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Use of Peyer's Patch and Lymph Node Fragment Cultures To Compare Local Immune Responses to *Morganella morganii*

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Lymphoid tissue fragment cultures were established to analyze the differentiative processes among B cells in Peyer's patches (PP) and peripheral lymph nodes (PLN), especially those in germinal centers. PP cultures from both conventionally reared mice and formerly germ-free mice colonized with *Morganella morganii* could be maintained for >12 days with continued B-cell division, especially among cells binding high levels of peanut agglutinin, a characteristic of germinal center cells. PLN cultures from conventionally reared mice injected with a heat-killed vaccine of *M. morganii* could be maintained for the same amount of time. Over this period, PP cultures continued to secrete immunoglobulin A (IgA) as well as smaller amounts of IgM. PP cultures from formerly germ-free mice colonized with *M. morganii* showed net increases of IgA antiphosphocholine (anti-PC) antibodies with avidities as high as those of the prototypic T15 monoclonal antibody. Similar PLN fragment cultures from conventionally reared mice given footpad injections of *M. morganii* showed net increases of IgM and IgG anti-PC antibodies in the culture fluid. Thus, although *M. morganii* stimulated lymphoid tissues in vivo to produce an anti-PC response in vitro when given by either the oral or the parenteral route, the antibody isotypes differed between PP and PLN fragment cultures. Fragment culturing may offer a complementary and simpler way to detect a local secretory IgA response than does either measuring IgA antibody in secretions or detecting IgA antibody in the cytoplasm of plasma cells in the lamina propria of gastrointestinal or respiratory tissue.

The germinal centers (GC) in the Peyer's patches (PP) of conventionally reared mice are constantly activated, presumably because of the presence of gut antigens and mitogens, and therefore are chronically present. In germ-free (GF) mice, GC are absent or quiescent in PP, since there is a lack of gastrointestinal microflora. Peripheral lymph node (PLN) GC of conventionally reared mice are usually only transiently present in response to various local antigenic stimuli (14, 15). The GC are considered to be the sites of generation of memory cells and preplasmablasts within PP and PLN, but their precursors have been neither precisely distinguished nor functionally characterized (3, 7). It is also not yet clear whether the microenvironment of chronically present GC in PP differs from that of transient GC in PLN with respect to the influences the microenvironment may have on the immunoglobulin (Ig) isotype switching process that occurs among B-cell blasts localized to either type of site (7, 15).

We sought to compare local specific antibody responses that resulted from oral versus parenteral immunization at a time at which GC development was maximal. Since it is cumbersome and not very quantitative to compare specific antibodies in blood and secretions and to relate the titers to local GC development, we decided to attempt to compare antibodies secreted in PP and PLN fragment cultures. We hoped that these cultures would permit the continued growth of GC and, eventually, the contribution of the GC B cells to the in vitro production of antibodies. We had previously found that isolated GC cells did not continue to divide or develop into antibody-secreting cells when cultured without added factors or accessory cells. However, when cloned T_H cells and dendritic cells were added to such cultures, GC cells could be induced to divide and/or secrete Ig (10, 22). Thus, we hoped that the fragment cultures would provide a similarly supportive environment for specific antibody secretion.

To compare the differentiative processes among B cells in GC leading to Ig secretion, we compared murine PP and PLN antibody responses in nonmanipulated conventionally reared mice, orally inoculated GF mice, and footpad-injected conventionally reared mice. The antigenic stimulations were done with *Morganella morganii*, an occasional gut commensal previously reported to induce an antiphosphocholine (anti-PC) response when administered parenterally (19, 25). Cebra et al. (4) have shown that young adult GF mice can be effectively monoassociated with *M. morganii* given orally and that this colonization results in the development of PP and the generation of memory anti-PC B cells in PP.

In addition to analyzing the potential of lymphoid tissue to secrete anti-PC antibodies following local antigenic stimulation, we also assayed for perturbations in the GC B cells in vivo and the persistence of these cells in vitro by using as markers the binding of a lectin, peanut agglutinin (PNA), and the surface density of Ig (3, 21).

MATERIALS AND METHODS

Mice. Adult BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and maintained in the specific-pathogen-free animal colony of the Department of

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Biology, University of Pennsylvania. GF BALB/c mice were originally obtained from the Small Animal Section, National Institutes of Health, Bethesda, Md., and were maintained in a sterile environment within a flexible film isolator in the GF facility of the Biology Department, University of Pennsylvania, both before and after colonization.

Antigens and inoculations. *M. morganii* was kindly provided by Michael Potter, National Institutes of Health. A 1.0-ml aliquot of the organism, which had been stored at −70°C, was thawed, inoculated into a sterile broth of 10% sucrose and brain heart infusion agar, and propagated at 37°C for 16 h. Organisms were harvested and washed, and the GF mice were inoculated per os with a 0.2-ml solution containing 2×10^8 organisms and 5% sodium bicarbonate. Following colonization, the mice were kept in their formerly sterile isolator. Groups of conventionally reared specific-pathogen-free mice received footpad injections of 6×10^8 heat-killed organisms. Typically, 60 GF mice were orally inoculated with *M. morganii* in each of three experiments. Noninoculated GF littermates were kept in a separate isolator and comparatively analyzed at 0, 8, and 12 to 15 weeks as control groups.

PP fragment cultures. PP were recovered by dissection from the small intestines of mice and placed in a cold sterile medium consisting of RPMI 1640, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 5% fetal calf serum, gentamicin (50 μg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml). Typically, 10 mice were sacrificed at each time, yielding about 80 PP. These PP were washed by five successive transfers through fresh medium, remaining in each wash for 10 min before each transfer. About 20 PP, randomly selected from the pool, were used to establish fragment cultures. Each of two PP were halved with a sharp, sterile razor blade and placed into 2.0 ml of conditioned HY medium (Kennett's HY Medium; Hazleton Biologics, Inc., Lenexa, Kans.) in an atmosphere of 90% O_2–10% CO_2 in a 24-well plate at 37°C. Sampling of the cultures involved drawing off about 1.5 ml of supernatant at day 1 or days 5 to 10 of culturing. Thereafter, the remaining cells and tissue were dispersed by vigorous pipetting and the viable cells were separated from the dead cells and debris by centrifugation on a Ficoll gradient. Typical yields of viable cells per 10 cultures were 6.5×10^6, 5.2×10^6, 5.2×10^6, and 0.8×10^6 cells at days 1, 3, 6, and 12, respectively. Portions of these cells were stained and analyzed with a fluorescence-activated cell sorter (FACS) (Becton Dickinson, Mountain View, Calif.). Other portions were returned to cultures and pulsed with 1.0 μCi of [^{3}H]thymidine per 2 ml of culture for 16 h before harvest.

PLN fragment cultures. Popliteal and inguinal lymph nodes were recovered by dissection, and the procedure for culturing was essentially the same as that for PP. Typically, five mice inoculated in hind footpads were sacrificed for analysis at each time in each of three experiments.

FACS analysis. Preparation of fluorochrome-labeled PNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and rat anti-mouse κ chain has been described previously (16). Fluorescein isothiocyanate-labeled goat anti-mouse IgA was purchased from Southern Biotechnology Associates, Birmingham, Ala. After single-cell suspensions from freshly isolated or cultured lymphoid tissue were stained and washed (10), they were analyzed or preparatively sorted with a FACS IV flow cytometer (Becton Dickinson, Sunnyvale, Calif.) equipped with 4-decade logarithmic amplifiers. Fluorescein was excited by a 488-nm argon laser operating at 300 mW, and Texas red was excited by a 568-nm krypton laser operating at 200 mW. The FACS was calibrated each day with fluorochrome-labeled microbeads and nonstained control cells. All one- and two-dimensional profiles are presented relative to these standards. The use of fluorochrome-labeled PNA presents a special difficulty not generally encountered with labeled antibodies. Lymphoid cells are not uniformly positive or negative when stained with PNA, but GC B cells can be distinguished from nonactivated B cells by the higher level of PNA binding by the former: PNA^{high} versus PNA^{low}, respectively (3, 21). PNA is reactive with asialyl oligosaccharides bearing terminal galactosyl residues associated with glycolipids or glycoproteins at cell surfaces, but apparently asialyl versions of CD45 (leukocyte common antigen) account for the increased binding by activated (8) and GC (2) B cells. Typically, in vitro culturing of B cells or even slight variations in preparing single-cell suspensions from freshly isolated tissues can lead to a generalized increase in PNA binding and an overall increase in the fluorescence signals from both PNA^{low} and PNA^{high} populations. Because there is no other adequate control except for nonstained cells and no other general marker for murine GC cells except for PNA binding in conjunction with a B-cell marker (surface κ-chain positive) [$sκ^+$]), we have elected to present the FACS profiles without any attempt at normalization. However, a detailed comparison of the appearance of PNA^{high} $sκ^+$ cells detected by the FACS with the development of GC as assessed by immunohistochemistry shows a close correlation (24).

Immunoperoxidase staining. Cytocentrifuge spots of cells from the PP fragments at various stages of culturing were stained for cytoplasmic IgA. Cells were collected at 3×10^4 cells per slide. The spots were blocked with a solution of phosphate-buffered saline with 10% fetal calf serum. After the spots were rinsed with phosphate-buffered saline, the primary antibody, biotinylated goat anti-mouse IgA (Southern Biotechnology), was added to the spots, and the complex was incubated. After another rinse, an avidin-biotinylated peroxidase conjugate (12) (Vector Laboratories, Burlingame, Calif.) was added to the spots, and the complex was incubated. After an additional rinse, the substrate, a solution of 3 μl of 30% H_2O_2 and 15 μl of 8% nickel chloride in 3 ml of 0.05% diaminobenzidine (Sigma, St. Louis, Mo.), was added to the spots, and the complex was incubated. After the final rinse to remove the substrate, the spots were dehydrated by a 2-min dip each into 70, 95, and 100% ethanol and then xylene.

RIA and hapten inhibition. The radioimmunoassay (RIA) was performed as described previously (13), except that the plates were coated in the first step with 1% PC–bovine serum albumin (PC-BSA). The assay is linear for Ig concentrations in the range of 0.5 to 10 ng. For the determination of the relative avidities of the antibodies produced in the fragment cultures of colonized GF mice, 20 μl of each positive supernatant detected during screening was added to the wells of RIA plates previously coated with PC-BSA and containing 20 μl of a solution of free hapten (PC) at a concentration of 2×10^{-5} M, 2×10^{-4} M, or 2×10^{-3} M. These concentrations were used to cover a range of inhibitions typical of murine anti-PC antibodies. This procedure (23) examines the ability of low-molecular-weight free PC to block the uptake of standard antibodies or antibodies from culture supernatants by the PC-BSA-coated plates. To ensure that only a single isotype, IgA, was scored, all captured antibodies were developed with isotype-specific, iodinated rabbit anti-mouse IgA.

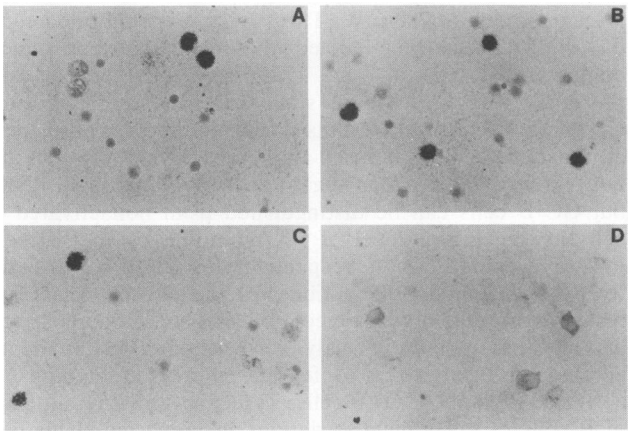

FIG. 1. Incorporation of [^{3}H]thymidine by cells from PP fragment cultures and appearance of plasmablasts containing cytoplasmic IgA. After various times of fragment culturing, cells were dispersed and viable cells were isolated on a discontinuous Ficoll gradient before being cultured with [^{3}H]thymidine for 16 h. Washed cells were deposited on slides by cytocentrifugation either before or after staining with Texas red-PNA and isolation of positive cells by FACS. The cytospots were subjected either to radioautography or to staining with biotinylated anti-IgA and then with avidin-biotinylated peroxidase. (A) Unfractionated cells after 5 days in fragment cultures. (B) PNA^{high} cells after 5 days in fragment cultures. (C) Unfractionated cells after 12 days in fragment cultures. (D) Same as panel C but with staining for cytoplasmic IgA.

RESULTS

Nonfractionated and PNA^{high} B cells from PP fragments continue to divide in vitro. PP from conventionally reared mice that had not been deliberately immunized were placed in fragment cultures. Within a few days, large blast cells emerged from the fragments and eventually covered the bottoms of the culture wells. At various times during the course of culturing, viable cell populations were prepared from the fragments and pulsed with [^{3}H]thymidine to determine whether cell division was occurring and what cell type(s) was dividing. Radioautography revealed that both the nonsorted population and the PNA^{high} subpopulation of B cells obtained from fragment cultures by FACS continued to divide through day 12 in cultures (Fig. 1). At day 5 of culturing, about 14 and 11% of the viable cells incorporated [^{3}H]thymidine in the PNA^{high} and nonsorted populations, respectively.

Changes in proportions of subpopulations of cells in PP fragment cultures as determined by surface phenotypes and cytoplasmic Ig. The sκ$^+$ B-cell population increased over the days of culturing from about 60 to >90% of the viable cells (Fig. 2, left). The fragment cultures maintained 15 to 20% PNA^{high} B cells over this period (Fig. 2, right), although the overall fluorescence signals from the PNA^{low} and PNA^{high} populations steadily increased with time of in vitro fragment culturing. These PNA^{high} cells were markedly enlarged and showed a conspicuous shift from the sκ^{low} (low levels of surface κ chain) to the sκ^{high} (high levels of surface κ chain) phenotype. It remains to be determined whether the PNA^{high} B cells recovered after fragment culturing can display any functional characteristics of GC cells, i.e., whether some of them are nonsecretory preplasmablasts that can be induced to secrete IgA (10).

The proportion of cells bearing both the PNA^{high} and surface IgA (sIgA) markers also increased with time of fragment culturing (Fig. 3). At day zero, two distinct populations of cells stained for one of these markers were evident; one was PNA^{high} sIgA$^{low/neg}$ (low levels of sIgA or no sIgA), and the other was PNA^{low} sIgA^{high} (high levels of

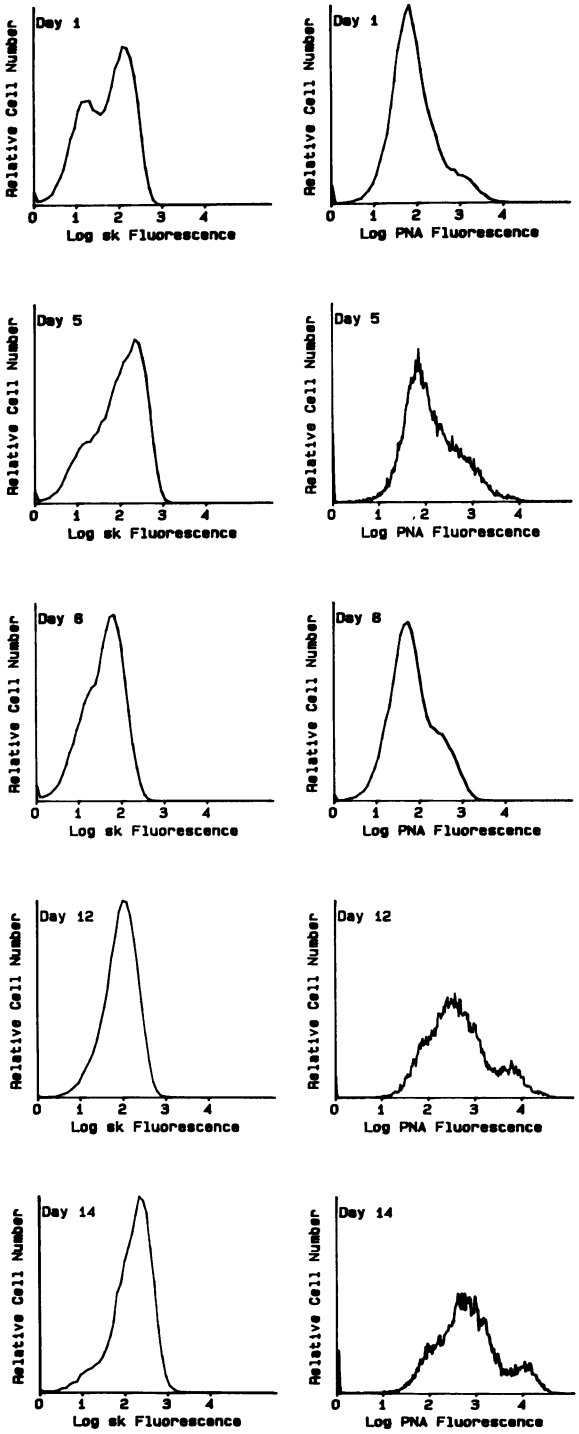

FIG. 2. Analysis of viable cells prepared from cultured PP fragments from conventionally reared mice that had not been deliberately immunized. Cells were analyzed by FACS after PP fragments had been cultured for 1, 5, 8, 12, and 14 days. Left, Cells stained with fluorescein-labeled rat anti-mouse κ chain. Right, Cells stained with fluorescein-labeled PNA.

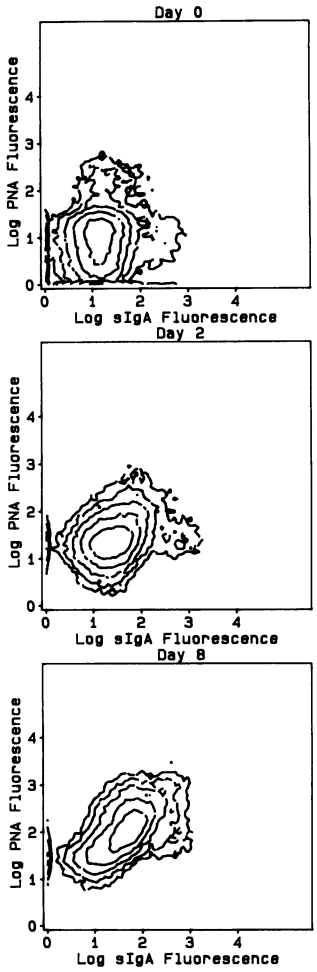

FIG. 3. Analysis by two-color fluorescence with FACS of viable cells from PP fragments (see Fig. 2) after culturing for 0, 2, and 8 days. Cells were stained with fluorescein-labeled anti-IgA and Texas red-labeled PNA.

sIgA). After 8 days of culturing, a population of cells which was both PNA^{high} and $sIgA^{high}$ became prevalent. The appearance of B cells with such a surface phenotype in vivo following acute oral infection of GF mice with reovirus can be correlated with isotype switching of GC cells to IgA expression (24).

An immunoperoxidase stain was used to detect cytoplasmic IgA in cells at various times during culturing of PP fragments. Figure 1 shows that IgA could be detected in the cytoplasm of some of the cells through day 12 of culturing. Many of the large blast cells were positive. Thus, at least some of the sIgA-positive ($sIgA^+$) B cells appeared to mature into plasma cells and presumably accounted for the net increase in IgA secreted by the cultures (see below).

Secreted Ig in PP fragment cultures detected by reverse RIA. Fragment cultures of PP from normal, conventionally reared mice produced total IgA at about 3.5, 8.6, and 11.2 μg on days 1, 5, and 10, respectively (Table 1). Thus, these cultures displayed a net increase in secreted IgA over the culture period. This net increase in secreted IgA supports the continued viability of IgA-secreting cells and their precursors over the culture period. The production of total IgM by cultures also increased over 10 days but was significantly

TABLE 1. IgM and IgA production in two PP from conventional BALB/c mice in an organ fragment assay[a]

Days in culture	Amt (μg) of:	
	Total IgA	Total IgM
1	3.4	<0.05
5	8.6	0.64
10	11.2	0.64

[a] No anti-PC IgA or IgM could be detected in culture media. Amounts of Ig were typical of PP from conventionally reared mice and were determined in pooled culture media from 10 PP in five individual cultures.

lower than that of total IgA. Only a small amount of IgG was detected in the culture fluid, and this did not increase with time (data not shown). There was no detectable anti-PC IgA or IgM antibody in supernatants from cultures of PP taken from conventionally reared mice that had not been deliberately immunized.

GC development and fragment cultures of PP from formerly GF mice. GF BALB/c mice were colonized orally with *M. morganii* to determine whether GC developed in PP and whether a specific local antibody response could be stimulated. PP were removed at various times following inoculation, specifically, at 2, 8, 12, 15, 16, and 20 weeks. The cells were dispersed, stained, and analyzed by FACS for various cell surface markers (Fig. 4). We were especially interested in the PNA binding and $s\kappa^+$ markers.

During the first 10 weeks, there was little change in surface phenotypes indicative of GC cells among the PP, although the PP themselves were somewhat enlarged. At 12 weeks, a population of cells that was PNA^{high} $s\kappa^{high}$ appeared (Fig. 4). These cells made up about 10% of the total population. In other experiments, this minor population of PNA^{high} $s\kappa^{high}$ cells became evident by 8 to 10 weeks after colonization. At 15 weeks after colonization, the PNA^{high} population grew to about 29% of the total and comprised both $s\kappa^{low}$ cells typical of GC B cells in conventionally reared mice and $s\kappa^{high}$ cells. The PNA^{high} population then began to decrease, and at 20 weeks, the PNA^{high} cells made up only 17% of the total; again, most were $s\kappa^{high}$. Culturing of gut contents indicated that *M. morganii* colonized both large and small intestines by 1 week after oral inoculation and remained there until the termination of the experiment at 20 weeks.

Fragment cultures of PP from formerly GF mice were prepared at 12, 15, and 20 weeks after colonization, when GC cells (PNA^{high}) were evident. The fragment cultures showed incremental increases in anti-PC IgA antibody secretion over 5 to 6 days of in vitro culturing. Fragment cultures of PP taken 12, 15, and 20 weeks after colonization showed net increases of 9.5, 19.5, and 45 ng of anti-PC IgA (versus 689, 1,401, and 1,103 ng of total IgA). Surprisingly, anti-PC IgM antibody was barely detectable in the cultures, and no anti-PC IgG1 antibody was found (Table 2). The net increases in both total IgA and anti-PC IgA secreted over 5 to 6 days attest to the continued viability of the cultures. The production of total IgA serves as a rough internal standard against which the production of specific IgA antibody can be judged to compensate for any variability in cultures due to size and choice of PP used. For instance, the production of total IgA by PP fragment cultures from GF littermates at 0, 8, and 12 to 15 weeks after inoculation of the experimental group was similar to that by PP fragment cultures from the latter, but no anti-PC antibodies could be detected. In other experiments, no anti-PC IgA could be detected in PP frag-

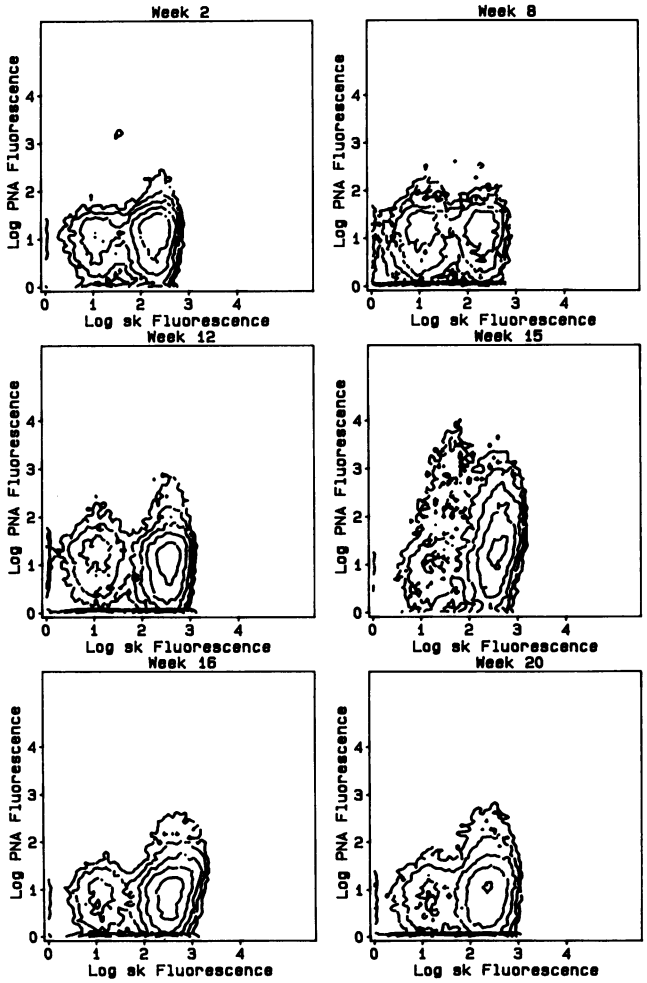

FIG. 4. Analysis by FACS of PP cells from formerly GF mice at 2, 8, 12, 15, 16, and 20 weeks after intestinal colonization with *M. morganii*. Cells were stained with fluorescein-labeled rat anti-mouse κ chain and Texas red-labeled PNA.

ment cultures initiated before 8 to 10 weeks following oral inoculation of donor mice with *M. morganii*.

Measurement of avidity of anti-PC IgA produced by PP fragments. To determine whether the anti-PC IgA secreted by the PP of the colonized formerly GF mice was of the same order of affinity as a standard for a typical anti-PC antibody (monoclonal antibody T15), we performed hapten inhibition

TABLE 2. Total IgA and anti-PC IgA secreted in vitro by two PP 12, 15, or 20 weeks after colonization of GF BALB/c mice with *M. morganii*[a]

Wk after colonization	Amt of anti-PC IgA (ng) on day(s):		Net increase in anti-PC IgA (ng)	Amt of total IgA (ng) on day(s):		Net increase in total IgA (ng)
	1	5 to 6		1	5 to 6	
12	6.5	16	9.5	359	1,048	690
15	6.5	26	19.5	364	1,765	1,402
20	14.5	60	45.0	405	1,510	1,103

[a] Only traces of IgM and no IgG1, IgG2, or IgG3 anti-PC antibodies were detectable in culture media. PP fragments were cultured for 1 day or 5 to 6 days before collection of culture media.

TABLE 3. Avidities of anti-PC antibodies made by PP fragments from GF BALB/c mice colonized by *M. morganii*

Antibody or antibody source	PC concn (M)	% Inhibition in culture medium on day(s):	
		1	5 to 6
T15 standard (1.25 ng)[a]	10^{-5}	22	
	10^{-4}	74	
	10^{-3}	98	
Culture medium[b]			
12 wk	10^{-5}	28	29
	10^{-4}	89	88
	10^{-3}	97	94
15 wk	10^{-5}	44	26
	10^{-4}	83	69
	10^{-3}	100	98
20 wk	10^{-5}	35	29
	10^{-4}	74	69
	10^{-3}	94	100

[a] T15 gave a signal with radiolabeled anti-IgA antibody similar to that given by aliquots of culture media of PP fragments.
[b] Cultured PP from mice 12, 15, or 20 weeks after colonization.

studies; i.e., we sought to determine whether these mice had produced a typical anti-PC antibody or a lower-affinity antibody with cross-reactivity for PC. The studies (Table 3) showed that the IgA antibody produced by the PP fragment cultures of the colonized mice at 12, 15, and 20 weeks after colonization was inhibited by free PC as well as or better than was the T15 standard. Thus, the antibodies produced by fragment cultures have about the same affinity as or a higher affinity than a typical anti-PC IgA antibody made by BALB/c mice.

GC development in lymph nodes following footpad injection of *M. morganii*. PLN (popliteal and inguinal) were harvested from conventional mice which had received footpad injections of *M. morganii*. The dispersed cells exhibited various cell populations, detected by FACS analysis; these changed over a 4-week period following immunization, indicating the waxing and waning of GC. Initially, on day zero, there was a negligible PNA^{high} B-cell population and hence no detectable GC by this criterion; there was also only a small $sκ^{high}$ B-cell population. At 6 days postimmunization, a PNA^{high} population was barely discernible; there was also a slight increase in the number of $sκ^{high}$ cells. At 14 to 19 days postimmunization, two distinct PNA^{high} populations appeared: one was $sκ^{high}$ and the other was $sκ^{low}$. Also, there was a significant increase in the total proportion of B cells as compared with that in PLN 6 days after injection. After 21 days, the PNA^{high} populations had subsided but the $sκ^{high}$ (B-cell) population (Fig. 5) was still elevated.

Anti-PC antibodies secreted by PLN fragment cultures and detected by RIA. Fragment cultures prepared from PLN from mice 7, 11, and 14 days after footpad injection with *M. morganii* were maintained for 8 to 10 days. The outcome of the local response in the draining lymph nodes was different from that in the PP of orally colonized, formerly GF mice. The main non-IgM isotype of anti-PC antibody secreted by the PP fragments was IgA; very little IgG was secreted. On the other hand, most of the anti-PC antibody secreted by PLN fragments was IgM, and the principal non-IgM isotypes

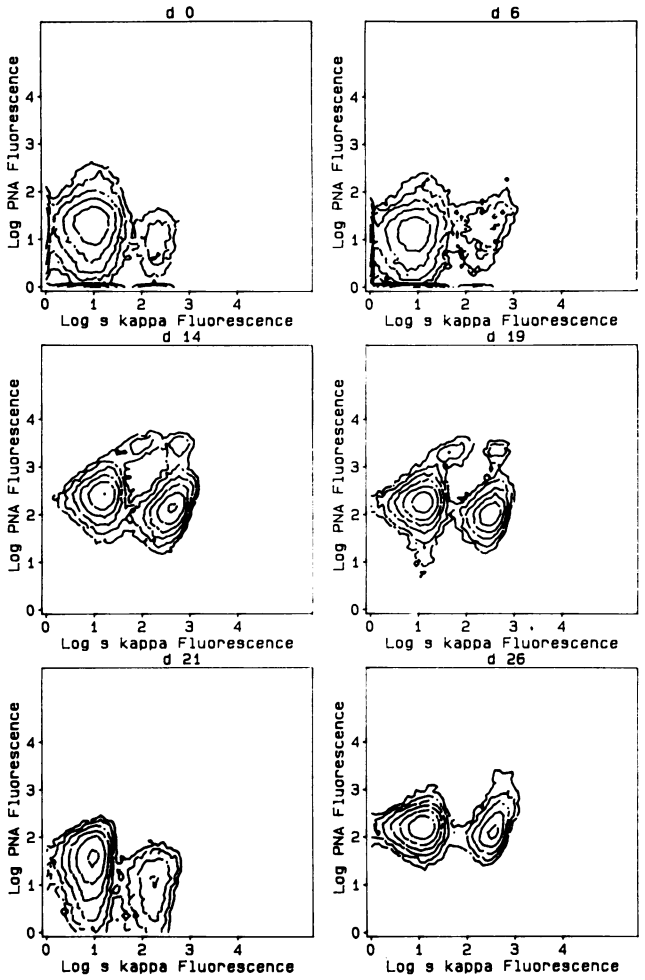

FIG. 5. Analysis by FACS of draining PLN cells from conventionally reared mice at 0, 6, 14, 19, 21, and 26 days (d) after local footpad injection with a heat-killed *M. morganii* vaccine. Cells were stained as described in the legend to Fig. 4.

detected were IgGs; very little IgA was detected in the culture fluid from the PLN fragment cultures (Table 4).

DISCUSSION

We have used the appearance of B cells with the PNA^{high} marker to evaluate the waxing and waning of GC in PP of GF mice colonized with *M. morganii* and in PLN of mice given a footpad injection of a killed vaccine. In general, the binding of high levels of PNA by B cells has been rather closely correlated with most but not all B cells situated in GC (3, 21). More recently, we have compared GC development in PP of formerly GF mice and in PLN of conventionally reared mice after local stimulation with infectious reovirus by both immunohistochemical staining of tissue sections and analysis by FACS for PNA^{high} cells (24). We have found an excellent quantitative correlation between the proportion of PNA^{high} cells and the magnitude (number and size) of the GC reaction, although the FACS method is somewhat less sensitive, especially at the outset of this response, when most GC cells are PNA^{high} $s\kappa^{high}$ and are not well resolved from a major B-cell population. In the present study, the development of the GC reaction in PP was slow following colonization and required weeks, even though bacterial colonization of the gut was easily detectable at 1 week. The GC reaction in PLN developed and waned rapidly upon injection of the bacterial vaccine, as described for other antigens (7, 15). We cannot account for the delay in GC development in PP, although both GF and neonatal mice require 10 to 12 weeks after bacterial colonization of the gut before they develop the IgA memory cells that are specific for bacterial antigens and that are typical of adult, conventionally reared mice (5). It is possible that the slow development of necessary specific T cells or cross-reacting natural antibodies produced by $CD5^+$ B cells in the gut tissue is involved in the apparent delayed responsiveness of PP to gut bacterial antigens.

The use of fragment cultures of PP to detect a secretory IgA antibody response after in vivo mucosal antigenic stimulation should prove to be a useful complement to previously described and somewhat cumbersome methods for the detection of such a response. If fragment cultures are sampled on both day 1 and days 5 to 10 after initiation, a net increase in specific antibody and nonspecific Ig attests to the continued viability of the secretory cells. Also, the net production of total Ig provides an internal standard for judging fluctuations in specific antibody with time after local immunization. Pierce and Gowans (18) developed the fluorescent sandwich technique for detecting a specific gut mucosal IgA response. The intestines are sectioned, and the lamina propria is stained for both IgA-containing and antigen-binding cells. This method allows the enumeration of IgA plasma cells, which contribute to a local secretory immune response, but it is also tedious and time-consuming. An alternative method is to assay IgA antibodies in secretions from the gut after local immunization (20). The gut is flushed with saline or an appropriate medium, and intestinal fluid containing the locally produced antibodies is obtained. There are two disadvantages of this method. First, because of intestinal proteolytic degradation, an accurate quantitation of IgA antibody produced is difficult. Second, it is difficult to quantify the initial volume of gut secretions, including mucus, when flushing the intestinal contents. Consequently, the antibody concentration therein is diluted to an unknown extent, and one must normalize to some other component, for instance, the total IgA concentration. Therefore, fragment culturing, which involves the simple removal of lymphoid tissue and the placement of this tissue into supportive medium, may be an easier and more convincing method for the detection of a secretory IgA response after local antigenic stimulation.

During the course of fragment culturing there is a continuous increase in the proportion of B cells and B blasts. These cells include elements with the GC characteristic of binding high levels of PNA. Some of the B cells can be shown to continue to divide. Fragment culturing shows that at least some of these cells become secretory plasma cells and continue to secrete antibody over the period of in vitro culturing. It would be informative to analyze both PNA^{high} and PNA^{low} cells from PP and PLN fragment cultures for functional potential with clonal microculture assays (22). Such an analysis would help to determine whether the B-cell differentiation associated with GC development also proceeds in fragment cultures. We have previously found that PP contain subsets of B cells distinguishable by surface phenotype and functional potential. For instance, $sIgA^+$ PNA^{low} cells act like memory cells, yielding clones in vitro that secrete exclusively IgA upon interaction with T_H2 cells and dendritic cells (10). Another subset, present in GC, is $sIgA^+$ PNA^{high} and behaves like a preplasmablast commit-

TABLE 4. Anti-PC response in *M. morganii*-immunized PLN

Day(s) in culture	Day immunized in vivo	Site of PLN	Isotype distribution (cpm ± SD)[a]				
			Fab	IgM	IgA	IgG1	IgG2a+2b
1	7	Popliteal	672 ± 222	357 ± 261	0	0	0
	7	Inguinal	601 ± 222	372 ± 317	0	0	0
	11	Popliteal	1,534 ± 377	7,530	0	0	0
	11	Inguinal	2,677	9,783	0	394	367
	14	Popliteal and inguinal	1,805 ± 464	1,080	0	898 ± 339	854
8–10	7	Popliteal	2,602 ± 1,368	5,593 ± 3,505	160 ± 961	52 ± 314	3 ± 17
	7	Inguinal	1,994 ± 662	5,269 ± 1,438	70 ± 418	84 ± 156	41 ± 243
	11	Popliteal	3,978 ± 65	18,256 ± 2,926	0	39 ± 55	196 ± 112
	11	Inguinal	4,067 ± 531	16,473 ± 2,125	489 ± 847	165 ± 101	184 ± 91
	14	Popliteal and inguinal	4,482 ± 779	5,636 ± 2,380	186 ± 557	5,294 ± 2,351	5,364 ± 2,112

[a] Counts per minute above the background in 100 μl of undiluted culture fluid tested on PC-BSA-coated plates with radiolabeled antibodies specific for different isotypes. The signals given by these isotype antibodies are roughly equivalent (13).

ted to secretion. If such cells are blocked from dividing by X-irradiation or aphidicolin and are stimulated by alloreactive T_H2 cells in the presence of dendritic cells, they mature to secretory plasma cells that make IgA (10). It would be of particular interest to determine whether both specific IgA or IgG preplasmablasts and memory cells continue to be generated in vitro in fragment cultures and to determine whether precursor-progeny relationships exist among GC subsets of B cells that may arise in vitro from PP or PLN after chronic or acute antigenic stimulation in vivo. The approach would be to purify subsets of B cells by FACS from cultured fragments and assess their functional potential in single-cell microcultures (22).

M. morganii stimulates GC development and anti-PC responses in PP of formerly GF mice and in PLN of conventional mice. These demonstrations encourage us to analyze possible PC-specific GC cells for the occurrence of early genetic events associated with isotype switching and V-region gene segment point mutations. Both Gearhart and co-workers (9) and Claflin and co-workers (6) have shown that parenteral antigenic stimulation of mice with PC-hemocyanin or *M. morganii* yields productive fusion partners which produce hybridomas expressing V_H genes for anti-PC antibodies that vary from the germ line V_{HS107} gene by point mutations. Since an early phase of T-cell-dependent stimulation of B-cell proliferation likely occurs in GC (11, 17), the in vivo system described here (stimulation of PP to generate PC-specific cells by colonization with *M. morganii*) may offer an opportunity to determine whether the GC microenvironment of PP is a site of generation of point mutations in expressed V_H genes, as has been recently been demonstrated for splenic GC (1). It is possible that the cultured fragments from colonized mice also support ongoing somatic variations among PC-specific B blasts.

Finally, these experiments represent an approach to support our belief that differences in the isotype potentials of GC and memory B cells generated at a given site (PP versus PLN) more likely are due to intrinsic differences in the microenvironment than to differences in physiologic state. Our observations suggest that differences in isotype expression in GC of PP versus GC of PLN do not merely reflect qualitative or quantitative differences due to the superimposition of a deliberate mucosal antigenic challenge upon the ongoing, chronic stimulation of PP with other antigens and mitogens versus the relatively acute but transient antigenic stimulation that most commonly occurs in normally quiescent PLN. However, because of the considerable differences in the time courses of GC development in PP versus PLN observed in the present experiments, more convincing support for microenvironmental differences must await means to stimulate acute, de novo GC development in PP and PLN within the same period of time following local exposure to antigens.

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