in ppIgR /− and wild-type mice**

A consequence of S IgA deficiency in ppIgR /− mice may be increased Ag penetration through the intestinal epithelium, resulting in heightened immune induction, possibly explaining the increased number of IgA-secreting blast cells present in the intestinal LP. To test this hypothesis, the expansion of OVA-specific CD4+ T cells following oral administration of 20 mg of OVA was measured in mice that had been adoptively transferred with lymph node cells from OT-II × GFP double-transgenic mice. The relative expansion of OVA-specific CD4+ T cells in the spleens, MLN, and PP was determined by measuring the number of CD4+ GFP+ cells in these organs by flow cytometry (Fig. 5A). Significant Ag-specific expansion of OT-II/GFP cells was detected in the MLN and PP of both C57BL/6 and ppIgR /− mice fed OVA compared with mice that received only PBS (Fig. 5B). The OT-II/GFP lymphocyte population increased 2.2-fold in the MLN of C57BL/6 mice (from FIGURE 3. ppIgR−/− mice have increased numbers of IgA ASC. A, Mononuclear cells isolated from the LP of three C57BL/6 (●) or three ppIgR−/− mice (●) were pooled, and the number of IgA ASC was determined by ELISPOT assay. The results presented show data obtained from three independent experiments. B, Lymphocytes were isolated from LP, PP, MLN, and spleen of C57BL/6 and ppIgR−/− mice, and the number of IgA ASC was determined by ELISPOT assay. The results are expressed as the ratio of the number of IgA ASC detected in ppIgR−/− mice to C57BL/6 mice. The bars in the histogram presented here represent the mean ratio detected in three to five independent experiments; the individual points represent individual experiments.

FIGURE 4. Flow cytometric analysis of intestinal LP cells in C57BL/6 and ppIgR−/− mice. Mononuclear cells obtained from intestinal LP were stained with B220-PE (surface) and IgA-FITC (intracellular). Flow cytometry was used to analyze the relative populations of small lymphocytes (R1) and blasts (R2) and to analyze the expression of IgA and B220. The numbers represent the percentages of gated cells in the quadrants, and those in brackets represent these cells as a percentage of the total cell number. Shown are the results of one representative of three independently performed experiments.

**FIGURE 5.** Ag-specific expansion of CD4+ T cells following oral administration of protein Ag is comparable in C57BL/6 and ppIgR−/− mice. Lymph node cells (2 × 10^6) obtained from OT-II × GFP transgenic mice were adoptively transferred into C57BL/6 or ppIgR−/− mice, which were fed either OVA or PBS 1 day later. Three days after feeding, lymphocytes were isolated from MLN, PP, and spleen and were surface-stained with CD4-APC. Flow cytometry was used to determine the number of CD4+ GFP+ cells in the gated lymphocyte population of each sample. A. Representative dot plot of PP lymphocytes indicating the CD4+ GFP+ cells in the gated lymphocyte population of each sample. B. The percentage of OVA-specific CD4+ T lymphocytes which were detected in the spleen, MLN and PP of C57BL/6 mice fed either PBS (●), or OVA (●), and of ppIgR−/− mice fed either PBS (●) or OVA (●). Shown are the mean ± SD of groups of five mice.
numbers of circulating CFSEhigh, peptide-pulsed target cells, indi-

temically immunized with OVA-coated spleen cells had lower

Oral tolerance can be induced to an equivalent degree in

cytometry. A, Representative profiles from each group. B, The specific elimination of peptide-pulsed target cells was
calculated and expressed as the percent specific lysis for each group. Shown are the mean ± SD of C57BL/6 mice (■) and plgR−/− mice (■) of five mice per group.

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oral Ag administration could not confirm the hypothesis that the elevated numbers of LP IgA-secreting blasts in plgR−/− mice was a consequence of increased Ag uptake. An alternative approach to investigating this possibility was to examine the in-
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We next examined whether the SIgA deficiency and elevated se-
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determine whether the abundance of IgA in the serum of plgR−/− mice could interfere with the development of a normal Ab re-
sponse against systemically administered Ag, the induction and

FIGURE 6. Lack of a functional plgR does not affect the induction of oral tolerance. Groups of five C57BL/6 and plgR−/− mice received either four doses of 5 mg of OVA in PBS or PBS alone by gastric lavage and were subsequently immunized i.v. with OVA-coated spleen cells. The presence of OVA-specific CTL responses in these mice was assayed using an in vivo CTL assay. As a negative control, naive mice were used. Twenty-
hour after the injection of target cells, the mice were bled, and the relative depletion of each population of target cells from the blood lym-
phocyes was analyzed by flow cytometry. A, Representative profiles from each group. B, The specific elimination of peptide-pulsed target cells was
calculated and expressed as the percent specific lysis for each group. Shown are the mean ± SD of C57BL/6 mice (■) and plgR−/− mice (■) of five mice per group.

FIGURE 7. Systemic immunization induces normal affinity maturation of anti-NP IgG1 serum Abs in plgR−/− mice. C57BL/6 (■) and plgR−/− mice (□) were immunized i.p. with 100 μg of NP17-KLH, and serum was
collected at weekly intervals. Serum titers of total NP-specific IgG1 (A) and high affinity anti-NP IgG1 (B) were determined by ELISA. No NP-specific Abs were detected in naive mice.
true for both C57BL/6 and pIgR<sup>−/−</sup> mice, which indicated that the induction and maturation of a systemic Ab response were not affected in pIgR<sup>−/−</sup> mice.

The ability of pIgR<sup>−/−</sup> mice to generate a mucosal anti-influenza A virus CTL response in the respiratory tract was next investigated. The lungs, spleen, and MLN were removed from C57BL/6 and pIgR<sup>−/−</sup> mice 7 days after an i.n. infection with 10<sup>6.5</sup> PFU Mem71. Cells isolated from the lung were assayed immediately for the presence of virus-specific CTL using a 51Cr release assay. The ability of lung-derived effector cells to lyse influenza virus-infected target cells was comparable between C57BL/6 and pIgR<sup>−/−</sup> mice and resulted in a 14 ± 5 and 11 ± 5% specific lysis, respectively, at an E:T cell ratio of 100:1 (Fig. 8A). In addition, with decreasing E:T cell ratios, the percent specific lysis decreased in an equivalent manner in both strains of mice.

Cells isolated from the spleens and mediastinal lymph nodes of the infected mice were restimulated for 5 days in vitro in the presence of irradiated, Mem71-infected spleen cells, after which the level of virus-specific cytolysis was determined. A high degree (56 ± 3%) of specific lysis of influenza virus-infected target cells was measured in spleen cell suspensions from pIgR<sup>−/−</sup> mice at an E:T cell ratio of 100:1, which was equivalent to the 52 ± 4% detected in spleen cultures from C57BL/6 mice (Fig. 8B). Similar results were obtained with mediastinal lymph node cells (Fig. 8C). These results indicated that the pIgR<sup>−/−</sup> phenotype did not affect the induction of virus-specific CTL following i.n. infection with influenza A virus.

**Discussion**

Since the realization several decades ago that external secretions contain a striking predominance of IgA, in contrast to the prevailing IgG in blood, SlgA has been considered critical to the defense of the mucosal surfaces against infection. Several murine models have been developed to study the importance of the IgA system in protection against mucosal infections. Firstly, IgA<sup>−/−</sup> mice have been generated, which have no IgA in serum or secretions and therefore lack all IgA-associated immune effector functions (6). J chain<sup>−/−</sup> mice, on the other hand, have only circulating monoclonal IgA, which cannot be transported to the secretions by the pIgR (7). Finally, pIgR<sup>−/−</sup> mice are unable to secrete dIgA into the mucosal lumen and, in contrast to J chain<sup>−/−</sup> mice, accumulate polymeric IgA in serum (Ref. 8 and this study). Recent studies, however, have suggested that adequate immunity against many pathogens can be achieved through vaccination in IgA<sup>−/−</sup> mice (9–13). While these reports do not exclude the role of SlgA in infection immunity, they challenge the absolute nature of this theory and raise the question of exactly what the major role of SlgA is, particularly in the gastrointestinal tract where SlgA is constitutively secreted in large amounts.

In this study mice lacking a functional plgR were used to investigate the effect that an absence of dimeric IgA secretion into the mucosal lumen has on several aspects of mucosal homeostasis and immune induction. The plgR<sup>−/−</sup> mice have been generated previously, and consistent with these reports, we showed that pIgR<sup>−/−</sup> mice had almost undetectable levels of intestinal IgA in conjunction with elevated concentrations of dimeric serum IgA (8, 30). In addition to those previous observations, however, we observed a significantly larger number of IgA-secreting intestinal LP blast cells in the pIgR<sup>−/−</sup> mice compared with their wild-type counterparts. While these results indicated a disturbance in the mucosal homeostasis of pIgR<sup>−/−</sup> mice, this could not be attributed to any apparent alteration in Ag sampling across the epithelium and subsequent Ag-specific activation of T cells. Moreover, there was no detectable difference in any other investigated parameter of immune induction.

Previous studies describing the generation of pIgR<sup>−/−</sup> mice attributed the elevated serum IgA concentration to a lack of transport of dIgA to external secretions and bile by the pIgR (8, 30). The plgR-mediated hepatic IgA pump operating in rodents may be most important in this respect (31). While such a lack of external IgA transport remains the most probable factor responsible for the accumulation of dIgA in the serum of plgR<sup>−/−</sup> mice, our observations indicate that an overall increase in the number of intestinal LP cells that secrete dIgA also contributes to the elevated serum IgA levels in plgR<sup>−/−</sup> mice. It is likely that the increased number of IgA ASC in plgR<sup>−/−</sup> mice detected by ELISPOT assays in both MLN and spleen are mucosal ASC destined for accumulation in the LP. A 3-fold increase in LP IgA ASC in plgR<sup>−/−</sup> mice equates with an increase of >10<sup>9</sup> cells that, assuming there is circulation of even a small proportion of these cells, would result in the large relative increase in IgA ASC observed in the spleens of pIgR<sup>−/−</sup> mice.

The fact that there was no apparent difference between the number of IgA ASC in the LP of C57BL/6 mice and pIgR<sup>−/−</sup> mice may reflect the fact that the B cells homing to this site are naive. After priming, they migrate rapidly from the PP through MLN and via peripheral blood to the LP (32). As such, the B cells in the PP that are secreting IgA are mostly in the process of migration and do not accumulate at this inducive site. Alternatively, the population of B cells responsible for the elevated numbers of IgA ASC in the LP of pIgR<sup>−/−</sup> mice could be B-1 cells derived from the peritoneal cavity, which normally constitute approximately half the IgA PC in the LP of mice (33, 34). Significant, B-1 cells are virtually absent from the PP (35). No dependency on T cells or germinal centers has been revealed for the generation of SlgA Abs against commensal intestinal bacteria by this LP PC population. Nevertheless, such Ab production is clearly an instructive (Ag-driven) process because it depends on bacterial colonization of the gut (36).

Further analysis of intestinal LP PC by flow cytometry revealed that IgA ASC in both plgR<sup>−/−</sup> and C57BL/6 mice were predominantly IgA<sup>−</sup>B220<sup>−</sup> plasma blasts. The increased number of IgA<sup>+</sup>B220<sup>−</sup> cells in plgR<sup>−/−</sup> mice coincided with a decreased percentage of IgA<sup>−</sup>B220<sup>−</sup> cells within the blast cell population relative to that in C57BL/6 mice. Interestingly however, the IgA<sup>−</sup>B220<sup>+</sup> population was approximately equivalent in both strains of mice when expressed as a percentage of the total number of cells in the LPMC preparation. Recent studies have indicated that IgA<sup>+</sup>B220<sup>+</sup> LP blast cells are morphologically similar to PC (37), while
IgA⁺ B220⁺ LP cells have just completed class switch recombination and somatic mutation (38). Thus, it appears that while there is approximately the same number of undifferentiated precursor LP IgA⁻ B220⁺ cells in C57BL/6 and plgR⁻/− mice, more of these cells have been stimulated to undergo class switch recombination and differentiate into IgA⁺ B220⁺ PC in plgR⁻/− mice.

These observations lead to the hypothesis that one of the consequences of a lack of transepithelial transport of SlgA was a heightened level of immune induction due to a decreased mucosal barrier function. Exogenous Ags could thus induce proliferation and/or terminal differentiation of IgA ASC in several ways. Increased Ag sampling by M cells would presumably induce an immune response involving conventional B-2 cells in the PP. These activated B cells migrate to the MLN, then follow the efferent lymph into peripheral blood before homing to the intestinal LP (32). Alternatively, in plgR⁻/− mice, luminal Ags may breach the mucosal lining over its entire expanse. Columnar epithelial cells, which express classical and nonclassical MHC molecules, probably have a role in Ag sampling and presentation to mucosal T cells (39), thereby perhaps contributing to the elevated numbers of LP IgA ASC in plgR⁻/− mice.

Furthermore, a small degree of epithelial irritation and mucosal inflammation could arise from an increased association between luminal Ags and enterocytes. This might, in turn, result in decreased epithelial integrity, allowing Ags enhanced access to the mucosal tissues and the systemic immune system. The slightly increased fecal IgG in plgR⁻/− mice observed in this study and in both previously published reports of plgR⁻/− mice (8, 30) suggested breakdown of epithelial integrity and subsequent leakage of proteins to the intestinal lumen. An immune response with more systemic characteristics, such as IgG Ab production, may be induced as a result of Ag access to both the mucosal and systemic immune system. Increased anti–Escherichia coli IgG levels were detected in the sera of plgR⁻/− mice in a separate study (8), indicating that there was enhanced contact between enteric bacteria and the systemic immune system in these mice. Despite these indications of increased immune activation in plgR⁻/− mice, attempts to demonstrate this directly with Ag-specific CD4⁺ T cells proved unsuccessful. More discrete systems may be needed to address this issue, including various Ags at a range of doses. Ags from the commensal intestinal flora, rather than other antigenic matter administered through the gut intermittently and at high doses, might be responsible for the altered IgA ASC phenotype in plgR⁻/− mice, which could reflect stimulation of B-1 cells, as discussed above. IL-15 is secreted by irritated intestinal epithelial cells and has been shown to selectively regulate the differentiation of B-1 cells into IgA ASC (40–42).

Although the precise mechanisms could not be established in this study, our results show that SlgA has a role in maintaining mucosal homeostasis. This regulatory role for SlgA has been previously inferred from correlations of IgA deficiency in humans with various autoimmune diseases and inflammatory conditions, such as celiac disease (14, 15) and IBD (43). It has been suggested that IgA deficiency might result in undue triggering of the immune response to gluten in the case of celiac disease (19) and to the intestinal flora in the case of IBD (18, 44), Ags to which the immune system is usually tolerant. While oral CD8⁺ T cell tolerance to OVA was induced in plgR⁻/− mice in this study, the propensity of these mice to abrogate a previously established tolerance was not investigated. It would be interesting to determine whether a state of nontolerance could be more readily induced in plgR⁻/− mice, such as that observed following dextran sulfate administration to mice (45).

Another generally attributed function for SlgA is protection of mucosal surfaces against invading pathogenic microorganisms. Although the induction of mucosal immunity often correlates with protection against infection, recent studies in IgA⁻/− mice have shown that vaccine-elicited protection against infection with mucosal viral or bacterial pathogens is not necessarily dependent on the presence of IgA. In a recent study Asahi et al. (46) showed that cross-protection against nasal influenza virus infections in the upper respiratory tract correlated with the presence of virus-specific IgA in nasal washings. This cross-protection was reduced in plgR⁻/− mice, suggesting that SlgA is an important mediator of cross-protection against influenza virus infections. On the other hand, SlgA did not play a role in protection against influenza virus in the lower respiratory tract. In our study we only investigated the CD8⁺ T cell response against influenza virus in a lower respiratory tract model. Our results showed that normal influenza virus-specific CD8⁺ CTL responses were elicited in the absence of plgR, suggesting that anti-viral CTL responses were not defective in the absence of SlgA in the respiratory tract. Therefore, it is conceivable that the lack of SlgA and the increased levels of circulating IgA in plgR⁻/− mice do not result in compensatory effects affecting the induction of anti-viral CTL responses. Indeed, it would be interesting to study influenza virus-specific T cell responses in plgR⁻/− mice in a comparable model of upper respiratory tract infection.

Further studies are clearly required to determine whether one of the main roles of intestinal SlgA is to act as a blanket to protect the epithelium from constant antigenic stimulation, rather than primarily acting as the major protective mechanism against acute pathogenic challenge. This might account for the high levels of SlgA that are constitutively generated on a daily basis. Thus, SlgA would be partially responsible for the maintenance of mucosal homeostasis, such that the immune response to otherwise innocuous luminal Ags is no longer appropriately regulated in the absence of SlgA. Elucidating which factors may be involved in the alterations in mucosal homeostasis observed in plgR⁻/− mice, such as the nature of the possible antigenic stimulation and the cytokines induced, may provide further insight into not only the roles of SlgA in intestinal immunity, but also the possible clinical consequences of a breakdown of its function.

References


