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Immunoglobulin G (IgG) and IgA Subclass Pattern of Human Antibodies to *Shigella flexneri* and *Salmonella* Serogroup B and D Lipopolysaccharide O Antigens

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The subclass distribution of human serum antibodies to the O-antigenic lipopolysaccharides of *Salmonella* serogroups B and D and to *Shigella flexneri* serotypes 1b, 2a, and 4a lipopolysaccharide antigens were analyzed in an enzyme-linked immunosorbent assay with monoclonal antibodies to the immunoglobulin subclasses. The patients had culture-verified *Salmonella* (17 Swedes) or *Shigella flexneri* (23 Vietnamese; 11 children and 12 adults) infections. Consecutive samples drawn during 1 year postinfection were investigated. Antibodies to the *Salmonella* antigens were mainly of the immunoglobulin G1 (IgG1), IgA1, and IgA2 subclasses. For the *Salmonella* serogroup B O polysaccharide, the IgA1 and IgA2 subclasses had peak values earlier than (6/9) or coinciding with the IgG1 (3/9) peak value. Furthermore, the IgA2 response to *Salmonella* serogroup B was positively correlated to the duration of the carrier state ($P < 0.001$); the corresponding IgA1 response was less well correlated but was still significant ($P < 0.02$). In the case of the *Shigella flexneri* O polysaccharide, specific antibodies appeared mainly in the IgG1 and IgA1 subclasses. Some IgG2 was also found, surprisingly even in very young patients. No subclass shift with time within the immunoglobulin classes was noted in any of the groups.

Human immunoglobulin subclasses are identified by different antigenic determinants (8, 12, 31, 33) and are also characterized by different physiochemical and biological properties. The antibodies appear restricted in response to various antigens (for reviews, see references 26 and 27 and F. Shakib, Monogr. Allergy, in press), but little is known about the influence of time and route of introduction of the antigen on the changes in subclass pattern of specific antibodies in humans. A subclass shift with time has been proposed for beekeepers who were repeatedly immunized (1); indications for a similar immunoglobulin G1 (IgG1) to IgG4 shift have also been reported for certain autoantibodies (29). In these studies, specific antibodies to protein antigens were investigated. Therefore, it was of interest to analyze consecutive serum samples for specific antibodies to polysaccharide antigens because carbohydrate antigens mainly induce IgG2 antibodies. We report here on subclass distribution patterns of specific antibodies to *Salmonella* serogroup B and D and *Shigella flexneri* O antigens in patients with infections verified by positive fecal cultures of the corresponding agents. The follow-up time was approximately 1 year after infection.

MATERIALS AND METHODS

Antigens. *Salmonella* serogroup B and D lipopolysaccharide O antigens were extracted, chemically characterized, and adsorbed to enzyme-linked immunosorbent assay microtiter plates as previously described (16, 20). The purification of *Shigella flexneri* lipopolysaccharide O antigens 1b, 2a, and 4a is described elsewhere (4). The antigens were coated to microtiter plates as previously described (16).

Antibodies. The following monoclonal antibodies to human IgG1-4 (21) were purchased from Seward Laboratories, Bedford, England: clone NL16 (anti-IgG1), GOM1 and GOM2 (both anti-IgG2), ZG4 (anti-IgG3), and GB7B (anti-IgG4). They were purified from ascitic fluid as previously described (25). Purified monoclonal antibodies to human IgA1-2 (5) were obtained from Becton Dickinson Europe, Mechelen, Belgium; they were clones 1-155-1 (anti-IgA1) and 14-3-26 (anti-IgA2). Rabbit anti-mouse immunoglobulins were purchased from DAKOPatts A/S, Copenhagen, Denmark, as product no. Z 109. The antiserum was extensively absorbed by glutaraldehyde-fixed human serum proteins. Alkaline-phosphatase-conjugated goat anti-rabbit IgG was purchased from Sigma Chemical Co., St. Louis, Mo., as product no. A 8025.

Enzyme-linked immunosorbent assay for subclass determination of specific antibodies. The method used for subclass determination has been described in detail elsewhere (25). In short, the following steps were included. (i) Serum samples were added to antigen-coated wells and allowed to incubate, followed by the addition of (ii) subclass-specific monoclonal antibodies, (iii) rabbit anti-mouse immunoglobulin antiserum, (iv) alkaline-phosphatase-conjugated goat anti-rabbit IgG, and (v) substrate. The optical density (OD) of the plates was read at 405 nm. Between each step, there were four washings. Incubation time after steps (i) and (iv) was overnight and after steps (ii) and (iii) was 4 h; all incubations were done at room temperature. Reagents and serum samples were diluted in phosphate-buffered saline with 0.05% Tween 20. The IgG and IgA subclass-specific monoclonal antibodies were titrated so as to give roughly comparable OD values when reacting with a fixed amount of the corresponding myeloma protein; in our initial work with the assay system, we found it beneficial to use two monoclonal antibodies to

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TABLE 1. Subclasses of antibodies to *Salmonella* group B O antigen which appeared during the observation period (4 to 360 days postinfection)

Patient no.	Range of OD_{405} values minus background (0.10) for subclass:						Day postinfection of peak value for subclass:					
	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
1	0.01–0.76	0	0	0	0–0.19	0–0.02	34				15[a]	15[a]
2	0–0.02	0	0	0	0	0–0.05	24					24
3	0–0.19	0	0	0	0–0.11	0–0.15	40				8[a]	8[a]
4	0–0.67	0	0	0	0–0.89	0–>1.90	94				13	13
5	0–0.81	0	0–0.01	0	0.07–0.79	0–0.52	22				14	8[a]
6	0–0.10	0	0–0.03	0	0.05–0.18	0–0.18	23				23	4, 23[b]
7	0–0.06	0–0.03	0	0	0–0.39	0–1.56	211				29	29
8	0–0.37	0–0.55	0	0.06–0.20	0–0.44	0–0.71	7	7		7, 31[b]	7	7
9	0.17–>1.9	0–0.09	0–0.13	0.08–0.29	0–0.30	0–0.01	60	25[a]	25[a]	25[a]	25[a]	25[a]

[a] First sample postinfection.
[b] Two samples equally high.

IgG2 because they are less avid for their antigen than are the other IgG-subclass-specific monoclonal antibodies (25), although after completing this study we found no disadvantage if the clone GOM1 was used separately. The plates were read after 30 (IgG1-4) or 60 (IgA1-2) min. The results are given as net OD_{405} values with the background, the absorbance value plus two standard deviations for an immunoglobulin-negative sample run two to four times for each antigen, subtracted.

Patients. A total of 17 Swedish patients with culturally verified *Salmonella* infections were analyzed. Nine of the patients suffered from a *Salmonella* serogroup B infection (five females and four males; ages, 17 to 64 years; mean age, 37; median age, 32), whereas eight of the patients had a *Salmonella* serogroup D infection (three females and five males; ages, 20 to 71 years; mean age, 38; median age, 37). Four and six patients, respectively, were treated with antibiotics. The main symptom was diarrhea, although two of the patients with a *Salmonella* serogroup D infection had uncommon manifestations (thyroiditis and orchitis). Sera from 13 *Shigella*-infected Vietnamese patients were assayed for antibodies to the O antigen of the corresponding serotype; there were eight patients with serotype 1b (four females and four males; ages, 1 to 28 years; mean age, 6.5; median age, 2.5), three patients with serotype 2a (one 27-year-old male and two 1-year-old females), and two patients with serotype 4a (two 1-year-old children, one of each sex). Samples were drawn at approximately 10, 30, 100, 180, and 360 days postinfection.

Identification of bacteria. The *Salmonella* and *Shigella* isolates were identified by the method of Edwards and Ewing (9). In the Vietnamese group, no subsequent fecal samples were taken after the initial one. For the *Salmonella*-infected individuals, consecutive fecal samples were cultured until three to five negative cultures were reported, at which time the patient was declared a noncarrier.

RESULTS

Subclass distribution of specific antibodies to *Salmonella* serogroup B and D O lipopolysaccharide antigens. Antibodies to the serogroup B antigen were of the IgG1, IgA1, and IgA2 subclasses (Table 1). The IgA1 and IgA2 peaks appeared within 30 days postinfection, and in six cases (out of nine) they occurred before the IgG1 peak (Fig. 1A through C); for three patients the IgA1 and IgA2 peaks and the IgG1 peak coincided (Fig. 1D).

Specific antibodies to the serogroup D O antigen were not found in all individuals in the group, but if found, the specific antibodies detected were mainly IgG1, IgA1, and IgA2 isotypes; IgG2, IgG3, and IgG4 were seen in occasional samples (Table 2). The order of appearance of the specific antibodies of the different subclasses was not consistent.

Subclass distribution of specific antibodies to the *Shigella flexneri* O lipopolysaccharide antigen. Antibodies to *Shigella* serogroups 1b, 2a, and 4a appeared mainly in the IgG1, IgG2, and IgA1 subclasses in patients infected by bacteria of the corresponding group. In addition, a few cases had specific antibodies of the IgG3, IgG4, and IgA2 subclasses (Table 3). IgG1 against antigen 1b, if present, remained at elevated levels (OD_{405} >80% of the peak value) in the latest sample at approximately 360 days postinfection (7 of 8 patients); this was less frequently the case for IgG2 (3 of 8 patients). Such a persistence in serum was not seen for the IgA subclasses (data not shown).

We later analyzed sera from 10 Vietnamese adults, drawn at approximately 10 and 180 days postinfection (*Shigella flexneri* serotype 1b). The subclass pattern found was identical to that for the children initially analyzed, mainly IgG1, IgG2, and IgA1, with a few individuals also having IgG3 and IgG4 (data not shown). Additionally, in six Swedes (ages 18 to 48 years) we recently found the same subclass pattern in serum samples, one from each individual, taken 21 to 119 days postinfection with *Shigella flexneri* serotype 1b (data not shown). None of these Swedes had any reinfection, as determined by negative fecal samples subsequent to the acute disease.

Correlation of specific antibodies and duration of the carrier state. The time from suspected infection to declaration as a noncarrier was positively correlated to the antibody response in patients infected by *Salmonella* serogroup B bacteria. A mean absorbance value for the specific antibodies from the first three consecutive samples, drawn approximately 10, 30, and 100 days postinfection, was calculated, and this value was correlated to the duration of the carrier state. Linear regression of these values on a log/linear basis (mean absorbance/carrier state duration) showed that the specific IgA2 responses were significantly positively correlated ($r = 0.92$, $P < 0.001$) to the duration of the carrier state, the IgA1 responses were slightly less correlated ($r = 0.79$, $0.02 > P > 0.01$), and the IgG1 responses were not correlated ($r = 0.48$, $P > 0.20$).

The corresponding calculations could not be done for the serogroup D analysis because too many of our samples were negative in the subclass assay.

Antibody response in atypically infected patients. No spe-

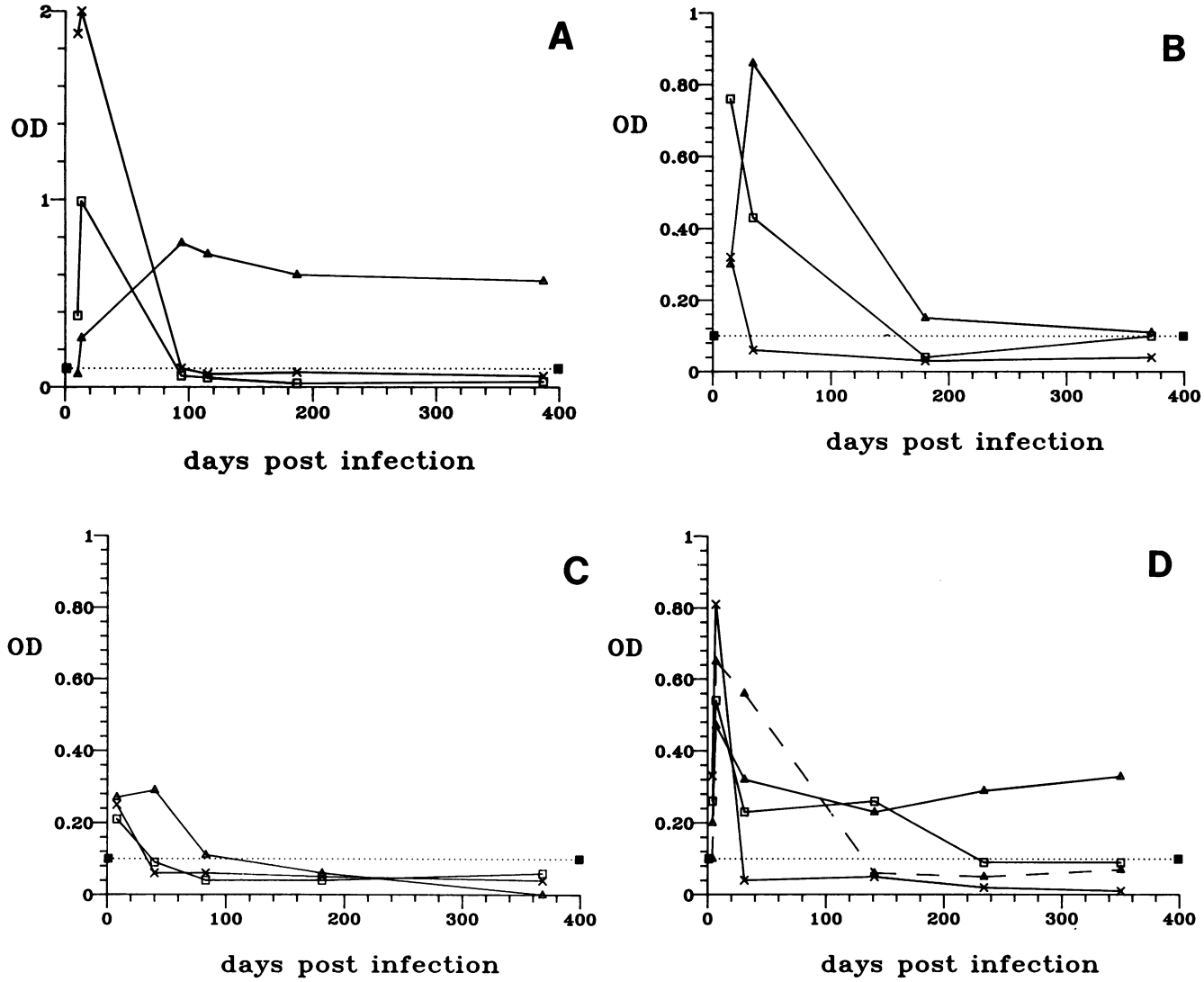

FIG. 1. OD_{405} values for subclasses of specific antibodies to *Salmonella* group B O antigen in samples from four individuals. Symbols: ■, background level; △—△, IgG1; ▲ - - -▲, IgG2 (positive only in 1D); □, IgA1; ×, IgA2.

cial features regarding the subclasses of the specific antibody response were observed for the patients with atypical manifestations (orchitis and thyroiditis) compared with the patients with only enteritis.

DISCUSSION

In the present study, our aim was to analyze whether a subclass shift occurred with time in antipolysaccharide antibodies, as has been reported for human antiprotein specific antibodies (1, 29). Three major sets of results were obtained. (i) The subclass distribution for specific antibodies to *Salmonella* serogroup B and D O lipopolysaccharides in serum was IgG1, IgA1, and IgA2. The corresponding pattern for antibodies to *Shigella flexneri* O lipopolysaccharides was IgG1, IgG2, and IgA1. (ii) For specific antibodies to *Salmonella* serogroup B O lipopolysaccharides, IgA appeared in serum before IgG in a majority of cases. (iii) The IgA2 response and, to a lesser extent, the IgA1 response to the *Salmonella* serogroup B O antigen correlated to the duration of the carrier state; for specific IgG1, no such correlation was found.

The basis for the subclass restriction of specific antibodies remains elusive. Most investigations dealing with the subclass pattern of specific antibodies have analyzed the pattern found in serum (for reviews, see reference 27 and Shakib, in press); a few studies have looked for production of specific antibodies in cell culture supernatants (30, 32). However, several recent studies have examined the influences on the immunoglobulin-heavy-chain switch of cells in vitro, analyzing the total immunoglobulins produced (3, 7, 14, 22, 23, 34). Several factors are known to influence the subclass pattern of the total immunoglobulin production and of specific antibodies. The type of antigen is one factor; proteins mainly elicit IgG1 and IgG3 or IgG4, whereas polysaccharide antigens mainly elicit IgG2. The pattern of IgA subclasses does not seem to be uniform for either of these two major types of antigens (27; Shakib, in press). A second factor is the age of the individual; pneumococcal polysaccharide responses are confined to the IgG1 subclass in very young children, whereas later a shift towards IgG2 is seen and in adults all IgG against the antigen may be of the IgG2 subclass (10). Repeated immunizations have also been shown to change the subclass pattern of protein-specific antibodies (1, 29). The locale for the immune reaction is a third factor which is

TABLE 2. Subclasses of antibodies to *Salmonella* group D O antigen which appeared during the observation peiod (4 to 360 days postinfection)

Patient no.	Range of OD_{405} values minus background (0.10) for subclass:						Day postinfection of peak value for subclass:					
	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
1	0	0	0	0	0	0						
2	0.02–1.90	0	0	0	0–0.03	0	29				29	
3	0.08–0.88	0–0.02	0–0.84	0	0–0.27	0	91[a]	116	116		116	
4	0.28–0.47	0	0	0	0	0–0.03	27[a]					118
5	0–0.03	0	0	0	0	0–0.05	6[a]					6[a]
6	0–0.02	0	0	0	0	0–0.01	200					200
7	0	0	0	0	0	0						
8	0–0.07	0.37–1.37	0	0–0.15	0–0.41	0–0.28	94	39		20, 39[b]	6[a]	6[a]

[a] First sample postinfection.
[b] Two samples equally high.

probably of importance because T cells from Peyer's patches have been shown to preferentially induce an IgM-to-IgA switch in immunoglobulin-bearing cells in mice (17, 18). Furthermore, the distribution of cells containing immunoglobulins of different subclasses is known to vary in different organs (2, 28; K. Kett, P. Brandtzaeg, J. Radl, and J. J. Haaijman, J. Immunol., in press). Finally, because T cells and T-cell-derived products influence which subclasses are produced by B cells after polyclonal activation (3, 7, 14, 22), the degree of T-cell dependence of the immune response to an antigen may be an important factor for the subclass pattern.

The subclass pattern found for the polysaccharide-specific antibodies analyzed in our study is consistent with earlier reports on polysaccharide antibodies. However, our findings of substantial amounts of IgG2, in addition to IgG1, to the *Shigella* antigens even in very small children are somewhat surprising in view of our earlier studies on pneumococcal polysaccharide-specific antibodies in Swedish children, in which IgG1 was the first subclass to appear (10). It is possible that the nature of the antigen (the *Shigella* antigen is a glycolipid, the pneumococcal antigens are pure polysaccharides), the way of introduction of the antigen, repeated contacts with the antigen beginning very early in life, or a combination of these factors was the main basis for these findings. The difference in the genetic background of the individuals studied could also have been a reason for the different patterns in children, although Swedish and Vietnamese adults had identical patterns of these specific antibodies.

The possibility that the locale where the immune response takes place influences the subclass pattern may find support in our results on the kinetics of the responses. IgA appeared before IgG, and IgA was also significantly correlated to the duration of the *Salmonella* serogroup B carrier state, whereas IgG was not. Furthermore, the antigens used in the present work were polysaccharides, which may be less dependent on T-cell help for an immune response to occur. The lack of any subclass shift within the immunoglobulin classes could be a result of this; thus, protein antigens may be more likely to induce a switch with time, because they have been shown to be T cell dependent.

The differing IgA subclass patterns for *Salmonella* and *Shigella* antibodies is surprising. From recent data on the distribution of immunoglobulin-containing cells, it would be assumed that infections in the colon may give rise to IgA2 antibodies to a greater extent than would an infectious process engaging the more proximal parts of the gastrointestinal tract because the proportion of IgA2-containing cells increases distally (Kett et al., in press). *Shigella* infection, known to affect mainly the colon and the terminal ileum, would therefore elicit an IgA2 response. Our results contradict this idea. However, Conley and Koopman (6) have recently shown that certain IgA2-producing cells may lack the capability of secreting this immunoglobulin, a phenomenon that could explain the observed dichotomy. Further-

TABLE 3. Subclasses of antibodies to *Shigella flexneri* 1b, 2a, and 4a antigens which appeared during the observation period

Antigen	Patient no.	Age (yr)	Range of OD_{405} values minus (0.15 for antigen 1b and 0.10 for antigens 2a and 4a) for subclass:						Day postinfection of peak value for subclass:					
			IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
1b	1	1	0.23–0.45	0	0	0	0	0	40					
	2	28	0.65–0.87	0.24–0.54	0	0–0.04	0–0.03	0	18[a]	111		48	18[a]	
	3	3	1.39–1.59	0.54–1.31	0–0.16	0	0.01–0.30	0	43	43	13[a]		13[a]	
	4	2	0.97–1.26	0–1.14	0	0	0–0.14	0	47	15[a]			15[a]	
	5	7	1.16–1.28	1.02–1.09	0	0	0–0.003	0	8[a]	39			8[a]	
	6	2	1.52–1.57	1.17–1.42	0.02–0.53	0–0.09	0–0.21	0	146	146	6[a]	6[a]	6[a]	
	7	7	0.22–0.54	0.07–0.30	0–0.07	0.03–0.19	0–0.05	0	42	42	42	115	9[a]	
	8	2	0.97–1.33	0–0.83	0–0.01	0–0.13	0–0.05	0	99	46	46	99	6[a]	0
2a	9	1	0.89–1.11	0.23–1.06	0–0.003	0–0.11	0–0.11	0	24[a]	24[a]	24[a]	24[a]	54	
	10	1	0–0.46	0	0	0	0	0	36					
	11	27	0.15–0.45	0.03–0.19	0	0	0	0	99	52				
4a	12	1	0.32–1.43	0	0–0.01	0	0	0	19[a]		19[a]			
	13	1	0.59–1.03	0–0.44	0–0.14	0	0–0.22	0–0.21	9[a]	41	41		9[a]	9[a]

[a] First sample postinfection.

more, the different patterns of invasion of the intestinal tissues may play a role, with *Shigella* spp. infecting and damaging the epithelial cells and *Salmonella* spp. invading beyond these cells (for a review, see reference 19).

For specific antibodies to *Salmonella* serogroup B O antigens, IgA appeared before or with the specific IgG. This can be explained in two ways: (i) totally different clones were active, some giving rise to the IgA response and others being IgG producers, or (ii) one IgA- and one IgG-producing clone developed from the same original B cell. The latter explanation may imply that IgG production is regulated differently from IgA production because we clearly saw different kinetics. However, the correlation of specific IgA2 and IgA1 responses (in contrast to IgG responses) to the duration of the carrier state is compatible with the idea that IgG and IgA are produced at different sites. Distinct immunoglobulin class patterns of specific antibodies in serum depending on different routes of antigen introduction have previously been shown (11, 13, 24).

Taken together, our data may suggest the following hypothesis. The specific IgA1 and IgA2 analyzed in serum stem from local production in the gut mucosa near the site of infection. These IgA antibodies appear immediately after infection and are probably produced by cells already committed to IgA production. The prominence of the response is influenced by the presence of bacteria in the gut. The specific IgG appears later and is therefore produced by B cells distinct from those which produce the initially seen IgA. It is perhaps not produced in the intestinal immune tissues and reflects more the subclass restriction found for any polysaccharide antigen, IgG1 appearing first in ontogeny and IgG2 later. The different subclass patterns seen for antibodies to the various antigens may be based on a selective switch in committed cells, with certain VDJ combinations preferentially combining with certain immunoglobulin-heavy-chain genes, because the IgA subclass patterns for the antibodies analyzed deviated from what was expected from studies on immunoglobulin-containing cells. Furthermore, the subclass pattern of specific antibodies has not been shown to be influenced by the mitogen used for stimulation of peripheral, already committed cells in culture, although the mitogens as such give rise to different amounts of total IgG subclasses (23, 32, 34).

Further investigations regarding the mitogenic and differentiating effects on lymphocytes of products from the cell walls of bacteria (15), T-cell influence on the immune response to intestinal bacteria, and in vitro-produced specific antibodies may help us in further elucidating these questions.

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