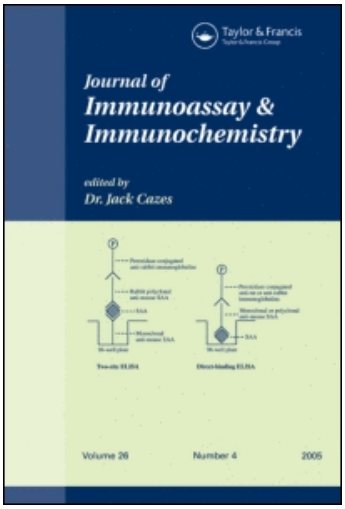

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QUANTITATIVE ANALYSIS OF IgA-SUBCLASS ANTIBODIES AGAINST TETANUS TOXOID

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ABSTRACT

Sera were analysed for levels of IgA- and IgG-class and IgA-subclass antibodies against tetanus toxoid and synthesized, tetanus-toxoid, β-chain peptides. Forty-five peptides, deduced from the amino-acid sequence of the tetanus-toxoid β-chain, were synthesized, in order to analyse epitope-binding of antibodies. Monoclonal, human, anti-tetanus antibodies were used to evaluate the synthesized peptides. Five synthesized peptides, bound by high, medium or low levels of IgA antibodies, were selected for the quantification of IgA1 and IgA2 antibodies. A set of chimeric, IgA-subclass antibodies with NP-specificity were used to quantify the antigen-specific IgA-subclass antibodies in a novel assay. Antibodies against the synthesized peptides were predominantly of IgA1 subclass.
(KEY WORDS: ELISA; IgA subclasses; Specific antibody; Tetanus toxoid; Monoclonal antibody)

INTRODUCTION

Immunoglobulin A (IgA) may be divided into two distinct subclasses, IgA1 and IgA2 (1-3), encoded by separate genes (4). An enzyme-linked, immunosorbent assay (ELISA) for the determination of the IgA-subclass distribution of antigen-specific antibodies has earlier been described (5). A chimeric, IgA2 antibody directed against 4-hydroxy-3-nitrophenacetyl (NP) was later used as a standard for estimating levels of IgA2 antibodies (6), whereas the monoclonal, IgA1 antibody with PPS 8 (pneumococcal polysaccharide 8) specificity, used as A1 standard, gave rise to erroneous results, possibly due to a low affinity of the antibody making it unsuitable in the quantitative assay.

In this study, we report a method of quantifying specific IgA1 antibodies, using a chimeric, IgA1 antibody with NP-specificity as standard, which, in addition to the earlier-developed method for IgA2 antibodies, gives a quantitative method for the determination of both specific, A1 and A2 antibody levels. In addition, we also used synthetic peptides of the tetanus-toxoid β-chain to estimate IgA-subclass antibodies against defined epitopes.

MATERIALS AND METHODS

Antigens

Tetanus toxoid was purchased from the National Bacteriological Laboratory, Stockholm, Sweden, and used at a concentration of 5 lf/ml in the semi-quantitative estimations. NP_2BSA [(4-hydroxy-3-nitrophenacetyl)$_2$-bovine serum albumin], used at a concentration of 20 μg/ml, was a gift from Prof. O. Mäkelä, of the Department of Bacteriology and Immunology, University of Helsinki, Helsinki,

Finland. Peptides were synthesized (7), according to the amino-acid sequence of the tetanus-toxoid β-chain (8). The peptides are depicted in Table 1. A five amino-acid overlap was made between the peptides (Table 1). They were diluted in bicarbonate buffer, pH 9.6, and added to polystyrene microtitre plates. In the quantitative estimations, they were used at a concentration of 10 μg/ml.

Serum

Sera from five, healthy, adult, blood donors, five individuals negative according to serum IgG antibodies against tetanus toxoid, one IgA-deficient individual and two individuals with homozygous, Cα1- or Cα2-gene deletions, i.e. individuals lacking of IgA1 or IgA2, were used in this study. The samples were stored at -20°C until used in dilutions between 1/25 and 1/1000.

Monoclonal, anti-tetanus-toxoid antibodies

Monoclonal, human anti-tetanus-toxoid antibodies, TT-1 and TT-2, were gifts from Dr. R. Tiebout, of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands (9, 10).

Antibody measurements

Antigen-coated plates were rinsed (0.15 *M* NaCl with 0.05% Tween 20, Polysorbatum, Apoteksbolaget, Stockholm, Sweden) five times, after which serum or human hybridoma antibodies were added. After incubation, unbound antibody was removed by washing and the plates were incubated with alkaline-phosphatase (ALP)-conjugated, rabbit, anti-human IgA and ALP-conjugated, rabbit, anti-human

TABLE 1

Deduced amino-acid sequence of the tetanus-toxoid β-chain peptides and levels of antibodies against the peptides[a)]

Peptide no	Amino-acid	IgG	IgA
1	SLTLGGELCIKIKNEDLTFIAEK	++++	++++
2	FIAEKNSFSEEPFQDEIVSYNTK	+++	++++
3	SYNTKNKPLNFNYSLDKIPVTKG	+	++
4	PVTKGIPYAPEYKSNAASTTEIH	++	+++
5	TTEIHNIDDNTIYQYLYAQKSPT	++	+++
6	QKSPTTLQRITMTNSVDDALINS	++	+++
7	ALINSTKIYSYFPSVISKVNQGA	+	+
8	VNQGAQGILFLQWVRDIIDDFTN	++	++
9	DDFTNESSQKTTIDKISDVSTIV	+	++
10	VSTIVPYIGPALNIVKQGYEGNF	+++	++++
11	YEGNFIGALETTGVVLLLGYIPE	++	+++
12	GYIPEITLPVIAALSIAESSTQK	++	++
13	SSTQKEKIIKTIDNFLEKRYEKW	+	++
14	RYEKWIEVYKLVKAKWLGTVNTQ	+	++
15	TVNTQFQKRSYQMYRSLETQVDA	++	+
16	TQVDAIKKIIDYEYKIYSGPDKE	+	+++
17	GPDKEQIADEINNLKNKLEEKAN	+	+++
18	EEKANKAMININIFMRESSRSFL	++	++
19	SRSFLVNQMINEAKKQLLEFDTQ	+	++
20	EFDTQSKNILMQYIKANSKFIGITEL	+	++
21	KNLDCWVDNEEDIDVILKKSTIL	++	++
22	KSTILNLDINNDIISDISGFNSS	++	++++
23	GFNSSVITYPDAQLVPGINGKAI	++	+++
24	NGKAIHLVNNESSEVIVHKAMDI	+++	+++
25	KAMDIEYNDMFNNFTVSFWLRVP	++++	++++
26	WLRVPKVSASHLEQYGTNEYSIE	++	++
27	EYSIESSMKKHSLSIGSGWSVSL	++	++
28	WSVSLKGNNLIWTLKDSAGEVRQ	++	++
29	GEVRQITFRDLPDKFNAYLANKW	+	+

(TABLE 1, continued)

Peptide no	Amino-acid	IgG	IgA
30	LANKWVFITITNDRLSSANLYIN	++	++
31	NLYINGVLMGSAEITGLGAIRED	++++	++++
32	AIREDNNITLKLDRCNNNNQYVS	++	++
33	NQYVSIDKFRIFCKALNPKEIEK	+	+
34	KEIEKLYTSYLSITFLRDPWGNP	++	+++
35	PWGNPLRYDTEYYLIPVASSSKD	+	+++
36	SSSKDVQLKNITDYMYLTNAPSY	++	++
37	NAPSYTNGKLNIYYRRLYNGLKF	+	+
38	NGLKFIIKRYTPNNEIDSFVKSG	+	+
39	FVKSGDFIKLYVSYNNNEHIVGY	+++	+++
40	HIVGYPKDGNAFNNLDRILRVGY	++	++
41	LRVGYNAPGIPLYKKMEAVKLRD	+	+
42	VKLRDLKTYSVQLKLYDDKNASL	++	+
43	KNASLGLVGTHNGQIGNPPNRDI	++	++
44	PNRDILIASNWYFNHLKDKILGC	+	+
45	KILGCDWYFVPTDEGWTND	+	++

[a]IgG and IgA antibodies from individual C. Serum diluted 1:100.
Absorbance value 0.0-0.5, +; >0.5-1.0, ++; >1.0-1.5,+++; >1.5, ++++.

IgG (DAKOPATTS A/S, Copenhagen, Denmark, both diluted 1/500) for IgA and IgG antibody determinations or with mouse, anti-human, IgA-subclass, monoclonal antibodies from Nordic Laboratories (Tilburg, The Netherlands) (11) to analyse the IgA-subclass distribution (anti-IgA1, diluted 1/5000; anti-IgA2, diluted 1/2000). After addition of monoclonal, anti-Ig-subclass antibodies, Fc-specific, rabbit, anti-mouse Ig (Jackson ImmunoResearch, Avondale, PA, USA, diluted 1/1000) and thereafter ALP-conjugated, sheep, anti-rabbit IgG $F(ab')_2$-fragments (Sigma Chemical Co., St. Louis, MO, USA, diluted 1/1000) were used. The plates were washed and incubated with the substrate disodium p-nitrophenyl phosphate (Sigma

Chemical Co.), diluted to 1 mg/ml in 10% diethanolamine buffer. The plates were read in a multichannel spectrophotometer (BIO-TEK Instruments Inc., Winooski, VT, USA).

Supernatants from a mouse hybridoma producing chimeric, IgA1 antibodies (a human constant, heavy-(H)-chain , a mouse heavy-chain variable region (VH) and a complete, mouse κ, light-(L)-chain), a gift from Dr. J.M. Woof, of the Department of Molecular Biology, Sheffield, Yorkshire, U.K., and chimeric, IgA2 antibodies (a human constant H chain, a mouse VH and a complete, mouse κ, L chain) directed against NP, purchased from the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology Research, Vaccine Research and Production Laboratory, Wilts., U.K. (12), were used as standards for the quantitative determination of specific, IgA-subclass antibodies. A human, standard serum (lot. 041015F, Behringwerke AG, Germany) was used as standard for specific IgA and IgG antibodies.

RESULTS

Tetanus toxoid and tetanus-toxoid β– chain peptides bound by antibodies

Two sera with high levels of IgA against tetanus toxoid, two sera with low levels and one serum with an intermediate IgA level, were chosen for the analysis of specific IgA antibodies of both subclasses against tetanus toxoid (Fig. 1).

Forty-five peptides, covering the tetanus-toxoid β-chain, were synthesized (Table 1). Monoclonal, anti-tetanus-toxoid antibodies were used to evaluate the synthesized peptides for binding of antibodies. Increased absorbency value was found for monoclonal antibody TT-1 for peptide 41 and monoclonal antibody TT-2 recognized the whole tetanus toxoid but failed to recognize any specific peptide,

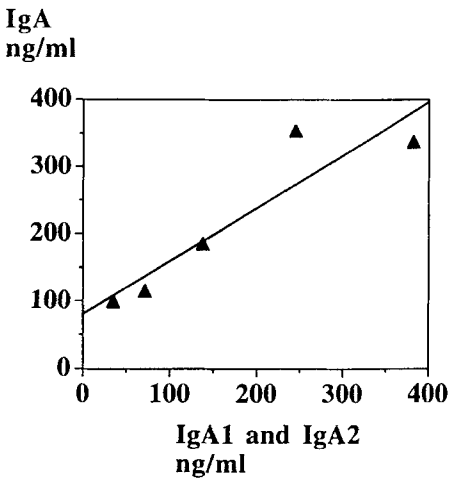

FIGURE 1. Relation between values in ng/ml of tetanus toxoid specific IgA antibodies and specific IgA subclass antibodies from five individuals.

TABLE 2

Human antibodies against tetanus-toxoid β-chain peptides[a)]

Peptide no	Donor A		B		C		D		E	
	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2	IgA1	IgA2
2	41	0	33	0	64	19	20	0	260	0
3	25	0	40	0	150	0	0	0	39	0
4	38	18	12	0	88	0	4	0	31	0
10	17	21	63	0	157	23	33	0	44	0
11	32	0	0	0	154	0	0	0	70	0
15	0	0	0	0	93	0	0	0	19	0
TT[b)]	45	26	349	33	224	21	35	0	138	0

[a)]Given for detectable levels of specific IgA1 and IgA2 antibodies in ng/ml.

[b)]Whole tetanus toxoid.

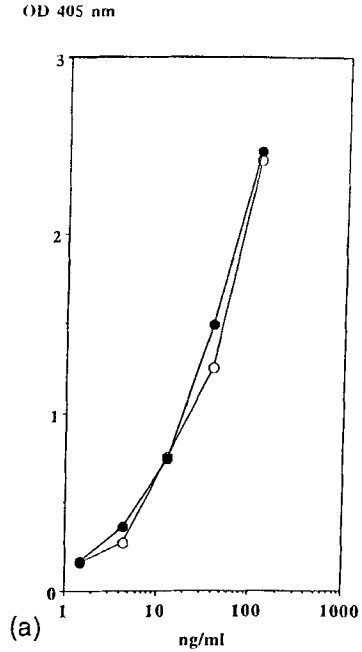

FIGURE 2a. Absorbency, as measured after 60 min, using an IgA1 monoclonal antibody with NP-specificity ● , and an IgA2 monoclonal antibody with NP-specificity ○ , against a plate coated with anti-IgA. Evaluated using an ALP-conjugated anti-IgA antibody.

suggesting that this monoclonal antibody may be directed against a conformational epitope or a determinant on the α chain (data not shown).

For further analysis of Ig levels against specific epitopes, four sera were analysed for IgA and IgG antibodies against the 45 synthesized peptides. The sera showed both IgG and IgA reactivity against the synthesized peptides (shown for individual C in Table 1). Five synthetic peptides, bound by high, medium or low levels of IgA antibodies, were selected for the quantification of specific IgA1 and IgA2 antibodies. IgA-subclass antibodies against these peptides were preferentially of A1 subclass (Table 2).

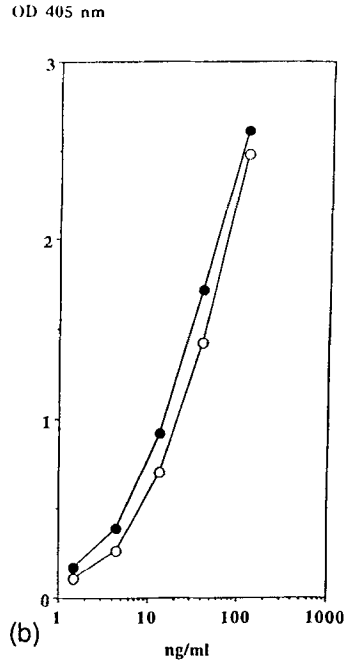

FIGURE 2b. Absorbency, as measured after 60 min, using an IgA1 monoclonal antibody with NP-specificity ● , and an IgA2 monoclonal antibody with NP-specificity ○ , against a plate coated with NP_2BSA. Evaluated using an ALP-conjugated anti-IgA antibody.

Quantification of human IgA-class and subclass antibodies

The IgA1 and IgA2 monoclonal, chimeric antibodies were added to plates coated with NP_2BSA or anti-IgA and an ALP-conjugated, anti-IgA antibody was thereafter added. The absorbency values for fixed amounts of the IgA1 and IgA2, monoclonal antibodies (standardized against normal human serum) were approximately the same for the monoclonal antibodies on plates coated with NP_2BSA or anti-IgA (Fig. 2*a* and 2*b*).

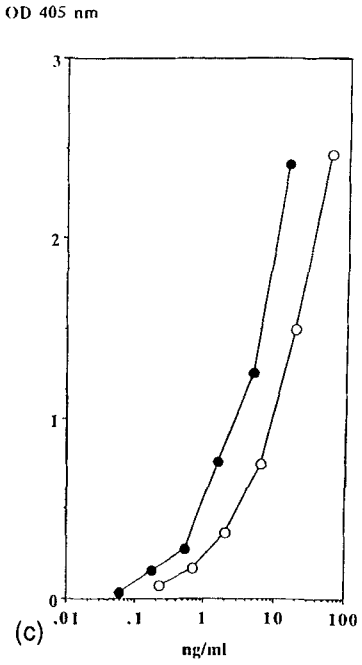

FIGURE 2c. Absorbency, as measured after 100 min, using an IgA1 monoclonal antibody with NP-specificity ● , and an IgA2 monoclonal antibody with NP-specificity ○ , against a plate coated with NP_2BSA. The samples were evaluated with anti-IgA1 or anti-IgA2 monoclonal antibodies followed by a Fc-specific, rabbit, anti-mouse Ig and thereafter an ALP-conjugated, sheep, anti-rabbit IgG.

The IgA1 and IgA2, monoclonal antibodies were also added to plates coated with NP_2BSA and evaluated with monoclonal, anti-IgA1 or anti-IgA2 antibodies, followed by a Fc-specific, rabbit, anti-mouse Ig and thereafter an ALP-conjugated, sheep, anti-rabbit IgG. The absorbency values for fixed amounts of the IgA1 and IgA2, monoclonal antibodies detected by the respective, anti-IgA-subclass, monoclonal antibody yielded parallel curves (Fig. 2*c*). The concentration of the monoclonal, anti-IgA1 and anti-IgA2 antibodies (both IgGκ isotype) also showed parallel curves and overlap when analysed on plates coated with anti-κ and

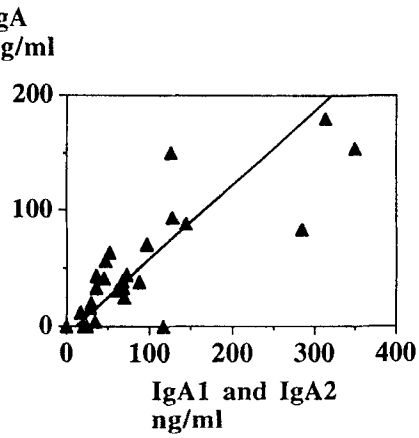

FIGURE 3. Relation between values in ng/ml of IgA antibodies and IgA subclass antibodies (from five individuals) against each of five, tetanus-toxoid, β-chain peptides.

examined using a rabbit, anti-mouse, ALP-conjugated antibody, suggesting that they contained the same amount of monoclonal antibody (data not shown).

The quantified specific, IgA-subclass antibodies against tetanus-toxoid β-chain peptides for five individuals are shown in Table 2.

The correlation between the levels of IgA and IgA-subclass antibodies (IgA1 plus IgA2) against tetanus toxoid is shown in Fig. 1 (r=0.92), and the correlation between IgA and the sum of IgA-subclass antibodies against five, tetanus-toxoid, β-chain peptides is shown in Fig. 3 (r=0.84).

DISCUSSION

In an earlier study, we analysed the sensitivity and specificity of our assay for the determination of antigen-specific antibodies of the two IgA subclasses (5). Using a chimeric, IgA2 antibody with NP-specificity, a method of estimating levels of

specific, IgA2 antibodies in a semi-quantitative fashion was also developed earlier (6). In this study, we used a chimeric, IgA1 antibody with the same specificity as the chimeric, IgA2 antibody to improve the assay for the determination of specific, IgA-subclass antibody levels.

The anti-IgA1, monoclonal antibody and anti-IgA2, monoclonal antibody used were further analysed in this study. The ELISA results suggest different affinities of the two, anti-IgA-subclass, monoclonal antibodies, as earlier reported for different anti-IgA-subclass antibodies by Reimer *et al.* (1989) (13). However, this does not influence the quantitative method for the determination of specific, IgA-subclass antibodies and, thus, a complete method of semi-quantifying IgA-subclass antibodies is now available.

The IgA-subclass distribution against tetanus toxoid has earlier been shown to be of both IgA subclasses (14). The IgG-subclass distribution against tetanus toxoid has been reported to be IgG1, followed by IgG4 in sera from army recruits, obtained after booster immunization with tetanus toxoid (15). A highly restricted, IgG1 and IgG4 antibody response to tetanus toxoid was also found to be predominant in a study by Devey *et al.* (1987) (16).

In addition to the use of whole tetanus toxoid, we used synthetic peptides based on the sequence of the tetanus-toxoid β-chain, with the intention of quantifying IgA1 and IgA2 antibodies against protein as well as peptides and of studying epitope restriction of IgA subclasses. The peptides were bound by both IgA1 and IgA2 antibodies with a clear predominance of IgA1.

The sum of the levels of IgA antibodies against peptides exceeded that of IgA antibodies against the whole protein. A different epitope display of the peptides, being linear rather than conformational, may lead to a recognition of the antigen by an increased number of antibodies, compared with the number of antibodies which detect the epitope as the whole protein structure.

In this study, protein and synthesized peptides from tetanus toxoid were used as a model system in an attempt to quantify IgA-subclass antibodies. Synthesized peptides, bound by high, medium or low levels of IgA antibodies, were selected for the quantifications of IgA1 and IgA2 antibodies. The specificity of the IgA1 and IgA2 assay was tested using sera from individuals lacking of total IgA or the subclasses IgA1 or IgA2. The quantitative, IgA-subclass assay may be used for further analysis of peptide-specific, IgA-subclass antibodies and may also be useful as a standardization system for the quantification of mucosal and systemic immune responses.

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