Commensal Enteric Bacteria Engender a Self-Limiting Humoral Mucosal Immune Response while Permanently Colonizing the Gut

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We have employed a germfree mouse model to study the development and persistence of a humoral mucosal immune response to a gram-negative murine commensal organism, *Morganella morganii*. *M. morganii* bacteria rapidly colonize the gut, resulting in hypertrophy of Peyer's patches (PP), including germinal center reactions (GCR), and the development of specific immunoglobulin A (IgA) responses detected in vitro in PP fragment cultures and by ELISPOT assays of lamina propria cells. The GCR peaks 14 days after infection and begins to wane thereafter. Upon colonization, the organisms successfully translocate to the mesenteric lymph node and spleen, but the number of translocating bacteria begins to drop with the onset of a specific IgA response. A clonal B-cell microculture technique was used to determine the frequency of specific IgA plasmablasts and IgA memory cells. The frequencies of preplasmablasts were seen to be higher in the earlier stages of germinal center development, whereas the frequencies of antigen-specific memory cells appeared to remain at a relatively constant level even after 193 days postmonoassociation. We suggest that a successful secretory IgA response can attenuate chronic stimulation of GCR even though the bacteria persist in the gut. The observed developing hyporesponsiveness to a chronically present commensal organism may be relevant to the use of bacterial vectors for mucosal immunization.

Efforts to target antigens (Ags) directly to the mucosal lymphoid tissues, i.e., those associated with the gut and the respiratory tract, have led to novel approaches. One such approach is to employ attenuated species of otherwise pathogenic gram-negative bacteria (6, 10, 11, 20, 41) or nonpathogenic gram-positive bacteria (16, 31, 46) as vectors for delivering immunogenic protein Ags or epitopes of viruses, bacteria, or parasites. This approach is based on our present understanding of cellular and molecular interactions that lead to a successful mucosal immunoglobulin A (IgA) response. This understanding is far from complete (27). For bacterial vectors to succeed as mucosal vaccines, we need to establish the following: (i) whether acute or chronic gut mucosal exposure to commensal enteric bacteria or to food Ags ever leads to a secretory IgA response and, if so, how this is so obviously attenuated in normal, adult animals, and (ii) the mechanisms by which systemic hyporesponsiveness may be initiated and maintained following exposure of the gut mucosa to Ag. Although immune responses to frankly pathogenic bacteria, efficiently colonizing the gut mucosa, consist of both a rapid secretory antibody response, dominated by IgA, and a strong serum antibody response (13), hyporesponsiveness of the host to copious, ever-present commensal bacteria and food Ags continues to remain an enigma.

Schaedler et al. made the first series of observations on the role of indigenous and autochthonous bacteria on the development and well-being of their host (34, 35). They employed germfree (GF) mice to study the effect each individual bacterial isolate had on the development and well-being of its host. Later, Pollard and Sharon (29) used the same GF model to demonstrate that there was a correlation between the development of germinal zones in the Peyer's patches (PP) and the appearance of specific serum antibodies as a result of an an-

tigenic stimulus. However, they did observe that while oral administration of *Salmonella paratyphi* A resulted in a germinal center (GC) response around day 16 postimmunization accompanied by the presence of serum antibodies, they failed to observe any response to an organism that belonged to the normal flora of the host, namely, *Streptococcus faecalis*. In other studies to understand immune responses of specific-pathogen-free and GF mice to indigenous and nonindigenous microorganisms, it was observed that parenteral immunization of specific-pathogen-free mice or oral immunization of GF mice with nonindigenous organisms resulted in a greater response than that generated when indigenous organisms were used (3). In our own laboratory, we have encountered bacteria which are indigenous to the host gastrointestinal tract and colonize it successfully but are incapable of generating an acute immune response on their own (38). To expand our understanding of the various aspects of development and persistence of a humoral mucosal immune response, we chose to monitor the temporal development of the humoral mucosal immune response in the gut to a commensal microorganism and to quantitate the fluctuations of antigen-specific IgA memory cells and IgA preplasmablasts by use of clonal B-cell microcultures (15). We decided to use a gram-negative commensal bacteria called *Morganella morganii* (Potter strain) (30). This organism expresses immunodominant oligosaccharide-bearing phosphocholine (PC) (30) epitopes that have been reported to be an integral part of the bacterial lipopolysaccharide (LPS) (49). The immunodominant PC moiety has been reported previously to induce a distinctive anti-PC response. PC is ubiquitous in nature and is present on such vividly divergent cells as *Streptococcus*, *Lactobacillus*, *Aspergillus* (30), and parasitic nematode *Ascaris* (4) spp. Thus, the response to PC represents a normal immune function. Besides, virtually all of the induced murine antibodies can be related to one of the PC-binding myeloma proteins (8), thus allowing a careful dissection of the clonal response at the B-cell level.

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MATERIALS AND METHODS

Mice. Six-week-old C3H/HeN GF (certified free of any viral and/or bacterial organisms) mice were obtained originally from Taconic Farms (Germantown, N.Y.). The mice were fed a sterile (autoclavable) mouse diet (PMI Feeds Inc., St. Louis, Mo.). Separate GF isolators were dedicated for generation of pups, and the adult mice were maintained in a sterile environment within flexible film isolators in the gnotobiotic facility of the Biology Department, University of Pennsylvania, both before and after colonization.

Ags and inoculations. *M. morganii* was kindly provided by Ann Feeney (The Scripps Research Institute, La Jolla, Calif.) and was originally isolated by Potter from mouse feces (30). A 1.0-ml aliquot of the organism, which had been stored at -70°C , was thawed and cultured on sterile brain heart infusion agar and propagated at 37°C for 16 h. Organisms were harvested, washed, and resuspended in sterile phosphate-buffered saline (PBS). GF mice were inoculated per os with a 0.2-ml solution containing 2×10^8 organisms. These mice were housed in a formerly sterile isolator. Typically, groups of five mice were used for each time point studied. Noninoculated GF littermates were kept in separate isolators and used as controls.

PP organ cultures. The general method of the PP organ culture technique was described previously (21). Briefly, PP were dissected from the small intestine of mice and placed in cold RPMI 1640 medium containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.01% gentamicin, and 5% fetal calf serum. The PP were washed by five successive transfers through fresh medium, remaining in each wash for 10 min before each transfer. Each PP was halved with a sharp sterile razor blade and placed in a sterile flat-bottom well of a 16-well plate (Costar, Cambridge, Mass.) in 2.0 ml of conditioned Kennett's HY medium (JRH Biosciences, Lenexa, Kans.) containing 10% fetal bovine serum, 1% L-glutamine, antibiotic-antimycotic solution (100 U of penicillin per ml, 0.1 mg of streptomycin per ml, 0.25 mg of Fungizone per ml; Gibco, Grand Island, N.Y.) and cultured for 7 days under 90% O_2 -10% CO_2 at 37°C .

Fluorescence-activated cell sorting (FACS) and analysis. For analytical and preparative sorts, the cells were incubated with the appropriate dilution of fluorochrome-coupled reagent in PBS-5% fetal calf serum-0.04% sodium azide (Sigma) for 30 min on ice. The cells were then washed three times and analyzed or sorted on a FACS IV flow cytometer (Becton Dickinson). We used fluorescein-labeled peanut agglutinin (PNA) in conjunction with a phycoerythrin-conjugated anti-B-cell marker (surface kappa chain [kappa] positive) to stain GC B cells (9, 33). By use of the same labeled anti-kappa along with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgA (Southern Biotechnology Associates, Birmingham, Ala.), we stained surface IgA-positive (sIgA⁺) cells. Sorted cells were resuspended in RPMI 1640 medium-fetal calf serum-L-glutamine-gentamicin-mercaptoethanol (complete RPMI 1640 medium) and incubated for 1 h at 37°C to allow any residual azide to leach from the cells before they were cultured. PNA^{high} kappa⁺, PNA^{high} sIgA⁺, and sIgA⁺ cell populations from PP of mice that were monoassociated with *M. morganii* for 14 and 28 days were 85 to 90% pure; PNA^{low} kappa⁺ and sIgA⁺ cell populations from PP of mice at days 117 and 193 postmonoassociation with *M. morganii* were 80 to 90% pure on reanalysis (data not shown). We also employed FACS analyses to detect coating of fecal bacteria with endogenous IgA. This method has been described previously for the detection of IgA-coated fecal bacteria in humans (43, 44). Bacteria from the mouse gut were isolated by either flushing out the contents of the small intestine or homogenizing the fecal pellets in PBS. Mild vortexing of the fecal matter separates the insoluble solids, which are removed by decantation. The fecal homogenate is then passed through a cotton column. Bacteria are then washed twice and resuspended in PBS. The optical density of the suspension is adjusted to give an optical density of 1 when measured at 550 nm. Two hundred microliters of such a suspension is pelleted by centrifugation at $600 \times g$ for 15 min. The pellet is then stained with FITC-conjugated goat anti-mouse IgA. Stained bacteria were washed twice with PBS and then fixed overnight with 1% paraformaldehyde. Prior to analysis, propidium iodide was added to the samples to achieve a final concentration of 50 $\mu\text{l}/\text{ml}$ (25). FACS analyses were conducted with a FACScan and by gating on the propidium iodide-positive events.

Dendritic cells. Dendritic cells were isolated from the PP of C3HeB/FeJ mice by the procedure of Spalding et al. as described previously (39). By FACS analysis, preparations of dendritic cells were free of B cells and macrophages as judged by their B220-negative, Mac 1-negative phenotype.

T cells. The helper T-cell clone D10.G4.1 (D10) was obtained from the American Type Culture Collection (ATCC; TIB 224); it was maintained and prepared for use in microculture as described previously (18). D10 cells are specific for conalbumin presented in the context of *I-A^k* and are alloreactive for cells bearing *I-A^b* (18). Since the microculture was designed to detect specific responses by B cells from C3H/HeN (*I-A^k*) mice against the PC determinant that is predominant on *M. morganii* (30), we conjugated *p*-azophenyl PC to conalbumin and used PC-conalbumin at 1 ng/ml (36).

B-cell clonal microculture. B cells from PP of mice colonized with *M. morganii* for various time periods were enriched for PNA^{hi} kappa⁺, PNA^{high} sIgA⁺, PNA^{low} kappa⁺, or simply sIgA⁺ and plated at either 20 or 100 cells per well with 3,000 D10 cells (D10-Th2 cell) in 12 μl of complete RPMI 1640 medium in 60-well Terasaki plates (Miles). As controls, B cells from PP of GF mice were plated at 80 cells per well. The plates were incubated at 37°C in 5% CO_2 -air at

>95% humidity for 7 to 14 days. Culture supernatants were then collected into 140 μl of RPMI 1640 medium, and 20 μl was used in radioimmunoassays for each analysis.

Immunoperoxidase staining of frozen sections. Immunohistochemistry was performed with 5- μm frozen sections from tissues embedded in O.C.T. compound (Miles Inc., Elkhart, Ind.). Air-dried sections were rehydrated in PBS and then blocked in 1% bovine serum albumin in PBS for 1 h each. The staining was carried out by use of the prescribed method and reagents in the biotin-horse-radish peroxidase plus avidin ABC kit from Vector Laboratories (Burlingame, Calif.).

BrdU incorporation analyzed by immunohistochemistry. Mice were injected with 100 μg of bromodeoxyuridine (BrdU) in PBS per g of body weight 12 h prior to euthanizing them. PP were frozen in O.C.T. embedding compound (Miles) and then cut into 5- μm sections. Air-dried sections were fixed with 1% paraformaldehyde for 15 min and then incubated for 30 min in 2 N HCl-0.05% Tween 20 in a 42°C water bath. Sections were stained with mouse anti-BrdU monoclonal antibody clone BMC 9318 (Boehringer Mannheim) and then washed in PBS-Tween. Specific binding of the anti-BrdU antibody was detected by a second step with biotinylated goat anti-mouse IgG1 (Southern Biotechnologies). The specificity of this staining was determined by staining adjacent sections with the second step alone. Sections were then developed and counterstained with Giemsa.

Radioimmunoassay. The radioimmunoassay used in our laboratory has been described previously (19, 36). Appropriate myeloma proteins were used as standards. The assays are linear for immunoglobulin concentrations of 0.5 to 10 ng.

Enzyme-linked immunosorbent assay. The ELISPOT assay used in these studies was performed by the standard method described by others (17).

Translocation of bacteria. At different times following monoassociation with *M. morganii*, translocation of the bacteria to mesenteric lymph nodes (MLN) and spleen was detected by sacrificing the mice and removing their MLN and spleens aseptically and homogenizing them in 10 ml of sterile PBS. Each of the homogenates was serially diluted in sterile PBS and then plated (0.1 ml of the homogenate per plate) onto three nutrient agar plates. Plates were incubated at 37°C for 24 h, after which colony counts were recorded. At random, five colonies were inoculated individually into Enterotube II (Roche Diagnostic Systems, Montclair, N.J.) to confirm their identity. During the entire period that the mice were monocolonized, bacteria could be cultured from the feces, where they were seen to persist at a constant level of about 10^9 organisms per g of feces.

RESULTS

Development and resolution of GCR in PP. Ontogeny of the local humoral response to a single gram-negative commensal colonizer, *M. morganii*, was detected by monitoring the generation of germinal center reactions (GCR) in the PP by flow cytometry. The most encompassing GC marker for B cells of mice and several other mammalian species is the capacity to bind high levels of the lectin PNA (5, 32). By use of a combination of fluorescein-coupled PNA and phycoerythrin-coupled anti-kappa to detect B cells that were expressing the ligand for PNA, we observed, in five separate colonization experiments, that the generation of a GCR within the PP began at day 7 (data not shown) following monoassociation and that the maximum number of PNA^{high} kappa⁺ cells could be detected on day 14 (Fig. 1). Thereafter, the proportion of PNA^{high} kappa⁺ cells began to gradually decline. By day 56 following colonization, the GCR had subsided appreciably. Very few PNA^{high} B cells could be seen in the FACS analyses at later time points, in spite of the copious presence of the bacteria in the gut. Distinct subsets of PNA^{high} kappa^{high} and PNA^{high} kappa^{low} cells arise at the peak of the GCR (days 14 to 28). The later subset corresponds to centroblasts that are in rapid cell cycle and have diminished their surface immunoglobulin (22, 32). These have been reported to form the dark zone of the GC and to continually give rise to progeny cells called centrocytes that are nondividing, express high levels of surface immunoglobulin (22), and likely appear in our FACS analyses as the PNA^{high} kappa^{high} subset. We also analyzed the PNA^{high} cells for expression of sIgA and observed that PNA^{high} sIgA⁺ cells constituted only an appreciable fraction of sIgA⁺ cells on day 14 postcolonization. The development of sIgA⁺ cells was also seen to begin around day 7, peak at day 14, and gradually subside (Fig. 2). The sIgA⁺ cells appear to be either kappa^{low}

FIG. 1. FACS profiles of total PP cells taken from either GF or formerly GF mice colonized with *M. morganii*. Cells stained on days 14, 28, 56, 107, 168, and 314 postmonoassociation are shown, with GC cell marker PNA conjugated to FITC and counterstained with a phycoerythrin conjugate of anti-surface immunoglobulin (kappa) for monitoring the development of GCR.

or kappa^{high}, depending on the magnitude and presence of GCR. At day 14, the time of maximal GCR, about half of the sIgA⁺ cells were PNA^{high} and kappa^{low} (data not shown). These were the cells enriched by FACS and analyzed by microculturing (see Table 1).

Immunohistology of the lymphoid follicles of PP. To establish whether the waxing and waning of the PNA⁺ and sIgA⁺ cells seen in the FACS analyses of single-cell suspensions of pooled PP were an accurate reflection of PP GCR and the perturbations of B-cell subsets within individual follicles at any specific time point, sections of PP taken at various times, including times of each FACS analysis, were stained and examined. To monitor the development of the GCR, particularly the centroblasts, we injected BrdU into the peritoneal cavity of the colonized animals 24 h prior to harvest and assessed the in vivo incorporation during this period. Figure 3A shows the incorporation of BrdU by rapidly dividing cells within the GC at the peak of the GCR at day 14. Although smaller foci of dividing cells could still be seen within the follicle at day 28 postcolonization, there were none to be seen at these sites at later time points studied (Fig. 3B). Cells binding PNA could be seen clearly within the follicular region at days 14 and 28 (Fig. 3A); however, none were obvious at later time points (Fig. 3B). The sIgA⁺ cells (Fig. 3A) are seen prominently and pervasively within the GCs of follicles at days 14 and 28. At later time points, they seem scattered in the mantle zone and dome region as they are in GF mice (day 0) but are also prominently visible in the lamina propria (LP) and in the core of the villi. In the GF control (day 0) PP, there is no apparent Ag-driven process taking place and the follicles appear to be filled completely with recirculating sIgD⁺ small B cells (Fig. 3A). However, with the formation of GCs and exponential expansion of

PNA⁺ B-cell blasts, the small recirculating B cells are excluded from the follicular center and retreat to form the familiar follicular mantle or corona that surrounds the GC. After day 28, when the GCR subsides, the small recirculating B cells that are IgD⁺ re-infiltrate the core of the follicular zone. A few follicles at days 107 and 314 continue to display a core that remains free of IgD⁺ cells and thus seem activated, although no appreciable content of BrdU-positive or PNA^{high} cells is evident within them (Fig. 3B). By day 107 postcolonization, the histology of most follicles begins to resemble that of the quiescent GF PP. Thy 1.2⁺ cells are most obvious in the interfollicular region at most time points and later can be seen scattered prominently within some follicles as well. Figure 3B shows that the follicles still showing some characteristics of activation contain a conspicuous content of these T cells. Their increased presence within the follicle, especially at days 107 and 314, correlates well with earlier reports that demonstrated retention of likely antigen-specific T cells within the GC (14). The immunohistology clearly demonstrates the transient nature of most GCR in the PP, initiated by colonization with enteric gram-negative bacteria which then remain chronically present. Subsequent immunohistochemical analysis of PP from conventionally reared, 225-day-old C3H mice that were administered BrdU showed incorporation of BrdU by dividing cells in the GCs within the follicles. IgA⁺ and PNA⁺ cells were prominently present in the GCs. This normal appearance of well-developed GCR in aged mice clearly suggests that aging did not influence the capacity of the mice to generate GCR in PP.

Anti-PC-specific IgA antibody detected in PP organ cultures correlates with the development of GCR and an IgA response determined by FACS. We have shown before that our PP organ

FIG. 2. Histograms showing total PP cells stained with an FITC conjugate of anti-mouse IgA, on days indicated following monoassociation, to detect the development of sIgA⁺ cells in the PP. Since cells from all five animals were pooled, this result is representative for all five animals at each time point. The day zero controls (leftmost panels) represent cells from noncolonized mice that were 42 (top) and 170 (bottom) days old.

culture accurately reflects the status of the small intestine at the time the tissues are sampled (45). Thus, PP organ cultures were set up in triplicate, at various times following colonization. The supernatants from these cultures were analyzed specifically for IgA antibody against the PC determinant, which is present as a major epitope on the surface of the bacteria (30). Although anti-PC IgA antibody was not detected in supernatants from cultures of PP taken from conventional (CNV) mice (data not shown) as well as from GF controls, Fig. 4A shows that a vigorous anti-PC-specific IgA response could be detected from day 14 through day 70. Thereafter, the response seemed to decline but to persist up to the last time point studied, i.e., 314 days postcolonization. We also analyzed the supernatants for IgA antibody against *M. morganii* extract, prepared by the method described before by Williams and Claflin (47), to determine whether the antibody secreted in our cultures was capable of binding with the LPS-PC complex that is reported (49) to be intact in the extract. Figure 4B shows that the anti-*M. morganii*-specific IgA antibody response assayed against *M. morganii* extract also appears at day 14 and begins to decline beyond day 70. Anti-PC IgA antibody response in the serum (data not shown) was observed to follow the same pattern as that seen in the fragment culture supernatants.

Detection of antigen-specific IgA antibody-secreting cells in LP. We were interested in determining whether antigen-specific antibody-producing cells persisted in the LP. Their presence in the LP would then account for the endogenous IgA that could coat the surface of *M. morganii* present in the gut (see below). We approached this by using an anti-PC-specific ELISPOT assay. As shown in Fig. 5, anti-PC-specific IgA an-

tibody-secreting cells could be detected even at 314 days postcolonization. This result correlates with the endogenous IgA coating of the luminal bacteria observed.

Coating of the gut bacteria in vivo with host IgA antibodies. We employed a rather simple but elegant assay for the appearance, waxing, waning, and continued presence of gut mucosal IgA reactive against bacterial colonizers. This assay was developed by D. van der Waaij for human patients (43, 44). Fecal samples or intestinal washes are periodically collected and processed as described in Materials and Methods. The assay permits a nondestructive, constant monitoring of the gut mucosal IgA response. Figure 6 shows the results of analysis of bacteria recovered from the feces (gut wash) of monoassociated animals at different time points, for endogenous coating with IgA. At 24 h after colonization, the recovered bacteria are uncoated with IgA and resemble nonstained or in vitro-grown controls (data not shown). By day 14 after colonization, at the peak of the GCR in PP, >60% of bacteria are coated with IgA. Even after the GCR wanes, more than 90% of the bacteria in the sample tested around day 70 postcolonization appeared coated with endogenous IgA.

Translocation of bacteria to the MLN and spleen. We monitored translocation of *M. morganii* to the MLN and spleen and were able to culture the organism from both of the tissues up to days 56 and 28, respectively (Fig. 7). Viable counts at earlier time points indicated progressive translocation. However, by day 14, coinciding with the appearance of anti-PC-specific IgA antibody, the counts began to decline, until we eventually failed to culture any bacteria from the spleen on day 56 and from the MLN on day 107, implying that the mucosal immune

FIG. 3. Immunoperoxidase staining of frozen sections of PP for development of GCs (PNA⁺ cells), BrdU incorporation, IgA-, IgD-, and Thy 1.2-expressing cells in noncolonized (GF) and *M. morganii*-colonized mice after 14 and 28 (A) and 107 and 314 (B) days postcolonization. Five-micrometer serial frozen sections stained and developed with a horseradish peroxidase-biotin plus avidin kit, counterstained with Giemsa, are shown. The arrow in the day 28 BrdU panel highlights the presence of BrdU⁺ cells.

response could contain completely the subsequent translocation of the bacteria from the lumen of the gut.

Determination of frequencies of Ag-specific IgA memory cells and preplasmablasts. Having demonstrated before that

the functionality of different subsets of B cells that arise following the development of a GCR could be defined by use of a B-cell clonal microculture (15), we sought to compare frequencies of IgA preplasmablasts (GC cells) and IgA memory

B

FIG. 3—Continued.

cells over time. To accomplish this, we had to successfully analyze Ag-specific B cells. To achieve this end, we used GF *H-2^k* mice, and B cells from these mice were cocultured along with the conalbumin-specific, *I-A^k* restricted Th2 cells (cloned D10) and PP dendritic cells from *H-2^k* mice. A population of antigen-specific B cells could be activated in an antigen-dependent fashion with PC-conalbumin conjugate in the presence of

conalbumin-specific Th2 cells (cloned) D10 and dendritic cells. This population of B cells includes those primed in vivo by the immunodominant PC-LPS of *M. morganii* (see Table 1). It is both a limitation and a benefit of clonal B-cell microculture that only 1 to <100 B cells can be used per 10 μ l per well. Thus, very few B cells are required for a frequency determination, but they must be enriched in reactive cells. Previously, we have

FIG. 4. Detection of IgA antibody in supernatant of PP organ cultures. At various times after colonization with *M. morganii*, PP from groups of three mice for each time point were used to set up organ cultures. Seven days later, the culture fluids were harvested and tested for IgA antibodies against PC (A) or against the *M. morganii* whole-cell extract (B). Data represent means $\pm$ standard deviations in micrograms per milliliter.

either enriched B cells for Ag-specific ones by panning on haptenated gelatin (36) or stimulated most B cells in an Ag-independent fashion with alloreactive T cells (15). Here, since our aim was to compare frequencies of Ag-specific B cells in different PP subsets, we chose to take advantage of natural enrichment *in vivo* into particular subsets. Thus, we used FACS to enrich for particular IgA memory and IgA preplasmablast (GC) subsets, hoping that the frequencies of Ag-specific B cells within these subsets would increase sufficiently to allow determination at 20 or 100 B cells per well. To accomplish FACS for sufficient IgA memory cells and IgA-preplasmablasts from GC, noncolonized mice required many donors. *M. morganii*-colonized mice always provided sufficient sIgA⁺

cells for recovery by FACS and microculture at all time points. Cultured without aphidicholin, these cells provided an estimate of frequencies of IgA memory cells. However, cells bearing GC characteristics, PNA^{high} kappa⁺ or PNA^{high} IgA⁺, were available only in appreciable numbers for FACS from experimental groups sacrificed during the period of maximum GCR (days 14 and 28). Nevertheless, these GC preplasmablasts could be compared with those from GF mice for PC reactivity in the presence of aphidicholin.

Table 1 shows that at inputs of 100 FACS-enriched GC (PNA^{hi} kappa⁺) B cells per well, obtained from mice at days 14 and 28 after colonization, frequencies of preplasmablasts that secreted IgA anti-PC exclusively of 0.2 and 0.5%, respectively, were determined. It should be noted that the frequencies were dependent on the addition of aphidicholin to the cultures to inhibit cell division; in its absence, frequencies of only 0.08 and 0.13% were found. On day 14 after colonization, it was possible to select most rigorously for IgA preplasmablasts by sorting for PNA^{high} IgA⁺ cells. Table 1 shows that cells so selected, which are a subset of PNA^{high} kappa⁺ cells, have an even higher frequency of IgA anti-PC preplasmablasts (0.5%) and that these also are dependent on the addition of aphidicholin for expression of detectable, secreted IgA antibodies. For these same time points (days 14 and 28 postcolonization) as well as later times (days 117 and 193 postcolonization), it was possible to enrich for IgA memory cells by either sorting for sIgA⁺ cells only or by sorting for PNA^{low} kappa⁺ cells. At the later time points, GCR had been resolved and there were insufficient PNA^{high} GC cells to enrich by sorting. Table 1 shows that inputs of either 20 or 100 cultured, enriched sIgA⁺ or PNA^{low} kappa⁺ cells gave frequencies of exclusively IgA anti-PC-secreting clones of about 0.6 to 0.25% over the period from day 14 to day 193 postcolonization. These frequencies were recorded in the absence of aphidicholin in cultures. Indeed, Table 1 shows that the addition of aphidicholin to these cultures of PNA^{low} kappa⁺ cells, including IgA memory cells, results in a marked decrease in the frequency of productive clones, indicating their requirement for cell division prior to antibody secretion. Thus, these results demonstrate for the first time (i) that it is possible to monitor the development of specific mucosal IgA memory cells and (ii) that the frequency of antigen-specific memory IgA cells increases and remains relatively constant for long periods after the genera-

FIG. 5. Detection of anti-PC IgA antibody-secreting cells (ASC) by ELISPOT in the LP of formerly GF C3H mice after 14, 28, 107, and 314 days following colonization with *M. morganii*. Data represent mean frequencies $\pm$ standard deviations of numbers of spots formed in four or more replicate wells with cell suspensions from three mice per group. Tenfold dilutions were assayed, and the dilutions yielding between 5 and 50 spots per well were used to estimate the antibody-secreting cell frequencies shown.

FIG. 6. FACS profiles of IgA-coated intestinal bacteria freshly isolated from the gastrointestinal tract of formerly GF mice on days 1, 14, 28, 70, 107, and 314 following colonization with *M. morganii*. The bacteria obtained from the gut washing (see Materials and Methods) were stained for coating by mouse IgA with affinity-purified FITC-conjugated anti-mouse IgA antibodies, fixed in paraformaldehyde, and counterstained for nucleic acid with 50 mg of propidium iodide per ml. The histogram profile shown is gated on propidium iodide-positive events.

tion and resolution of GCR within the lymphoid follicles of the PP.

DISCUSSION

This study was aimed at understanding the development of a mucosal humoral immune response generated by a gram-negative commensal organism of the mouse intestine. We also sought to determine the longevity of the response, i.e., the frequencies of Ag-specific memory cells and preplasmablasts over time. Since GCs are chronically present in PP of CNV mice but are absent or quiescent in GF mice, we adopted the approach that many others had employed previously (34, 35, 40) to study the role of normal bacterial flora in the development and well-being of mammals. In our laboratory, we tried colonizing formerly GF mice with various different gram-negative and gram-positive organisms, including a different phylogenetically related strain of *M. morganii* (21, 38)—which was subsequently characterized, on the basis of its biochemical properties, to belong to the genus *Proteus* (*rettgerii*)—and observed what others have reported before (7, 26, 29, 48), i.e., that not all organisms that succeed in colonizing the gut are capable of inducing an efficient humoral mucosal immune response. For our present study, we chose a gram-negative bacterium that represents a typical enteric, possibly opportunistically invasive, organism, i.e., *M. morganii*, which normally cohabits with other facultative and many more obligate anaer-

obes in the gut of the mouse and is also capable of translocating. Indigenous bacteria have been thought to achieve a commensalism with the host through evolution, and those that fail to induce even a minimal detectable immune response have been termed autochthonous flora (12). Our data with *M. morganii* suggest that this state of unresponsiveness may be apparent only after a well-formed mucosal immune response has already transiently occurred. In our system, much of this response appears to be directed specifically against the PC determinant associated with the LPS of *M. morganii*. The developing mucosal antibody response directed against the bacteria seems to successfully contain them within the gut lumen of the animal and preserve a normal state of health. The humoral mucosal response persists in the absence of ongoing GCR, giving rise to questions concerning the cellular, cytokine, or

TABLE 1. Clonal microculture of FACS-enriched subsets from PP of formerly GF mice colonized with *M. morganii*

Phenotype of FACS-enriched cells	No. of input cells/well ^a	Time after colonization (days)	Aphidicholin in culture	No. of IgA-only positive cells/60	Frequency (%)
sIgA ⁺	80	0	—	1	0.02
	20	14	—	8	0.6
	100	14	—	28	0.5
	20	28	—	3	0.3
	100	28	—	19	0.3
	20	117	—	4	0.3
	100	117	—	13	0.22
	20	193	—	6	0.5
	100	193	—	4	0.07
PNA ^{high} kappa ⁺	80	0	—	0	0
	80	0	+	0	0
	100	14	—	5	0.08
	100	14	+	13	0.2
	100	28	—	8	0.13
	100	28	+	27	0.5
PNA ^{high} IgA ⁺	100	14	—	5	0.08
	100	14	+	30	0.5
PNA ^{low} kappa ⁺	20	117	—	2	0.17
	20	117	+	0	0
	100	117	—	18	0.3
	100	117	+	5	0.08
	20	193	—	3	0.25
	20	193	+	1	0.08
	100	193	—	10	0.16
	100	193	+	10	0.16

^a The indicated number of FACS-purified B cells were cultured with 3,000 D10 Th2 cells and 400 purified PP dendritic cells with PC-conalbumin plus Ag in 12 μ l in 60-well Terasaki plates. The anti-PC response was measured by radioimmunoassay.

FIG. 7. Translocation of *M. morganii* from the lumen of the gut to the MLN (□) and spleen (◆). The MLN cluster and spleen of formerly GF mice were homogenized and cultured to detect the presence of *M. morganii* at various times following colonization.

external stimuli that may provide the bases for its prolongation. The increase in the T cells within the B-cell follicles after the GCR raises the possibility of their continuing interaction with previously generated, perhaps memory, cells, in the presence of persisting Ag, perhaps maintained within iccosomes of follicular dendritic cells (42). Since the presence of the bacteria within the gut does not result in the maintenance of chronic GCR in the PP, unlike the status of PP of CNV-reared animals, it seems that novel antigenic stimuli may be required for the continuously activated state of the PP seen in CNV mice. The prolonged output of Ag-specific IgA and the slow decline of IgA antibody-secreting cells in the gut LP indicate the continuing presence of specific IgA antibody-secreting cells in the gut LP. This is borne out by our own ELISPOT data over 314 days postcolonization. What is the derivation of these cells in the absence of continuing GCR in PP? In the mouse model of enteric rotavirus infection, specific IgA-secreting cells appear to persist in the gut LP for many months after the resolution of infectious virus particles (37). In our case, the gut bacteria persist in the lumen at high density even though complete GCR subsides. In both types of infection, i.e., acute viral and chronic bacterial colonization, the long-term production of IgA by LP plasma cells may be explained by the gradual reactivation of IgA memory cells, on the basis of their longevity and the persistence of both sequestered Ag and specific Th cells in the B-cell follicles as described above. Indeed, our microculture results offer the first data available to date on the long-term persistence and only gradual decline of relatively high frequencies of Ag-specific, IgA memory B cells. Their presumed precursors (1, 15), the PNA^{high} IgA⁺ B blasts in the GCs, increase and disappear with the GCR. A complementary mechanism for the long-term persistence of IgA plasma cells in the gut LP may be a half-life of these which is appreciably longer than that which has been carefully estimated, i.e., about 4.5 days (24). Recently, data demonstrating that LP plasmablasts may cease high-level secretion but be reactivated to resume greater output of antibody secretion by interleukin-5 and/or LPS or interleukin-10 stimulation have become available (23, 28). Given this possible fluctuation in immunoglobulin output, and hence detectability by methods used in the turnover studies, perhaps the longevity of gut LP IgA cells should be reexamined.

The secretion of IgA antibodies into the gut lumen correlates with the cessation of bacterial translocation and the coating of bacteria within the gut with IgA antibodies. A lag in the decline of bacteria found systemically could be due to bacteria that had translocated prior to the appearance of an effective, blocking anti-*M. morganii* IgA response. These systemic organisms may continue to proliferate within the tissues and grow out of spleen and MLN cultures set up at times after IgA coating of luminal bacteria can be easily detected. Perhaps an effective systemic response is required to clear these early translocates. The PC determinant has been shown to include a repeating trisaccharide unit of D-galactose, D-galactosamine with the PC on C-2, and N-acetyl-D-galactosamine, partially acetylated at C-6. These trisaccharides are linked through glycerophosphate units to form a teichoic acid type of structure. At least three repeating units of this structure are needed to bring about effective inhibition of interaction with PC-specific myeloma protein MoPC 603 (49). Thus, the antibody response against the bacterial extract suggests an interaction not only with the PC moiety but also with an extended epitope that probably involves parts of the LPS. Although the relevance of endogenous coating to the translocation-inhibition is not clear at this time, the data do suggest a role for this phenomenon. The coating appears to be specific, since in vitro-grown *M.*

morganii shows very specific binding to the monoclonal antibody MoPC 603, which is an IgA plasmacytoma, while failing to bind with another anti-PC of another idiootype, monoclonal antibody T15 (data not shown).

Bacterial overgrowth in the intestine has been reported before (2) to be a major mechanism promoting bacterial translocation from the gastrointestinal tract. Thus, failure to detect any translocation beyond 107 days of bacterial colonization, even when copious amounts of bacteria continue to be present in the gut, supports a substantive role for mucosal IgA response in protection.

Earlier, we reported (45) that oral reovirus infection in GF mice results in transient GCR that peak between 12 and 14 days in PP and which generate specific IgA plasmablasts. The reovirus is cleared and the GCR wane by the end of the third week postimmunization. Thus, a comparison of the histology and cellularity of the lymphoid tissues of the gut-associated lymphoid tissue from GF mice after a chronic exposure to environmental antigens, such as commensal bacteria, or after an acute oral infection with an enteric virus such as reovirus T1/d, suggests that both chronic exposure of the gut mucosa to environmental antigens or to an acute oral infection with enteric viruses can contribute to the normal steady state of the gut-associated lymphoid tissue. Whether IgA memory B cells can be maintained after an acute gut mucosal reovirus infection and its resolution for the long periods found for such cells specific for permanently colonizing bacteria found in this study remains to be determined.

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