

Isotype Commitment of B Cells and Dissemination of the Primed State after Mucosal Stimulation with *Mycoplasma pulmonis*

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Live *Mycoplasma pulmonis* organisms were used to examine the immune response in the bronchus-associated lymphoid tissue after primary and secondary challenge with *M. pulmonis*, to study the dissemination of the primed state to distal tissues (i.e., spleen, peripheral blood, and Peyer's patches), and to determine whether the chronic antigenic stimulation accompanying infection influences the isotype potential and commitment of the primed B cells recovered from the various tissues. We have shown that exposure to *M. pulmonis* by a variety of routes results in a generalized rise in frequency of T-dependent, antigen-sensitive B cells in all lymphoid tissues. The route of secondary exposure to *M. pulmonis* was found to markedly increase the frequency of *M. pulmonis*-specific B cells in the bronchus-associated lymphoid tissue relative to that in the Peyer's patches after intraduodenal but not intratracheal challenge. A substantial rise in the number of *M. pulmonis*-sensitive B cells in the peripheral blood suggests that the dissemination of the primed state, at least in part, is due to B-cell migration via lymph and blood from local sites exposed to *M. pulmonis*. The majority of T-dependent clones generated by *M. pulmonis*-specific B cells secrete exclusively immunoglobulin G1 (IgG1). We have demonstrated that the exaggerated IgG1 response was not due to the accompanying viable donor T cells in the inoculum. The predominance of IgG1 was also demonstrated in clones from the bronchus-associated lymphoid tissue of athymic BALB/c mice that were primed with *M. pulmonis*. Thus, we can infer that functional T cells are not required for the development of specific B cells with the potential for IgG1 expression at the time of in vivo priming. When anti-trinitrophenyl- and anti-*M. pulmonis*-specific clones were generated in the same splenic fragment cultures stimulated by trinitrophenylated *M. pulmonis*, only the *M. pulmonis*-specific clones showed exaggerated IgG1 expression. Therefore, we conclude that the exaggerated IgG1 response accompanying *M. pulmonis* infection of euthymic mice seems to be dependent, at least in part, on an intrinsic property of the B cells that develop during this antigenic stimulation.

The mucosal surface of the mammalian respiratory tract is the first barrier encountered by inhaled pathogens. Pulmonary lymphoid nodules, which are called bronchus-associated lymphoid tissue (BALT) (1, 3) have been considered to be the pulmonary analog of the intestinal Peyer's patches (PPs) and may be involved in the immune response (28). In rabbits and mice, PPs are a rich source of immunoglobulin A (IgA) plasma cell precursors (9, 13). The existing high levels of IgA precursors in the PPs to epitopes like phosphocholine and ($\beta 2 \rightarrow 1$) fructosyl groups (inulin) are attributed to chronic exposure to these antigenic determinants (6). Rudzik et al. (28) used lymphocytes from BALT mucosal follicles in the adoptive transfer system to study the BALT of rabbits. They and their colleagues (24) have found that the predominant isotype expressed by plasma cells arising in the gut from bronchial lymphocytes is IgA. Pierce and Cray (26) observed that acute intratracheal (i.t.) administration of cholera toxin to rats led to some local priming of IgA plasma cell precursors. This route was not nearly as effective as gut mucosal exposure in generating and disseminating precursors for IgA plasma cells.

The following areas of uncertainty exist regarding the role of BALT during chronic stimulation: (i) whether it can display an anamnestic response, (ii) whether B-cell priming there leads to antigen-specific cells with particular isotype potentials, and (iii) the nature of the relationship of the respiratory response to the overall mucosal immune re-

sponse. To address these questions we have used *Mycoplasma pulmonis*, a murine respiratory pathogen, to establish chronic respiratory infections in BALB/c mice. This type of antigenic stimulation, in combination with an in vitro splenic fragment assay for T-dependent B cells, allowed us (i) to examine the frequency and isotype potential of primed B cells in the BALT after primary and secondary infections with *M. pulmonis* cells; (ii) to monitor the dissemination of the primed state to distal tissues (i.e., spleen, peripheral blood [PB], and PPs); and (iii) to investigate the possible role of antigen (Ag) in influencing the isotype potential of the primed B cells recovered from various tissues.

If the BALT in mice were similar to the gut-associated lymphoid tissue, we would expect to find priming for an IgA response after chronic stimulation with *M. pulmonis*. Our data indicate that *M. pulmonis*-specific B cells in the BALT, spleen, PPs, and PB give rise to clones in vitro which make predominantly IgG1 with little IgA. The i.t. priming followed by intraduodenal (i.d.) challenge is the most efficient route for increasing the frequency of *M. pulmonis*-sensitive B cells in the BALT. In contrast, we have not been able to detect any anti-*M. pulmonis*-specific precursors in the PP at 2 weeks after secondary i.d. challenge with *M. pulmonis* organisms.

We have also examined the role of donor T-helper cells in the response to *M. pulmonis* by using athymic BALB/c mice as donors or by eliminating T cells in a donor inoculum from euthymic BALB/c mice with anti-Thy-1 and complement.

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Our findings indicate that (i) the presence or absence of donor T cells does not have an effect on the isotype profile of anti-*M. pulmonis*-secreting clones, and (ii) T cells are not required at the time of in vivo priming for the generation of B cells with the potential for exaggerated IgG1 expression.

MATERIALS AND METHODS

Animals. BALB/cN female mice were raised in our breeding facility at the University of Pennsylvania, Philadelphia. Mice for primary immunizations were 5 to 8 weeks of age. Athymic (*nu/nu*) BALB/c females were purchased from Frederick Cancer Research Center, Frederick, Md., and were primed at 5 weeks of age. Preimmunization serum samples from all mice used were screened by radioimmunoassay for anti-*M. pulmonis* antibodies. Only mice that gave no evidence of previous exposure to *M. pulmonis*, relative to mice raised under germfree conditions, were used for deliberate infection.

Ag. *M. pulmonis* Ag was prepared as described previously (5). Briefly, 100 ml of Gabridge modified medium (12) was inoculated with 10^8 CFU and propagated at 37°C for approximately 72 h. Organisms were harvested later by centrifugation at $15,000 \times g$ for 20 min. The sediment was washed twice with phosphate-buffered saline (PBS) and finally suspended in 3 to 5 ml of PBS. Aliquots of concentrated Ag were stored at -70°C. Immediately before use, Ag was diluted in PBS to a concentration of 100 µg of protein per ml (22). *M. pulmonis* cells used as infectious inoculum were also propagated in Gabridge medium. *M. pulmonis* Barden was kindly provided by Michael F. Barile, Mycoplasma Branch, Bureau of Biologics, Bethesda, Md.

Preparation of TNP-*M. pulmonis* Ag. Trinitrobenzene sulfonic acid (10 mg) was dissolved in 15 ml of 0.28 M cacodylate buffer (pH 6.9) at room temperature. When the trinitrobenzene sulfonic acid was dissolved, 10.5 mg of *M. pulmonis* Ag suspended in 15 ml of PBS was added to the mixture. The container was covered with aluminum foil to avoid photodecomposition, and the mixture was stirred for 2 h at room temperature. The reacted mixture was dialyzed against PBS for 3 days to remove excess trinitrobenzene sulfonic acid and then concentrated by vacuum dialysis. The final product contained 0.3 µmol of trinitrophenyl (TNP) per mg of *M. pulmonis* proteins as established by spectrophotometric measurement of TNP hapten and colorimetric determination of *M. pulmonis* protein based on a bovine serum albumin standard.

Immunizations. Intraperitoneal immunizations were done with 50 µg of *M. pulmonis* antigen per mouse in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). The i.t. priming or boosting was accomplished by injecting 10^8 CFU of live *M. pulmonis* cells in 50 µl directly into the trachea. Animals were anesthetized with Avertin (2,2,2-tribromoethanol; Aldrich Chemical Co., Inc., Milwaukee, Wis.). The lungs of BALB/c animals immunized i.t. with *M. pulmonis* cells were examined for histopathological changes. Observed changes consisted of peribronchial lymphoid cuffing, interstitial pneumonitis, and paratracheal lymph node enlargement. Lung lesions were never observed, either in the uninfected controls or in infected *nu/nu* BALB/c mice. In addition, the weight of the spleens in euthymic mice immunized with *M. pulmonis* increased 1.5-fold compared with the weight of the spleens of control mice. The i.d. priming or challenge was done as described previously (11). Doses containing 50 to 100 µg of previously frozen *M.*

pulmonis Ag (see above) were injected in a volume of 0.15 ml.

Preparation of cell suspensions. Spleen and PP cells were obtained by mechanical dispersion of the tissues in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 10% agamma horse serum (GIBCO). Cell suspensions were filtered through a 3-ml syringe containing nonabsorbent cotton to remove clumps. To obtain BALT cells, donor mice were sacrificed and perfused by direct cardiac injection with PBS until the lungs were visibly whitened. The trachea and lungs were removed from each mouse, and peripheral lung tissues were dissected out and discarded. The remaining tissue was transferred to a petri dish that contained a solution of Dispase (1.5 mg/ml, grade II; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (17) in Hanks balanced salt solution. The mixture was stirred continuously at 37°C for 2 h. Dissociated cells were washed extensively in Hanks balanced salt solution with 10% agamma horse serum. To obtain mononuclear cells from PB, mice were exsanguinated and the blood was collected into Alsever solution (GIBCO). Mononuclear cells were isolated on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). The percentage of B cells in cell suspensions was determined by fluorescence microscopy with a rhodamine-conjugated goat anti-mouse Fab reagent (13). Typical yields of mononuclear cells per mouse from BALT and PB (and the B-cell content) were 10×10^6 (7% B cells) and 1.5×10^6 (10% B cells), respectively. T cells were depleted from cell suspensions by selective lysis with anti-Thy-1 and rabbit complement (4, 21).

Fragment cultures. A modification of the splenic fragment assay (19) was used to enumerate T-dependent, Ag-sensitive B cells in various tissues after different modes of priming and challenge. Dispersed cells from tissues to be scored were injected intravenously into lethally irradiated (1,600 rads) recipients that had been previously primed intraperitoneally with *M. pulmonis* cells in complete Freund adjuvant. After 16 to 22 h, recipient spleens were removed and diced. Individual fragments were cultured in microtiter wells in Dulbecco modified Eagle medium (4.5 g/liter of glucose; GIBCO) containing 0.001 mg of *M. pulmonis* Ag per ml, 0.001 mg of TNP-*M. pulmonis* Ag per ml, and 15% agamma horse serum. On day 4 (spleens diced on day 0), the fragments were washed to remove excess Ag, and culture fluids were subsequently collected on days 7, 10, and 13 after stimulation and assayed by radioimmunoassay for the presence of antibody. Antibody-secreting clones detected by this assay were dependent on previous priming of the recipient. No clones were ever detected in fragments of unprimed, irradiated recipients inoculated with *M. pulmonis*-primed cells.

Radioimmunoassay. The radioimmunoassay procedure was similar to that reported by Taylor (31). Polyvinyl v-bottom microtiter plates (Costar, Cambridge, Mass.) served as the solid phase for the assay. The wells were coated with 100 µl of solution (0.1 mg of protein per ml of suspension of *M. pulmonis* Ag) or with a 1-mg/ml solution of TNP-bovine serum albumin. Plates were incubated overnight at 37°C in a wet chamber. Ag was removed, and plates were washed 6 times with PBS containing 0.5% (vol/vol) Tween 20 (polyoxyethylene sorbitan monolaurate; Sigma Chemical Co., St. Louis, Mo.). ^{125}I -labeled rabbit anti-mouse Fab or rabbit anti-mouse isotypes (13, 14) were used to detect antibody bound to the Ags. The sensitivities of detection of all isotypes were comparable (0.25 to 0.5 ng) and have been presented previously (14).

TABLE 1. Serum response to *M. pulmonis* in euthymic and athymic BALB/c mice after different modes of priming

Mode of priming	cpm ^a obtained with rabbit anti-mouse:					
	Fab	Specific isotype				
		IgM	IgG1	IgG2	IgG3	IgA
Unprimed ^b	983 ± 31	636 ± 17.5	315 ± 2	618 ± 24	194 ± 9	331 ± 12
i.p. ^b	10,270 ± 380	1,500 ± 9.5	11,300 ± 23.5	1,140 ± 9		
i.d. ^b	5,570 ± 80	2,940 ± 14.8				1,300 ± 21
i.t. 1 (2 wks) ^b	2,844 ± 14	1,070 ± 1.4				
i.t. 1 (13 wks) ^b	12,100 ± 60	6,231 ± 19	3,166 ± 11	2,574 ± 8		1,270 ± 4
i.t. 1 + i.d. 2 ^b	4,400 ± 23	1,192 ± 2.3	1,113 ± 7			
i.t. 1 + i.t. 2 ^c	4,670 ± 213	4,840 ± 543				
i.t. 1 + i.d. 2 ^c	3,070 ± 80	2,390 ± 165				

^a Mean counts per minute (± standard deviation) above background of radiolabeled antibodies and 1% bovine serum albumin. All sera were used at 1:125 dilution. All bleedings were done 4 weeks after primary (1°) or secondary (2°) immunization, except that bleedings from i.t.-primed euthymic mice were made at 2 and 13 weeks. 1.25 ng of purified T15 protein used as a standard for PC-specific antibodies gives a signal 2,000 cpm above the background when tested with ¹²⁵I-labeled rabbit anti-mouse Fab.

^b Each group consisted of four to six BALB/c euthymic mice. Mice were primed as described in the text.

^c Each group consisted of six BALB/c *nulnu* mice.

RESULTS

Infection caused by *M. pulmonis* is clinically silent. We have monitored the development of the immune response in normal and athymic BALB/c mice by examining their serum titers (Table 1). There was an increase of anti-*M. pulmonis* antibodies in serum samples from normal and athymic mice after different modes of priming. The isotypes of serum antibodies reflect the route of Ag introduction. After intraperitoneal priming, IgG1 is a predominant isotype of anti-*M. pulmonis* antibody, whereas i.d. priming results in IgM and IgA in the serum, and i.t. priming gives a variety of isotypes, i.e., IgM, IgG1, IgG2, and IgA. Analysis of serum from the athymic BALB/c mice showed only IgM. From these data we can conclude that *M. pulmonis* functions as both a T-dependent and T-independent Ag (as defined operationally in vivo). The enumeration of Ag-specific clonal precursors was done by the splenic fragment assay. Frequencies and isotype potentials of Ag-sensitive cells to *M. pulmonis* and TNP were determined in the BALT, spleen PPs, and PB. The frequency of *M. pulmonis*-sensitive B cells in a normal, uninfected mouse is approximately 1 in 10⁶ B cells. This frequency is typical of that found for B cells specific for Ags not usually present in the murine environment. An increase over this base line after immunization with *M. pulmonis* cells is indicative of priming of the T-dependent B-cell compartment.

The frequency of *M. pulmonis*-sensitive B cells increased in all tissues after two i.t. infections (Table 2). The most dramatic increase was observed in the PB, and there was a persistent increase in the frequency in the BALT compared with the frequency after a single i.t. primary infection (25/10⁶ B cells). In lymphoid tissues distal to the site of priming, PP and spleen, B-cell priming was less pronounced. Table 2 also shows the effect of a secondary i.d. challenge with *M. pulmonis* cells. Although a single primary i.d. inoculation increased the frequency of Ag-sensitive cells in both the PPs and the spleen (spleen, 4/10⁶ B cells; PPs, 17/10⁶ B cells), it is generally more effective in raising frequencies in distal tissues when given after i.t. priming. However, *M. pulmonis* Ag-sensitive B cells were not conspicuously detectable in the PP at 2 weeks after secondary i.d. challenge, although they were found there after i.d. priming alone or after secondary i.t. infection. This decrease of precursors in the PPs could not be attributed to cell death or improper lodging, since the same splenic fragments were tested for both

anti-*M. pulmonis* and anti-TNP-specific B cells by using TNP-*M. pulmonis* cells as the in vitro stimulating Ag and yielded expected frequencies of anti-TNP-secreting clones (Table 2).

Isotype analysis of anti-*M. pulmonis* clones from different tissues is shown in Table 3. Euthymic BALB/c mice were sacrificed at 2, 4, or 8 weeks after a secondary i.t. or i.d. challenge with *M. pulmonis* cells. IgG1 was the predominant isotype of anti-*M. pulmonis* antibody secreted by B-cell clones from all tissues tested over an 8-week observation period (Table 3). The exception to this was the appearance at 8 weeks of clones from the BALT that were secreting IgA in addition to IgG1 (22% IgG1 + IgA, 8% IgA only). To control for cell viability in the donor inoculum and for proper lodging of donor cells we used TNP coupled to *M. pulmonis* for in vitro stimulation in splenic fragment assays. Frequency and isotype profiles of TNP-reactive cells are presented in Table 4. The frequency of anti-TNP precursors in spleen and PPs, where they occur in sufficient numbers for analysis, is within a range previously established by using hemocyanin as a carrier (20). IgM, not IgG1, is the predominant isotype in the anti-TNP response. Frequencies of anti-TNP cells were too low in PB and BALT to allow analysis by using the cell inoculum sizes practical for this assay. Thus, both i.t. and

TABLE 2. Frequency of T-dependent *M. pulmonis* or TNP-specific B cells after i.t. primary immunization^a followed by i.t. or i.d. challenge

Source of tissue	Frequency ^b of precursors/ 10 ⁶ B cells ^c :			
	<i>M. pulmonis</i>		TNP	
	After 2° i.t.	After 2° i.d. ^d	After 2° i.t.	After 2° i.d.
Spleen	10-45	19-20	18-19	20-25
PP	15-17	0	26-33	14-23
PB	200-1,600	330		
BALT	58-65	137-1200		

^a All donor mice received a primary i.t. injection with 10⁷ *M. pulmonis* organisms in 50 µl.

^b Assuming that 4% of donor cells lodge in the spleens of the recipients.

^c The numbers represent lower and upper limits for frequencies observed in tissues over an 8-week observation period beginning 2 weeks after secondary challenge.

^d Recipient mice were primed with *M. pulmonis* Ag and lethally irradiated before receiving donor B cells. TNP-specific B cells were enumerated after stimulation of splenic fragments with TNP-*M. pulmonis*.

TABLE 3. Isotype profile of *M. pulmonis*-sensitive B cells from donors that were immunized i.t. with *M. pulmonis* cells followed by i.t. or i.d. challenge

Time after secondary challenge (wk)	Isotype	% of clones ^a expressing isotype			
		Spleen	PB	BALT ^b	PP ^c
2	IgM only	9	0	21	0
	IgG1 only	81	100	42	94
	IgA only	2	0	0	0
	IgM + IgG1	0	0	37	0
	IgM + IgG2	2	0	0	0
	IgG1 + IgG3	2	0	0	6
	IgG1 + IgA	2	0	0	0
4-8	IgM only	5	0	0	0
	IgG1 only	88	100	70	100
	IgA only	5	0	8	0
	IgG1 + IgA	0	0	22	0
	IgG1 + IgG2	2	0	0	0

^a Total number of clones at 2 weeks after secondary challenge: spleen, 43; PB, 27; BALT, 19; PP, 13. Total number at 4 to 8 weeks after secondary challenge: spleen, 42; PB, 10; BALT, 49; PP, 16.

^b BALT clones at 2 weeks after challenge represent only mice that received i.t. immunizations followed by i.d. challenge. BALT cells at 2 weeks after secondary i.t. were toxic to the recipients.

^c PP clones at 2 weeks after i.d. challenge represent only mice that received two i.t. immunizations.

i.d. challenges after an i.t. primary infection result in a chronically increased frequency of *M. pulmonis*-specific B cells in the BALT and most other lymphoid tissues. Large proportions of *M. pulmonis*-sensitive B cells from all tissues give clones that exclusively produce IgG1. In contrast, anti-TNP cells from the spleen and PPs, costimulated in the same splenic fragment cultures with TNP-*M. pulmonis*, make IgM along with other isotypes.

TABLE 4. Isotype profile of TNP-sensitive B cells from donors that were infected i.t. with *M. pulmonis* followed by i.t. or i.d. challenge

Time after secondary challenge (wk)	Isotype	% of clones ^a expressing isotype	
		Spleen	PP
2	IgM only	62	66
	IgG1 only		3
	IgG2 only		
	IgA only	24	9
	IgM + IgG1	3	6
	IgM + IgG1 + IgG2	3	
	IgM + IgA	5	9
	IgG1 + IgA		3
	IgG2 + IgA		3
4-8	IgM only	71	64
	IgG1 only	5	
	IgG3 only		5
	IgA only	13	10
	IgM + IgG1	3	5
	IgM + IgG2		7
	IgM + IgA	3	
	IgM + IgG1 + IgA		
IgG1 + IgG2	5		

^a Total number of clones at 2 weeks after secondary challenge: spleen, 37; PP, 32. Total number at 4 to 8 weeks after secondary challenge: spleen, 40; PP, 39.

The predominance of IgG1 isotype expression by Ag-specific B cells after either chronic mucosal stimulation or stimulation via other routes has not been observed previously. In the experiments described below, we explored the question of whether the priming for an exaggerated IgG1 anti-*M. pulmonis* response was due to accompanying, viable *M. pulmonis*-specific T cells in the inocula. Donor splenic T cells from *M. pulmonis*-primed BALB/c mice were either treated with anti-Thy-1 and complement before being injected into scoring recipients or were injected without being treated. The donor T cells did not have an effect on the isotype profile of anti-*M. pulmonis*-secreting clones (Table 5). This observation has also been confirmed in an experiment involving the use of the fluorescence-activated cell sorter for separating B cells from other splenic mononuclear cells (data not shown). We also examined the question of whether the development of *M. pulmonis*-specific B cells that appeared to have an intrinsic potential for IgG1 expression required the presence of competent T cells during in vivo priming. Athymic BALB/c donors were given an i.t. immunization followed by either i.t. or i.d. challenge with *M. pulmonis* cells. At 2 and 4 weeks after secondary i.t. or i.d. challenge, their BALT, spleen and PPs were examined in splenic fragment cultures for frequency and isotype potential of anti-*M. pulmonis* precursors by using *M. pulmonis*-primed, lethally irradiated euthymic mice as recipients. The frequency of *M. pulmonis*-sensitive B cells in the BALT, spleen, and PPs after secondary challenge (Table 6) is comparable to the frequency of specific Ag-sensitive cells in similarly stimulated euthymic donors (Table 2). However, the increase of *M. pulmonis*-sensitive B cells in the BALT of athymic donors after i.d. challenge was not quite as dramatic as in euthymic mice. In contrast to our finding with euthymic mice, we did not observe the absence of clonal precursors from PPs after i.d. challenge at any time point. The frequency of *M. pulmonis* precursors in the PPs after i.d. challenge was similar to the previously observed frequency after i.t. challenge of euthymic mice.

Specific B cells from the BALT of athymic mice eventually also gave rise to clones expressing IgG1 as a predominant isotype. This predominance of IgG1 in athymic mice is similar at 4 weeks to the response observed in euthymic mice. However, after only 2 weeks, IgM was also a prominent isotype, while few clones from B cells taken from the BALT of euthymic mice at this time expressed IgM. An i.d. challenge resulted in the production of IgM by 80% of all clones in the BALT at 2 weeks, although this isotype profile changed to the exclusive production of IgG1 by most clones from BALT B cells taken at 4 weeks. The isotype profile of

TABLE 5. Isotype profile of anti-*M. pulmonis* clones from spleens after priming with *M. pulmonis* cells

Clonal product	% of clones ^a expressing isotype when donor spleens were treated with:	
	Nothing	anti-Thy-1+C ^b
IgG1 only	82	87
IgG2 only	0	4
IgM + IgG1	6	4
IgG1 + IgG2	6	4
IgG1 + IgG3	6	0

^a Number of clones: untreated, 17; treated, 23. Frequency per 10⁶ B cells: untreated, 17; treated, 15.

^b Spleens from BALB/c mice were used 2 weeks after i.t. challenge with *M. pulmonis* cells.

clones derived from spleen and PP B cells taken at 2 weeks resembled that for BALT cells: a rather large proportion of their clones only secreted IgG1 and another large proportion only secreted IgM. However, by 4 weeks after challenge via the i.t. or i.d. route, these two sets of precursors shifted relative to each other in a way opposite to that found in the BALT of these animals: B cells giving clones making only IgG1 became less common and those giving clones secreting IgM alone or with other isotypes became dominant. This latter shift was not observed over this period after challenge of euthymic mice.

Aside from these subtle differences, suggesting that athymic mice are not as intensively stimulated by *M. pulmonis* Ags during their milder infection, exaggerated IgG1 secretion was observed in clonal cultures from B cells derived from both athymic (Table 6) and euthymic (Table 3) BALB/c mice, although only IgM antibodies were detected in the sera of the athymic donors. We can infer from these results that functional T cells are probably not required at the time of in vivo priming with *M. pulmonis* cells for the generation of precursor B cells with the potential to display an exaggerated IgG1 response in *M. pulmonis*-primed euthymic splenic fragments. The observed IgM anti-*M. pulmonis* antibody in the sera of athymic mice was reflected in the isotype profile of *M. pulmonis* precursors from these donors.

DISCUSSION

The goals of this study were (i) to examine the immune response in the BALT after primary and secondary challenge, with *M. pulmonis* organisms; (ii) to study the dissemination of the primed state to distal tissues (i.e., spleen, PB, and PPs); and (iii) to determine whether the chronic antigenic stimulation accompanying infection influences the isotype potential and commitment of the primed B cells recovered from the various tissues.

We have shown that exposure to *M. pulmonis* organisms by a variety of routes results in generalized priming of T-dependent, Ag-sensitive B cells in all lymphoid tissues as reflected by a marked increase in their frequency. Although we were able to examine B cells from the murine BALT at the clonal level, the assay system is not without limitations. Murine respiratory mucosal follicles are too small for separation by microdissection. Therefore, our preparation of BALT may also contain cells from parabranchial lymph nodes. The low content of B cells (7 to 10%) makes it difficult to use BALT in adoptive transfer experiments unless the frequency of specific B cells is rather high. For instance, the frequency of anti-TNP precursors in our BALT preparation is usually too low to detect in the splenic fragment assay, although we were able to observe an increase in the frequency of *M. pulmonis*-specific B cells. This display of priming is particularly prominent at the site of chronic exposure to Ag and BALT and rises there continuously. Frequencies of *M. pulmonis*-specific B cells in the BALT and PPs were affected by the route of secondary challenge (i.e., i.t. versus i.d.). Although specific B cells are undetectable in PPs by 2 weeks, they increase in frequency in the BALT after exposure by either route. The apparent disappearance of *M. pulmonis* B cells from PPs is probably due to their migration from the patches and their subsequent sequestering in respiratory tissue (i.e., the primary site of infection). Pierce and Cray (26) reported that initial i.t. immunization of rats with cholera toxin primes them for a local secondary response in respiratory tissues but appears to cause little or no detectable priming for a secondary

TABLE 6. Clonal analysis of anti-*M. pulmonis* precursors after i.t. or i.d. challenge after i.t. priming of *nulnu* BALB/c mice

Time after 2 ^o challenge (wk)/route	Isotype	% of clones ^a expressing isotype		
		BALT	Spleen	PP
2/i.t.	IgM only	33	49	26
	IgG1 only	67	37	42
	IgM + IgG1		15	26
	IgG1 + IgG2			2
	IgM + IgG3 + IgG1			5
4/i.t.	IgM only	11	64	45
	IgG1 only	74		3
	IgG2 only			
	IgA only		2	
	IgM + IgG1	15		
	IgM + IgG3		4	11
	IgM + IgG2		9	26
	IgM + IgG2 + IgG3		2	3
	IgM + IgG1 + IgG3		4	
	IgM + IgA		15	
	IgG1 + G3			
IgG3 + IgG2			5	
2/i.d.	IgM only	80	14	18
	IgG1 only	20	50	50
	IgG2 only		7	
	IgG3 only		7	
	IgM + IgG1		21	32
4/i.d.	IgM only		60	60
	IgG1 only	100	15	35
	IgM + IgG1			
	IgM + IgG2		10	
	IgM + IgG3		5	
	IgG1 + IgG3		5	
IgM + IgA		5	5	

^a Total number of clones at 2 weeks after i.t. challenge: BALT, 14; spleen, 41; PP, 43; frequency per 10⁶ B cells: BALT, 42; spleen, 16; PP, 21. Total number at 4 weeks after i.t. challenge: BALT, 19; spleen, 53; PP, 38; frequency: BALT, 62; spleen, 36; PP, 16. Total number at 2 weeks after i.d. challenge: BALT, 12; spleen, 14; PP, 22; frequency: BALT, 45; spleen, 10; PP, 14. Total number at 4 weeks after i.d. challenge: BALT, 10; spleen, 20; PP, 20; frequency: BALT, 40; spleen, 13; PP, 10.

response in the distant intestinal mucosa. Since we were able to detect *M. pulmonis*-specific clonal precursors in PPs both before and 3 days after i.d. challenge (data not shown) and at the much later times of 4 and 8 weeks (Table 2), we would interpret the absence of specific B cells in PPs after i.t. infection and i.d. challenge as the result of cell migration rather than as the failure of the chronic respiratory infection to raise the level of specific B cells in the gut-associated lymphoid tissue.

The substantial rise of *M. pulmonis*-sensitive B cells in the PB suggests that the dissemination of the primed state is due at least in part to B-cell migration via lymph and blood from local sites exposed to *M. pulmonis* cells. Respiratory tissues were shown to be a part of the lymphocyte recirculation pathway (10). Hence, *M. pulmonis*-sensitive B cells from PPs may be expected to contribute to the recirculating pool of "memory" cells, to the increase in frequency of Ag-specific B cells in the BALT, and to the plentiful specific plasma cells known to appear in the respiratory lamina propria after infection (5, 32).

The majority of T-dependent clones generated by *M. pulmonis*-specific B cells secrete exclusively IgG1, an isotype that is not ordinarily predominant after mucosal

stimulation. We have demonstrated that the exaggerated IgG1 response was not due to the accompanying viable donor T cells in the inoculum, nor could it be exclusively due to T cells from "carrier"-primed recipients, since the isotype profile of clones from anti-TNP-specific B cells generated in the same splenic fragments by TNP-*M. pulmonis* was similar to that previously observed with hemocyanin as a carrier rather than resembling the isotype profile of the anti-*M. pulmonis* clones (20, 29). Therefore, the exaggerated IgG1 response seems to be dependent on an intrinsic property of the B cells. The predominance of IgG1 has also been demonstrated in the BALT of athymic BALB/c mice that were primed with *M. pulmonis*. Thus, functional T cells are apparently not required at the time of in vivo priming to generate the precursors for the exaggerated IgG1 response. A caveat is that *M. pulmonis* may contribute to differentiation of effector T cells in athymic mice (16), a possibility that we are presently examining. Isotype analysis of *M. pulmonis*-specific B cells from PPs and spleens of primed athymic mice indicates that our splenic fragment cultures allow expression of all isotypes and thus supports our suggestion that exaggerated IgG1 expression by B cells from euthymic mice is due at least in part to their intrinsic properties and is not imposed by the assay. Related observations by Speck and Pierce (30) indicate that both a T-independent antigen (2,4-dinitrophenol-Ficoll) and a T-dependent analog (dinitrophenol-hemocyanin) prime athymic mice to give increased frequencies of specific B cells that generate clones in a T-dependent splenic fragment assay. The majority of those clones (70 to 90%) from primed B cells express some IgG1, but always along with IgM. We found that *M. pulmonis* cells prime both euthymic and athymic mice to generate B cells capable of an even more exaggerated IgG1 response. All these observations argue against the necessity for functional T cells to generate B cells with IgG1 potential, while still being consistent with a role for T cells in promoting the secretion of non-IgM serum antibodies in euthymic mice.

In vitro studies do show that expression of IgG1 secretion can be influenced by T-helper-type cell lines (23) or by the factors that they produce (15). Martinez-Alonso et al. (23) showed that addition of hapten-specific T-helper cells to hapten-derivatized normal spleen cells results in the appearance of IgM, IgG1, and IgG2 plaque-forming cells. B-cell cultures simultaneously stimulated by lipopolysaccharide and alloreactive T-helper cells preferentially generate IgG1, whereas cultures that get lipopolysaccharide without helper T cells make mostly IgM, IgG2(a+b) and Ig3 plaque-forming cells. Isakson et al. (15) showed that B-cell differentiation factors produced by T-cell lines enhance IgG1 secretion by cultures of B cells lacking surface IgG (sIgG⁻) cells in the presence of lipopolysaccharide. Thus it may be that *M. pulmonis* stimulation somehow directly influences the T cells in the splenic fragments required to generate antibody-secreting clones to exaggerate the likelihood that plasma cells in the fragment secrete IgG1. There are many observations of exaggeration of IgG1 responses in vivo after chronic mucosal infection with various pathogens. For instance, Cassel et al. (5) studied the antibody response to *M. pulmonis* cells in serum and tracheobronchial secretions of pathogen-free mice. They found that most of the infiltrated plasma cells in the lungs were producing IgA, but the next largest number of plasma cells were producing IgG1 or IgG2. The respiratory lamina propria of all uninfected control mice contained IgA-producing cells but did not contain detectable numbers of IgM-, IgG1- or IgG2-producing cells. Blandford

and Heath (2) were surprised to find a 20-fold increase of IgG-containing cells in the lungs of mice after Sendai virus infection. Much has also been published to date on the predominance of IgG1 after chronic parasitic infections (25). In experimental *Nematosporoides dubius* and *Mes-socestoides corti* infections of euthymic mice, exaggerated IgG1 production is T-cell dependent and is not observed in heavily infected athymic mice (7, 27).

Our data and observations made by others (8) suggest that IgG1 may have a role in either the resolution of mucosal infections or the pathogenesis they initiate. We would like to know why after certain infections, e.g., *M. pulmonis* or parasite infections, there is a predominance of IgG1 expressed over all other isotypes. We propose the following possibilities. (i) *M. pulmonis* acts on a T-helper-cell population in the irradiated recipient, which is primed with *M. pulmonis* Ag. Such T cells may preferentially express Fcγ1 receptor and therefore may help IgG1 responses selectively. Other workers have provided evidence for T-cell clones that express Fcα receptors and selectively exaggerate IgA responses (17). (ii) *M. pulmonis* infection results in extensive tissue damage and causes the appearance of autoreactive antibodies (5). *M. pulmonis* may also stimulate proliferation of autoreactive T-helper cells that in turn can exaggerate IgG1 plaque-forming cells in specifically stimulated clones. Such cells may act polyclonally on B cells responding to *M. pulmonis*, much as allo(auto)reactive T cells can act on clones initiated by lipopolysaccharide to exaggerate their content of IgG1 plaque-forming cells (23). (iii) The IgG1 response results from a direct action of *M. pulmonis* cells on B cells. By virtue of the structure of IgG1, *M. pulmonis* cells may bind preferentially to B cells with that isotype and cause their proliferation. When T-cell help is supplied, those B cells undergo terminal maturation and secrete IgG1. Which, if any, of these mechanisms are operative, the exaggerated expression of IgG1 must also reflect an intrinsic property of the specific B-cell population, since *M. pulmonis*-primed B cells display the phenomenon whereas unprimed TNP-specific B cells do not when analyzed in the very same splenic fragment cultures. Our ongoing studies should help to elucidate the mechanism of IgG1 predominance and the relative roles of B-cell differentiation versus T-cell regulation that may contribute to this phenomenon.

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