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Lack of IgA subclass restriction in antibody response to phosphorylcholine, β lactoglobulin and tetanus toxoid

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Summary. Although there is IgG subclass restriction in the antibody responses to most antigens, our data indicate that the human IgA subclasses, IgA, and IgA_2, do not demonstrate a similar antigen specific restriction. We did not find evidence for IgA subclass restriction in the antibody responses to phosphorylcholine (PC), β lactoglobulin or tetanus toxoid. These antigens were chosen to represent carbohydrate-like versus protein antigens and antigens presented through the mucosal route versus the humoral route. For each of these antigens the proportion of antigen specific IgA that was IgA_1 and IgA_2 was similar to that of total serum IgA. IgA anti-PC, which is thought to be directed against the phosphorylcholine moieties found on certain bacterial polysaccharides, could be found in the serum of all individuals tested and constituted 0·063–0·088% of the total serum IgA. IgA anti-β lactoglobulin and anti-tetanus toxoid could be measured only in the serum of selected individuals, usually those with known milk protein sensitivity, or those recently immunized with tetanus toxoid. The lack of a marked subclass restriction of IgA responses to these antigens stands in contrast to results obtained by others for IgG antibodies, in which carbohydrates and proteins preferentially stimulate antibodies in different IgG subclasses.

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INTRODUCTION

IgA in human serum and secretions can be divided into two subclasses, IgA_1 and IgA_2, based on antigenic and biochemical differences (Kunkel & Prendergast, 1966; Vaerman & Heremans, 1966; Feinstein & Franklin, 1966; Abel & Grey, 1971; Tsuzukida, Wang & Putnam, 1979). Functional differences between the two subclasses have not yet been described. However, the predominance of IgA_1 in the serum, the lack of correlation between the serum concentrations of IgA_1 and IgA_2 (Conley & Koopman, 1983) and the observation that in certain clinical disorders, such as systemic lupus erythematosus, there is selective increase of IgA_1 (Conley & Koopman, 1983) indicate that there is independent regulation of IgA_1 and IgA_2. This further suggests that functional differences between the two subclasses probably do occur.

Distinguishing functional characteristics have been defined for the IgG subclasses in man and several other mammalian species (Spiegelberg, 1974). The IgG subclasses vary in their ability to fix complement (Ishizaka *et al.*, 1967), bind to Fc receptors (Huber *et al.*, 1971; Lawrence, Weigle & Spiegelberg, 1975) and enhance or suppress the primary immune response

(Gordan & Murgita, 1975). But perhaps one of the most intriguing differences relates to antigen specific responses. Certain antigens, most notably carbohydrates, elicit an antibody response of a particular isotype. The anti-carbohydrate response in man is IgG_2 (Yount *et al.*, 1968; Riesen, Skvaril & Braun, 1976) and in mice IgG_3 (Perlmutter *et al.*, 1978). In both species the major IgG anti-protein response is of the IgG_1 subclass (Yount *et al.*, 1968; Perlmutter *et al.*, 1978).

To determine if the IgG subclass restriction seen in response to protein and carbohydrate antigens is also seen in the human IgA subclasses, we measured the IgA subclass of antibody directed against three antigens, phosphorylcholine (PC), tetanus toxoid and β lactoglobulin. These antigens were chosen to represent carbohydrate-like versus protein antigens; T independent *vs* T dependent antigens; and antigens presented through the mucosal *vs* the humoral route. PC has many properties in common with carbohydrate antigens. It is the immunodominant group of pneumococcal C-polysaccharide where it occurs as a simple, repeated antigenic determinant (Leon & Young, 1970). Like most carbohydrates, it is a T cell independent antigen (Howard *et al.*, 1971) that elicits an IgG_3 response in mice (Perlmutter *et al.*, 1978). PC is common to several microbial species including lactobacilli which are part of the normal gut flora (Potter 1971). In contrast to most carbohydrate antigens, antibody against PC can be found in the serum of most children over 6 months of age (Gray, Dillon & Briles, 1983) and virtually all adults (D. E. Briles *et al.*, manuscript in preparation).

Tetanus toxoid elicits a well characterized IgG response in man (Stevens & Saxon, 1978; Stevens, Benveniste & Pineda, 1982) that is T cell dependent and mostly of the IgG_1 subclass (Yount *et al.*, 1968). This protein antigen is presented through the systemic route. In contrast, β lactoglobulin is a protein antigen presented through the mucosal route.

MATERIALS AND METHODS

Antibodies

The production and characterization of the monoclonal antibodies against IgA_1 (1-155-1, a mouse $\gamma_3\lambda$), IgA_2 (14-3-26, a mouse $\gamma_{2b}\kappa$), have been previously described (Conley *et al.*, 1980). The heterologous goat anti-IgA used in these studies has also been described (Conley & Koopman, 1983). To insure that the anti-IgA did not have any subclass specificity, antibody from a goat immunized with an IgA_1 κ myeloma protein was eluted off a column to which an $IgA_2\lambda$ myeloma protein had been fixed. Peroxidase labelled goat anti-mouse antibody was purchased from Cappel, Cochranville, Pa.

Affinity columns

Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated by cyanogen bromide (March, Parikh & Cuatrecasas, 1974) and 10-ml aliquots were coupled to 24 mg of tetanus toxoid (Wyeth Laboratories, Marietta, PA), 36 mg of β lactoglobulin (Sigma Chemical Co., St. Louis, Mo) and 27 mg of heterologous goat anti-human IgA. PC conjugated sepharose and PC-human serum albumin (HSA) were prepared by the method of Cheseboro & Metzger, 1972.

Purification of PC-specific IgA

IgA was purified from 2–3 ml samples of serum from four healthy adults by passage over a Sepharose column to which goat anti-human IgA had been fixed. The IgA was eluted with a glycine-HCl buffer, concentrated to 1–3 mg in 1 ml, dialysed against borate saline pH 8.2 and then passed over a 2 ml column to which PC-HSA had been fixed. When the protein concentration in the effluent had reached baseline, as measured by a continuous reading spectrophotometer, 0·1% PC in 0·1% bovine serum albumin (BSA) in borate saline pH 8·2 was added to the column to competitively elute the IgA-anti PC. Five 2-ml fractions were collected and the amount of IgA in each fraction was assayed by radioimmunoassay. The peak concentration of IgA was in the second fraction in all four experiments. The IgA containing fractions were then pooled, concentrated and dialysed against borate saline.

Radioimmunoassay

The radioimmunoassay (RIA) was performed as previously described (Conley *et al.*, 1983). Polystyrene wells of microtitre plates were coated with monoclonal anti-IgA_1 or anti-IgA_2 or heterologous goat anti-IgA. After blocking remaining protein binding sites with 0·5% BSA the test samples were added and incubated overnight. After washing the wells, ^{125}I-tagged heterologous anti-IgA was added and incubated for 4 hr. The wells were then washed and counted in a Beckman Gamma 4000. Concentrations of IgA_1, IgA_2 and total

IgA were derived from a standard curve generated by a known sample run in parallel.

Enzyme-linked-immunoabsorbant assay (ELISA)
Polystyrene wells of microtitre plates were coated with the antigens PC-human serum albumin, β lactoglobulin, or tetanus toxoid at 6–10 μg/ml. After blocking remaining protein binding sites with 0·5% BSA in borate saline, the test samples were added. In parallel, the same serum dilutions were added to HSA coated wells to control for non-specific protein binding. All samples were tested in duplicate at four dilutions. After an overnight incubation the wells were washed and supernatants from hybridoma cultures of anti-IgA_1 (1-155-1), or anti-IgA_2 (14-3-26) were added and incubated for 4 hr. The wells were then washed and peroxidase-labelled goat-mouse antibody was added and incubated for 2 hr. The wells were washed and enzyme substrate, *o*-phenyldiamine (Sigma Co., St Louis, Mo) was added. The optical density was read at 450 nm on a Dynatech MR 580 Micro ELISA Auto Reader. The optical density of the HSA-coated wells was subtracted from that of the antigen specific wells. The titre was taken as the highest serum dilution that gave an optical density twice as great as the background.

RESULTS

Direct binding assay for IgA anti-phosphorylcholine

A direct binding ELISA assay was used to measure anti-PC antibody of the IgA_1 and IgA_2 subclasses. Serum was added to PC-coated plates at various dilutions. After an overnight incubation, monoclonal murine antibody to human IgA_1 or IgA_2 was added, followed by peroxidase-labelled goat anti-mouse immunoglobulin and then the *o*-phenyldiamine substrate. IgA anti-PC of both IgA_1 and IgA_2 subclass could be measured in approximately equal titres in all ten individuals tested (Table 1). These results demonstrated that there was not a complete IgA subclass restriction in antibody response to PC. However, they suggested that there might have been an enrichment for IgA_2 in the antigen specific response, since IgA_2 constitutes only 10–30% of the total IgA (Conley *et al.*, 1983) and there were similar titres of IgA_1 and IgA_2 anti-PC. We recognized however, that the titre, or optical density, measured in this assay was dependent in part on the affinity of the monoclonal anti-subclass antibodies for their targets, and the affinity of the goat anti-mouse antibody for the particular mouse anti-IgA antibodies involved. We also knew that the anti-IgA_2 (14-3-26) was a very high affinity antibody. To partially control for these affinity differences a similar assay was used to quantitate total serum IgA_1 and IgA_2. Dilutions of serum were added to wells coated with heterologous goat anti-human IgA. The rest of the assay was performed in the same manner as the PC specific assay. Monoclonal anti-IgA subclass antibodies were added, followed by the peroxidase labelled anti-mouse immunoglobulin and the substrate. In most assay IgA_1 and IgA_2 could be diluted to the same titre. The curves generated in this part of the assay allowed us to normalize our IgA_1 and IgA_2 antigen specific titres, and demonstrated that the PC specific IgA_1 and IgA_2 both constituted approximately 0·05% of the total IgA_1 or IgA_2. These data did not show an enrichment for IgA_1 or IgA_2 in the PC specific antibody.

Table 1. Serum titres of IgA_1 and IgA_2 anti-PC as measured by a direct binding ELISA*

Serum donor	Titre of IgA_1	Titre of IgA_2
1	1:64	1:32
2	1:64	1:256
3	1:4	1:2
4	1:64	1:256
5	1:8	1:2
6	1:64	1:256
7	1:32	1:8
8	1:16	1:16
9	1:32	1:8
10	1:64	1:64

* The results are expressed as the highest serum dilution that gave an optical density twice as great as the background.

Quantitation of IgA_1 and IgA_2 in purified IgA anti-phosphorylcholine

A second method was also used to determine if there were IgA subclass restriction in the antibody response to PC. IgA anti-PC was purified, and the proportion of IgA_1 and IgA_2 in this PC specific IgA was measured. IgA was purified from serum by absorption and elution from an affinity column to which goat anti-human IgA had been fixed. This IgA was passed over a column to which PC coupled to human serum albumin

had been coupled. When the protein concentration in the effluent had reached baseline, 0·1% PC in 0·1% BSA in borate saline was added to the column to competitively elute the IgA-Anti-PC. IgA_1, IgA_2 and total IgA were measured by RIA in the purified IgA and in the PC-specific IgA isolated from it. IgA anti-PC was present in the serum of all four individuals tested and constituted between 0·063 and 0·088% of the total IgA (Table 2). In all cases the percentage of antigen specific IgA that was IgA_1 or IgA_2 was similar to the percentage of preabsorbed IgA that was IgA_1 or IgA_2. These results confirm those of the direct binding assay and provide further evidence that there is not an enrichment for IgA_1 or IgA_2 in PC specific IgA.

Characterization of IgA anti-β lactoglobulin and anti-tetanus

Using similar procedures, the subclass of IgA anti-tetanus toxoid and anti-β lactoglobulin were determined. The purified IgA that did not bind to the PC column was passed over a tetanus toxoid affinity column, and then a β-lactoglobulin affinity column. The antigen specific IgA was eluted with glycine HCl, neutralized, concentrated and dialysed. The IgA_1, IgA_2 and total IgA were then measured by RIA. From one of the four individuals tested, we were able to isolate detectable amounts of IgA against both tetanus toxoid and β lactoglobulin. From another individual, IgA against β lactoglobulin but not against tetanus toxoid could be measured. From the remaining two no IgA could be isolated on either the tetanus toxoid or the β lactoglobulin column. The amount of IgA against each antigen was not correlated with the total amount of IgA passed over the columns, making it unlikely that the isolated antibody was absorbed on the columns non-specifically. In all cases, the proportion of the antigen specific IgA that was IgA_2 was only slightly less than the proportion of IgA_2 in the preabsorbed samples (Table 3).

A direct binding ELISA assay was also used to examine the titre of IgA_1 and IgA_2 anti-β lactoglobulin and anti-tetanus toxoid. Serum samples from eight individuals were assayed for IgA antibody directed against β lactoglobulin; four were from babies with known milk protein sensitivity (Sera 15–18) and the other four (Sera 19–22) were from unselected adults. All of the babies had significant levels of IgA anti-β lactoglobulin. This antibody was comprised of both IgA_1 and IgA_2 in equal titres (Table 4). Three of the four samples from adults had no measureable IgA anti-β lactoglobulin, although all had total serum concentrations of IgA higher than the babies. One asymptomatic adult did have a low titre of IgA anti-β lactoglobulin. This antibody also consisted of IgA_1 and IgA_2 in equal titres (Table 4).

Serum samples from eight adults were also assayed for IgA antibody directed against tetanus toxoid; three were from individuals given a tetanus booster immunization 10–20 days prior to having the sample drawn (Sera 23–25); three were from subjects boosted 1–6 years earlier (Sera 26–28); and two were from adults

Table 2. Characteristics of PC-specific IgA eluted from a PC-HSA affinity column

Serum donor		Percentage of IgA that is IgA_2*	Percentage of total IgA that is PC-specific†	Amount of IgA passed over PC-HSA column
11	Preabsorbed	22		
	PC-specific	28	0·066	1·0 mg
12	Preabsorbed	24		
	PC-specific	24	0·088	1·2 mg
13	Preabsorbed	31		
	PC-specific	33	0·063	2·7 mg
14	Preabsorbed	21		
	PC-specific	20	0·083	2·7 mg

* IgA_1, IgA_2 and total IgA were measured by RIA in an aliquot of the purified IgA passed over the PC-HSA column (pre-absorbed) and in the PC specific IgA eluted from the column. The percentage of IgA that is IgA_2 was calculated as $100 \times IgA_2/\text{total IgA}$.

† Calculated as $100 \times \frac{\text{amount of IgA eluted from PC-HSA column}}{\text{amount of IgA passed over PC-HSA column}}$.

Table 3. Characteristics of β lactoglobulin and tetanus toxoid specific IgA eluted from affinity columns

Serum donor		Percentage of IgA that is IgA_2*	Percentage of total IgA that is antigen specific
13	Preabsorbed	31	
	β-lactoglobulin specific	14	0·004
	Tetanus-toxoid specific	17	0·0035
14	Preabsorbed	21	
	β-lactoglobulin specific	15	0·0031

* Calculated as in Table 2.

who had not received a tetanus booster in over 20 years (Sera 29, 30). IgA anti-tetanus could be measured in serum samples from all individuals who had been immunized within the preceding 6 years. In all cases, the titre of IgA_1 and IgA_2 anti-tetanus toxoid were similar (Table 4).

An ELISA assay was run in parallel to assay the serum IgA_1 and IgA_2 in all samples tested for antibody against tetanus toxoid or β lactoglobulin. The curves generated in this part of the assay were used to normalize the antigen specific titres. The normalized data are expressed as the percentage of the total IgA_1 or IgA_2 that was specific for the antigen in question (Table 4). In all samples in which IgA anti-β lactoglobulin or anti-tetanus toxoid could be measured, the proportions of total IgA_1 and IgA_2 that were antigen specific were similar. In some experiments there was a slight, but not significant enrichment for IgA_2.

DISCUSSION

To determine if certain antigens elicit an IgA subclass specific response in man, the IgA subclass of anti-phosphorylcholine, anti-β lactoglobulin and anti-tetanus toxoid was measured. IgA anti-PC could be detected in all samples assayed and constituted between 0·063 and 0·088% of the total IgA. IgA anti-β lactoglobulin and anti-tetanus toxoid could only be measured in the serum of selected individuals, usually those with known milk protein sensitivity, or those recently immunized with tetanus toxoid. In all cases, the subclass distribution of the antigen specific IgA was similar to that of the total serum IgA. There was not an enrichment for IgA_1 or IgA_2 in the antibody directed against PC, β-lactoglobulin or tetanus toxoid.

Several factors indicate that the IgA studied in these experiments was truly antigen specific. The method used to purify PC-specific IgA was the same as that used by Perlmutter *et al.* (1978), to demonstrate an enrichment for murine IgG_3 in anti-PC antibody. The percentage of total IgA that was PC specific was similar in the two assays used and was also similar to that of total IgM that we have found to be PC specific (Gray, Dillon & Briles, 1983). In the direct binding assays for β-lactoglobulin and tetanus toxoid, the amount of IgA bound to the antigens did not correlate with the amount of total IgA in the serum and antigen binding was seem most commonly in serum samples that might clinically be expected to have antigen specific antibody.

These results indicate that the mechanisms by which certain antigens elicit a subclass specific IgG response do not play a major role in the regulation of IgA subclasses. The identity of the factors that regulate the synthesis of the IgG subclasses remain controversial. T cells and T cell factors undoubtedly play a role. In athymic mice, the concentrations of serum IgG_2 and IgG_1 are decreased, whereas IgG_3 levels remain normal (Bloemmen & Eyssen, 1973; Bankhurst, Lambert & Miescher, 1975). In addition, providing athymic mice with T cells enhances the proportion of antigen specific antibody of the IgG_{2a} and IgG_{2b} isotypes, even when the antigen is T independent (TNP Ficoll) (Mongini, Paul & Metcalf, 1982). T cell factors have also been shown to play a role in IgA regulation. T cell clones that enhance IgA synthesis and secretion have been reported in both mice and humans (Kawanishi, Saltzman & Strober, 1982; Mayer, Fu & Kunkel, 1982). Whether these factors might have different effects on IgA_1 and IgA_2 is not known. We have found that altering T cell help in pokeweed mitogen stimu-

Table 4. Serum titres of IgA_1 and IgA_2 anti-β-lactoglobulin and anti-tetanus toxoid as measured by direct binding ELISA assays*

Antigen lactoglobulin	Serum donor	Titre of IgA_1	Titre of IgA_2	Percentage of IgA_1 that is antigen specific	Percentage of IgA_2 that is antigen specific	Serum IgA†
	15	1:80	1:80	0·10	0·10	29
	16	1:40	1:40	0·25	0·40	17
	17	1:640	1:640	0·4	0·55	54
	18	1:160	1:160	2·5	2·5	26
	19	<1:5	<1:5	—	—	130
	20	1:20	1:20	0·20	0·25	78
	21	<1:5	<1:5	—	—	165
	22	<1:5	<1:5	—	—	155
Tetanus toxoid	23	1:960	1:960	3·0	2·5	269
	24	1:2048	1:2048	0·8	3·2	189
	25	1:40	1:10	0·031	0·033	60
	26	1:256	1:256	0·03	0·10	138
	27	1:20	1:80	0·05	0·10	130
	28	1:320	1:320	0·80	1·07	305
	29	<1:5	<1:5	—	—	212
	30	<1:5	<1:5	—	—	105

* The results are expressed as the highest serum dilution that gave an optical density twice as great as the background.
† Expressed as mg/dl.

lated cultures, by adding limiting numbers of T cells or irradiated T cells, does not change the proportion of IgA plasma cells expressing IgA_1 or IgA_2 (M. E. Conley & M. S. Bantelt, submitted). This observation, along with the finding that IgA antibody against the T independent antigen phosphorylcholine is not enriched for IgA_1 or IgA_2, suggests that the IgA subclasses do not differ in their ability to respond to T dependent and T independent antigens.

Many of the current models for regulation of the relative proportion of each isotype synthesized, have focused on genetic aspects of antibody production. It has been suggested that the IgG subclass restriction of an antibody response to a particular antigen might be due to selective recombination of certain variable region genes with certain heavy chain genes (Slack *et al.*, 1980). Others have placed more emphasis on the gene order of the heavy chain isotypes. In the mouse this order is μ, δ, γ_3, γ_1, $\gamma_{2\beta}$, $\gamma_{2\alpha}$, ε, α (Shimizu *et al.*, 1981). Cebra *et al.* (1982) have proposed that the expression of the IgA isotype is related to marked B cell proliferation prior to terminal differentiation. As B cells divide, an opportunity is provided for sequential genetic recombinations of the variable part of the heavy chain gene to constant heavy chain genes more and more distal to the gene for μ chain. As the gene for α chain is most distal to the gene for μ chain in the mouse, chronic antigenic stimulation, as occurs in the gut with PC, would result in the accumulation of IgA producing cells. Mongini *et al.* (1982) have noted the importance of gene order for the proportion of each IgG subclass synthesized against TNP Ficoll in an *in vitro* system. They have proposed that T cells act to enhance isotype switching to genes further and further downstream from the μ gene. However, they have recently shown that regulation of murine IgA expression is inconsistent with a mechanism dependent on constant region gene order (Mongini, Paul & Metcalf, 1983).

Our own past studies also suggest that gene order may not be crucial in isotype switching to human IgA_1 or IgA_2. Virgin IgA B cells, as seen in the human newborn, are equally divided between IgA_1 and IgA_2 bearing cells (Conley *et al.*, 1980). This suggests that the predominance of IgA_1 B cells in the adult peripheral circulation and the predominance of IgA_1 molecules in serum (Conley *et al.*, 1983), are due to selective clonal expansion of IgA_1 bearing cells rather than selective isotype switching from IgM to IgA_1. The results presented in this paper indicate that this selective clonal expansion of IgA_1-bearing cells is apparently not explained by a unique ability of IgA_1 to respond to certain antigens.

Although we cannot rule out the possibility that other antigens may elicit an IgA subclass specific response our results suggest that route of antigen administration, the biochemical characteristics of the antigen and T cell dependence or independence of the antigen do not influence the IgA subclass of the antibody produced.

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