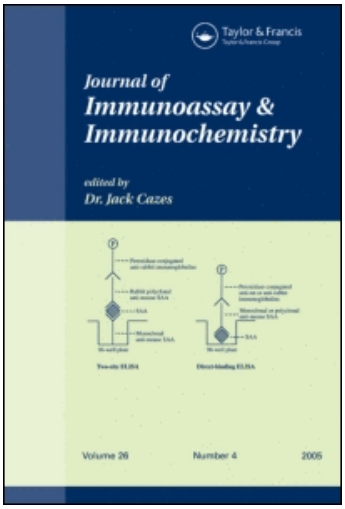

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Quantitative Analysis of Immunoglobulin G Subclasses in the Rat

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QUANTITATIVE ANALYSIS OF IMMUNOGLOBULIN G SUBCLASSES IN THE RAT

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ABSTRACT

Quantitative ELISAs have been developed for the four immunoglobulin G (IgG) subclasses (IgG1, IgG2a, IgG2b and IgG2c) of the rat. These assays were shown to have excellent sensitivity, reproducibility and adequate specificity for studies of natural IgG and antigen-elicited IgG responses. The sensitivity (working range for each isotype assay) was: IgG1, 1.6 to 200 ng/ml; IgG2a, 8 to 200 ng/ml; IgG2b, 1.6 to 200 ng/ml; and IgG2c, 1.6 to 1000 ng/ml. The isotype sum and the direct assay of total plasma IgG agreed closely. The utility of these assays was demonstrated in a study of the development of total IgG and of the specific IgG response following immunization with tetanus toxoid (TT). For total IgG, the predominant isotype was IgG2b (64% of total IgG) followed by IgG2a (29%), IgG1 (4%) and IgG2c (3%). In contrast, the anti-TT response was mainly of the IgG1 subclass (57% of total anti-TT). These quantitative assays should prove useful for investigating the response to experimental vaccines and the influence of cytokines on class switching in vivo. (KEY WORDS: immunoglobulin G; isotype; tetanus toxoid)

INTRODUCTION

The immunoglobulin G (IgG) class of man, the mouse and the rat each comprises four subclasses, designated IgG1, IgG2, IgG3 and IgG4 in humans (1,2), IgG1, IgG2a,

IgG2b and IgG3 in the mouse (3,4), and IgG1, IgG2a, IgG2b and IgG2c (5,6) in the rat. There is convincing evidence that the IgG subclasses have evolved independently in humans and mice (7,8). The heavy chain constant (C) regions of the four human IgG subclasses are thought to have risen through gene duplication of a primitive [Cγ-Cγ-Cε-Cα] gene cluster, producing four IgG isotypes as well as two isotypes of IgE and of IgA (9,10). In the mouse, genes for Cγ3, Cγ1 and Cγ2 are thought to have branched from an ancestral Cγ gene after which Cγ2a and Cγ2b were produced by duplication of a Cγ2 gene (11,12). Because of this evolutionary difference, the murine IgG isotypes are not strictly homologous to those of human IgG.

In comparison to this difference between the human and murine systems, there is striking homology in the order of the Ig heavy chain gene domains between the mouse and the rat (8,13). The germ-line gene order in the mouse, D-JH-Cμ-Cδ-Cγ3-Cγ1-Cγ2b-Cγ2a-Cε-Cα (12), is very similar to that in the rat, D-JH-Cμ-Cδ-(Cγ2c,Cγ2a)-Cγ1-Cγ2b-Cε-Cα (8). In addition to these similar gene arrangements, sequence analysis within the CH3 domain has revealed that rat Cγ2b shares greater homology with mouse Cγ2a/2b, whereas the rat Cγ1 and Cγ2a genes are very similar to each other and both are highly homologous to the mouse Cγ1 gene (8). Equivalence of rat IgG2c to mouse IgG3 was also deduced from comparison of nucleotide sequences (14) as well as functional properties (13,15).

After immunization or infection, the predominance of a particular immunoglobulin class or subclass is often observed. Factors known to influence class or subclass selection include the type of antigen, route of administration, age and strain of animal, and concomitant factors such as infection (16). Recent experiments have implicated cytokines as one type of factor that regulates immunoglobulin class and subclass expression (16,17).

The rat is being used increasingly as a model for studies of infection and immunity. However, there currently is very little information on the regulation of IgG isotype expression in the rat. This seems to be partly because of the lack of sensitive and specific quantitative assays for the rat IgG subclasses. In previous work (18,19), we used the rat to investigate the role that vitamin A plays in antibody production. Tetanus toxoid was used for these studies because 1) it

is often used as a model T cell-dependent antigen and 2) in humans, neonatal tetanus remains a very significant cause of morbidity and mortality in some of the same geographic areas where vitamin A status is poor (20). In the vitamin A-deficient rat, there was a marked decrease in both the primary and secondary (IgM and IgG) antibody responses to TT as compared to pair-fed control rats (18,19).

To enable further studies of IgG production in the rat, we have developed sensitive and specific quantitative ELISAs for the four rat IgG isotypes. Reported here are the characteristics of these assays and information on the age-related changes in plasma total IgG and IgG subclasses and the IgG subclass response following immunization with TT.

MATERIALS AND METHODS

The plasma samples used in this work were all obtained from male Lewis rats whose care followed procedures approved by the Institutional Animal Use and Care Committee. For longitudinal studies of total IgG and IgG isotypes between 20 and 80 days of age, 10 non-immunized rats were bled from the caudal vein into heparinized syringes at 10-day intervals from 30 to 80 days of age, while six 20 day-old rats were euthanized by carbon dioxide inhalation and bled from the vena cava.

Samples used for analysis of anti-TT IgG were from the control group of rats which had been fed a balanced, vitamin A-adequate, semi-synthetic diet in a previously reported study (19). These rats were immunized ip with 100 µg of TT in saline (lot TAS239, Connaught Laboratories, Canada) when they were 40 days old. The primary IgG response to TT was determined on plasma prepared from blood collected 10 days after immunization [the peak of the primary IgG response to this antigen (19)].

Because of the large number of individual plasma samples from each age or treatment group, samples were pooled to prepare 3 or 4 representative samples (plasma from 3-5 rats per pool) for isotype analysis. A preliminary study was conducted to ensure that the concentration of total IgG or IgG anti-TT of each pool was identical to the mean for the individual plasma samples included in each pool. All plasma samples were stored in aliquots at -20°C.

ELISAs for IgG Isotypes

Plasma IgG isotypes were assayed by a sandwich ELISA. Wells of polystyrene plates (Immunolon 4, Dynatec Laboratories, Inc., Chantilly, VA) were coated with 100 µl of affinity-purified goat anti-rat IgG (Cappel, Malvern, PA, lot No 32043; 2.5 µg/ml in 0.15 M Tris-HCl buffer, pH 7.6) and incubated at 4°C overnight. The wells were washed 5 times with washing buffer (0.015 M Tris-HCl buffer, pH 7.6 with 0.135 M saline and 0.05% of Tween 20) and incubated with 250 µl of incubation buffer (0.15 M Tris-HCl buffer, pH 7.6, containing 1% BSA) for at least 1 hr at room temperature to block any remaining active sites. After aspirating the incubation buffer, 100 µl of rat serum samples or standard samples appropriately diluted with incubation buffer were added to wells in duplicate and incubated at 4°C overnight. After washing the plate, horseradish PO-conjugated sheep anti-rat IgG1 antibody (Binding Site, Ltd., Birmingham, UK; lot No G1715), anti-rat IgG2a (Binding Site, lot No G1480), anti-rat IgG2b (Binding Site, lot No G1720), or anti-rat IgG2c (Binding Site, lot No G1482), diluted 1:2000 in incubation buffer, was added and further incubation was carried out for 1 hr at room temperature. After washing again, 100 µl of substrate solution containing 0.4 mg/ml of *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and 0.4 µl/ml of 31% H_2O_2 in 0.01 M citrate / 0.02 M phosphate buffer, pH 4.0, was added to the wells. The enzymatic reaction was stopped by adding 100 µl of 1N H_2SO_4, and absorbance values at 490 nm were read on an automatic ELISA reader (Dynatec). In each plate, known amounts of affinity-purified rat myeloma IgG1 (IR 595, Binding Site, lot No 028-B2724), IgG2a (Binding Site, lot No 028-B2725), IgG2b (IR 863, Binding Site, lot No 028-B2504) or IgG2c (Binding Site, lot No 028-B2199) were run as an inner standard, and concentrations of each IgG isotype in the plasma samples were calculated with a correlation formula between the concentrations of the purified IgG isotype and absorbance values. The average of two or three plasma dilutions that fell on the standard curve was used for final calculation, expressed as µg IgG/ml plasma.

In the ELISA for total IgG, either alkaline phosphatase (AP)-conjugated goat anti-rat IgG (Cappel, Malvern, PA; lot 32586) or PO-conjugated sheep anti-rat IgG (Binding Site, lot G1722) were applied as second antibodies.

Assay of Isotypes of IgG Anti-Tetanus Toxoid

The IgG isotypes of the antibodies against TT were quantified by ELISA, similar to an assay described previously for total IgG anti-TT (19), with minor modifications. Briefly, wells of Immunolon 4 plates were coated with TT (100 µl of 10 µg/ml in Tris-HCl buffer, pH 7.6) and incubated at 4°C overnight. After washing and blocking with incubation buffer, plasma samples diluted with incubation buffer were added to the wells and incubated at 4°C overnight. After additional washing, PO-conjugated isotype-specific antibody against each IgG isotype was added to the wells and the enzymatic reaction was developed as described above. The concentration of anti-TT IgG of each isotype in a standard sample, obtained from rats immunized twice with 100 µg of TT, was expressed in µg IgG/ml as described elsewhere (19).

Specificity of the PO-conjugated anti-rat IgG isotype antibodies was studied both by a direct binding assay and an absorption assay. In the direct binding assay, affinity-purified rat myeloma IgG1, IgG2a, IgG2b or IgG2c, diluted serially from 1000 to 0.32 ng/ml with incubation buffer, was added to the wells coated with goat anti-rat IgG and incubated at 4°C overnight. After washing, the binding of PO-conjugated anti-rat IgG isotype antibodies with each rat IgG isotype were evaluated by enzymatic color reactions as described above. In the absorption assay, PO-conjugated anti-IgG isotype antibodies were absorbed by each isotype as follows: wells were incubated with each IgG isotype in concentrations ranging from 3.9 to 1000 ng/well at 4°C overnight. After washing and blocking, 150 µl of each PO-conjugated anti-IgG isotype antibody, diluted 1:4000 in incubation buffer, was added to the wells and incubated for 4 hr at room temperature. 100 µl of each supernatant was carefully collected from the wells and served as absorbed antibodies.

The presence of IgM-rheumatoid factor (RF), which has potential to interfere in the sandwich ELISA, was assayed as described elsewhere (21). Briefly, wells were coated with 100 µl of rat IgG (200 µg/ml in Tris-buffer, pH 7.6) at 4°C overnight and appropriately diluted rat plasma samples were added in the wells after washing and blocking. IgM bound to IgG on the well was detected using AP-conjugated goat anti-rat IgM (Cappel, lot 32485).

Statistics

The data are presented as the mean ± SEM. Statistically significant differences were determined by a one-way analysis of variance, followed by Tukey's test; differences having a p value ≤ 0.05 are reported as statistically significant.

RESULTS

Establishing ELISAs for Individual Isotypes of Rat IgG

Our initial studies were designed to determine the optimal ELISA conditions and the specificity of the second antibodies (sheep anti-rat IgG1, IgG2a, IgG2b, and IgG2c). The results of direct binding studies are shown in Figure 1. In this assay, the concentration of goat anti-rat IgG selected for coating was 2.5 µg/ml, based on the optimal concentration for the assay of total IgG (19). Anti-rat IgG1 reacted strongly with rat IgG1 but did not react with IgG2a or IgG2b across a concentration range of 1.25 to 200 ng/ml (Figure 1A). There was some reaction of anti-IgG1 with IgG2c at concentrations greater than 200 ng/ml. Similarly, anti-IgG2a bound strongly with IgG2a and also showed some binding to IgG2c (Figure 1B). Only at concentrations of ~200 ng/ml and greater did binding of IgG1 and IgG2b to anti-IgG2a become significant. Anti-IgG2b (Figure 1C) and anti-IgG2c (Figure 1D) were each highly specific, reacting only with the homologous isotype. The working range for each isotype assay was: IgG1, 2 to 200 ng/ml; IgG2a, 10 to 200 ng/ml; IgG2b, 2 to 200 ng/ml; and IgG2c, 2 to 1000 ng/ml.

Using an absorption assay to further test specificity, the same tendencies were shown. Anti-IgG1 activity was absorbed by IgG1 even at low concentration, and was also partially absorbed by IgG2c, but not by IgG2a or IgG2b (Table 1). Anti-IgG2a was absorbed by IgG2a itself and to a lesser extent by IgG2c at high concentrations, but not by IgG1 or IgG2b. On the other hand, anti-IgG2b and anti-IgG2c were absorbed only by their corresponding isotype (IgG2b and IgG2c, respectively) and not by any other IgG isotypes.

Specificity was also tested by making two mock samples from pure IgG subclasses mixed in different proportions. The recovery of total IgG averaged 112% in the two mixtures. In mixture 1 with IgG1:IgG2a:IgG2b:IgG2c in ratios of 10:20:60:10 (resembling total IgG) the observed ratios were 14:22:57:6. In mixture 2

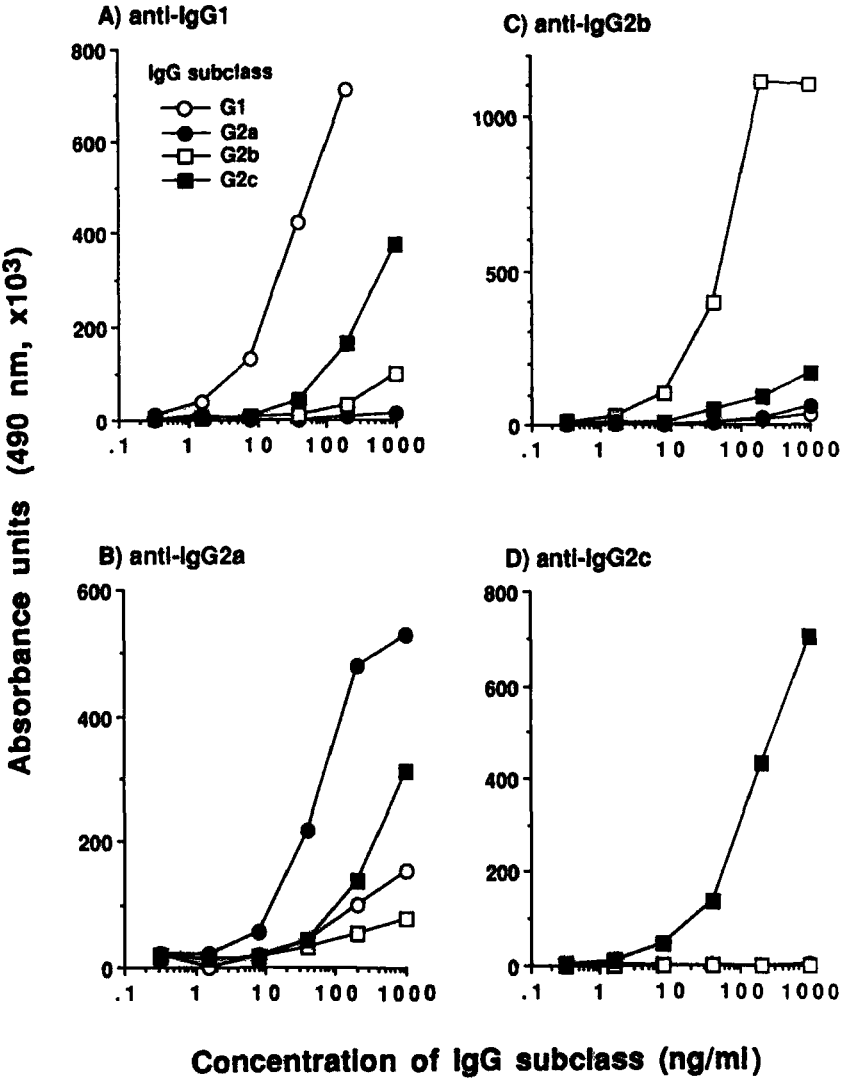

FIGURE 1. Binding activities of peroxidase (PO)-conjugated anti-rat IgG isotype antibodies with affinity-purified rat myeloma IgG proteins. Anti-rat IgG1 (A), anti-rat IgG2a (B), anti-rat IgG2b (C), and anti-rat IgG2c (D) were reacted with rat IgG1 (○), IgG2a (●), IgG2b (□) or IgG2c (■). Points for IgG1 and IgG2a in panel D were behind those of IgG2b. Myeloma proteins were diluted serially from 1000 to 0.32 ng/ml and incubated in wells coated with goat anti-rat IgG. Binding is expressed in absorbance units, A490 nm.

TABLE 1.

ELISA Specificity Determined by Absorption with IgG Subclasses

Isotype assayed:	Concentration of IgG subclass used for absorption, μg/ml: IgG1 10	IgG1 .63	IgG1 .04	IgG2a 10	IgG2a .63	IgG2a .04	IgG2b 10	IgG2b .63	IgG2b .04	IgG2c 10	IgG2c .63	IgG2c .04
	(% of maximum absorption[a])											
IgG1	100	80	23	0	0	2	0	0	1	37	16	6
IgG2a	14	16	9	100	94	29	0	0	3	69	32	4
IgG2b	0	0	13	14	10	11	100	65	3	14	9	8
IgG2c	6	10	9	0	12	10	0	4	0	100	61	9

a) The percent of the maximum absorption was calculated as follows: (ODc-OD) / (ODc-ODm) x 100, where ODc is the absorbance reading after the second antibody was absorbed with 1% BSA, OD is an absorbance unit of each sample, and ODm is the absorbance reading of the maximum absorption.

with IgG isotypes in ratios of 60:10:10:20, the observed ratios were 52:16:12:21. Thus, in these mixtures there was a slight tendency to overestimate IgG2a, and IgG1 when IgG2b was in excess. However, in other dilution tests rat IgG2a in a mixture agreed very well with pure IgG2a across a wide concentration range.

Thus, both the direct binding assay and the absorption procedure showed that anti-IgG1 and anti-IgG2a antibodies react strongly with their respective isotypes but also cross-react weakly with IgG2c. The binding properties of anti-IgG2b and anti-IgG2c were highly specific. As will be shown below, IgG2c comprised only a very small portion of plasma IgG (and the TT-specific IgG) as compared to the other three isotypes. Furthermore, because each sample was diluted to be in the range of 5 to 200 ng/ml in the assay, the cross-reactivity of anti-IgG1 or anti-IgG2a with IgG2c would not be expected to significantly affect the assay results. At this concentration, even if *equal* amounts

of IgG2c and IgG1 or IgG2a were present in a test sample, the observed cross-reactivity with IgG2c would result in an overestimation of only 5% and 9% for IgG1 and IgG2a, respectively. Tests with mock mixtures also revealed good overall agreement between expected and observed results. Thus, we concluded that these ELISAs have suitable sensitivity and specificity for the determination of plasma IgG isotypes as they occur in in vivo studies.

After ensuring the specificity of the second antibodies, the optimal concentration of goat anti-rat IgG used in coating was re-examined. The binding of PO-conjugated anti-isotype antibodies with each corresponding rat IgG subclass was tested on wells coated with goat anti-rat IgG in concentrations from 0.78 to 80 μg/ml. For each IgG isotype assay, a concentration of goat anti-rat IgG $\geq$ 2.5 μg/ml produced binding close to the maximum at any concentration of the test sample (data not shown). Based on these studies, 2.5 μg/ml was selected as an optimal concentration for coating. Under these conditions, the limits of reliable detection, defined as twice the background absorbance, were as low as 1.6 ng/ml for IgG1, 3.1 ng/ml for IgG2a, 1.6 ng/ml for IgG2b, and 0.8 ng/ml for IgG2c (Figure 2). Background absorbances were normally less than 0.01 A_{490} unit in the assays of IgG1, IgG2b or IgG2c, and about 0.025 unit in the IgG2a assay.

In a sandwich ELISA there exists the potential that rheumatoid factor (RF), an antibody against the Fc portion of IgG, might interfere in the assay system. As IgM RF could, if present, bind to both the IgG (i.e., goat IgG against-rat IgG) coated onto the plate and to the second antibody (sheep IgG against a rat-IgG isotype), it would be estimated as IgG, resulting in over-estimation of each IgG isotype. We examined the presence of IgM RF in the test samples and found these levels to be barely detectable (data not shown).

Comparison of IgG Subclass Concentrations with Total Plasma IgG

To determine whether the sum of the concentrations of independently determined IgG isotypes agrees satisfactorily with direct measurements of total plasma IgG, we compared the sum of plasma IgG1, IgG2a, IgG2b and IgG2c with the total plasma IgG concentration in 14 plasma samples. As is shown in Figure 3, there was good agreement between the isotype sum and the direct assay

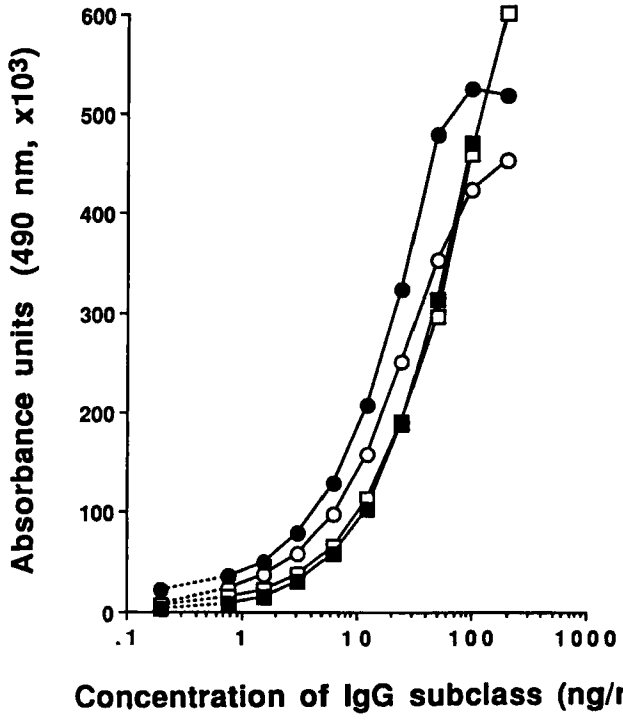

FIGURE 2. Sensitivity of the ELISAs for the IgG subclasses. The serially diluted affinity-purified rat myeloma IgG1 (○), IgG2a (●), IgG2b (□) or IgG2c (■) were assayed by the corresponding PO-conjugated anti-IgG isotype antibodies. Points connected with broken lines show background absorbance values in each isotype assay.

of total IgG. Data presented below have been expressed relative to both the sum of the individual isotypes and with respect to the directly determined total IgG.

The reproducibility of each assay was determined using three plasma samples chosen for a high, moderate, and low concentration of IgG or IgG anti-TT. The interassay coefficients of variation (Table 2) ranged from 2.8 to 14.2% for the four isotypes, whereas the intra-assay coefficients of variation equalled 3.9% to 10.9%.

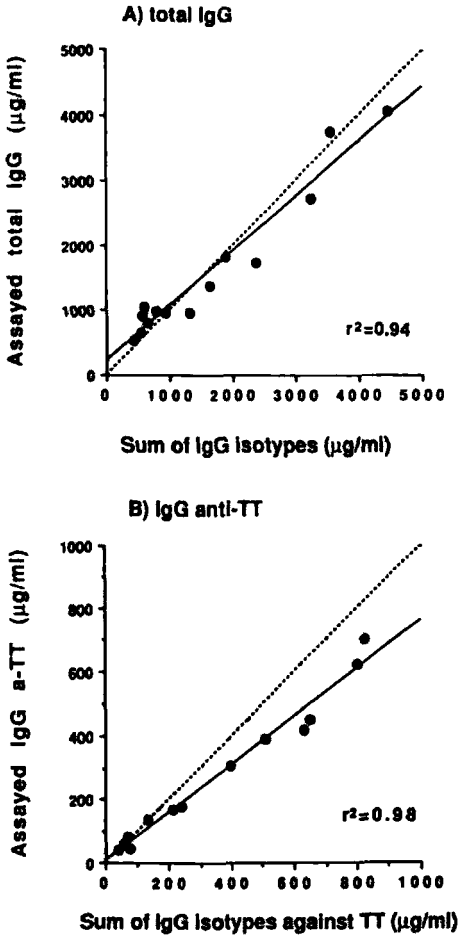

FIGURE 3. Comparison of the sum of independently determined IgG isotypes with direct measurements of total plasma IgG. Dotted line shows expected correlation.

Use of Assays to Measure Total Plasma IgG Isotypes

With this understanding of the strengths and limitations of the ELISAs for the four rat IgG isotypes, we conducted a study of the contribution of the various isotypes to total plasma IgG in the normal rat over the

TABLE 2.

Coefficients of Variation for Rat IgG ELISAs

Isotype determined	Interassay a)		Intra-assay b)	
	total IgG	anti-TT	total IgG	anti-TT
	(%)			
IgG1	8.2	3.4	4.0	4.9
IgG2a	9.8	2.8	10.9	3.9
IgG2b	10.1	14.2	4.9	8.5
IgG2c	6.3	ND	6.0	ND

a) Interassay variation was measured by assaying three plasma samples, with a high, moderate, and low concentration of IgG or IgG anti-TT, on three different days. The concentration of each sample was calculated in μg/ml and results of the assay were expressed as a coefficient of variation. The means of the coefficients of variation of the three plasma samples are shown above.

b) Three plasma samples were assayed in four replicates on the same plate.

age range of 20 to 80 days. Previously we reported that the total plasma IgG concentration in normal Lewis rats increases progressively between 40 and 70 days of age (19). In the present study, we examined the IgG isotypes in plasma from rats between 20 and 80 days of age. As is shown in Table 3, during development the concentrations of each subclass of IgG increased proportionally with the increase in total IgG concentration. Thus, the isotype *distribution* did not change significantly with age. The predominant IgG isotypes were IgG2b and IgG2a, which accounted on average for nearly 64% and 29%, respectively, of the total IgG. IgG1 and IgG2c were very minor components, comprising together less than 10% of total plasma IgG at all ages. The IgG2a and IgG2b concentrations increased significantly, and did total IgG, between 60 and 80 days of age.

TABLE 3.

Isotypes of plasma IgG in 20 to 80 day-old male rats

Age (days)	20	30	40	50	60	70	80	Ave. distribution (all ages)
				(μg/ml; mean ± SEM) [a]				
IgG1	6.7 ± 0.5 (1%)[b]	25.9 ± 2.9 (3%)	26.1 ± 5.7 (4%)	48.4 ± 12.4 (5%)	85.3 ± 12.0 (5%)	80.4 ± 7.1 (4%)	101.9 ± 9.3 (3%)	3.6 ± 0.5 %
IgG2a	263 ± 138 (26%)	267 ± 37 (36%)	190 ± 29 (31%)	240 ± 17 (27%)	405 ± 60 (25%)	556 ± 69 (28%)	953 ± 128 (30%)	29 ± 1.4 %
IgG2b	755 ± 237 (74%)	439 ± 63 (59%)	377 ± 75 (61%)	564 ± 54 (64%)	1018 ± 139 (64%)	1214 ± 277 (62%)	2062 ± 427 (64%)	64 ± 1.8 %
IgG2c	2.3 ± .02 (<1%)	10.1 ± 3.2 (1%)	27.2 ± 3.3 (4%)	31.1 ± 5.5 (4%)	77.9 ± 3.1 (5%)	104.4 ± 11.6 (5%)	91. 6 ±90. (3%)	3.3 ± 0.6 %
IgG [c]	1027 ± 98 (100%)	742 ± 102 (100%)	620 ± 109 (100%)	884 ± 57 (100%)	1586 ± 192 (100%)	1954 ± 345 (100%)	3208 ± 42 (100%)	

a) Plasma samples from 10 rats were pooled to prepare 3 representative samples for assays, except for day 20 for which 2 pooled samples were prepared from 6 rat plasma samples.

b) Percent of sum of IgG isotypes

c) Mathematical sum of IgG1, IgG2a, IgG2b and IgG2c

TABLE 4.

IgG Isotypes Following Immunization with TT

	anti-TT µg/ml; mean ± SEM [a]	total IgG µg/ml; mean ± SEM [a]	anti-TT % of total IgG
IgG1	4.8 ± 1.3 (57%) [b]	36.8 ± 5.1 (5%) [c]	14%
IgG2a	0.6 ± 0.2 (7%)	179.6 ± 19.2 (24%)	0.3%
IgG2b	3.0 ± 0.6 (36%)	528.5 ± 49.7 (71%)	0.6%
IgG sum [d]	8.4 ± 1.8 (100%)	744.9 ± 45.8 (100%)	1.2%
IgG direct [e]	8.3 ± 1.3 (99%)	668.9 ± 16.9 (90%)	1.3%

a) 17 rat plasma samples collected 10 days after immunization were pooled to prepare 4 representative samples for assays.

b) % of total IgG anti-TT sum

c) % of total IgG sum

d) IgG calculated by summing IgG1, IgG2a and IgG2b

e) IgG assayed directly using PO-conjugated anti-IgG

Use of Assays to Measure Isotypes of Anti-TT IgG

The utility of these assays for antigen-specific responses was demonstrated in plasma samples from rats immunized with TT. The isotype pattern of the primary IgG response to TT (Table 4) was remarkably different from that of total plasma IgG. The dominant isotype of anti-TT was IgG1 which equalled 57% of total anti-TT activity. In comparison, this subclass comprised only 5% of total (non-specific plus TT-specific) IgG in these

animals. Thus, at the time when the primary response to TT was maximal, anti-TT IgG1 accounted for 14% of total IgG1. The second population in rank was anti-TT IgG2b which comprised 36% of the total anti-TT activity, but accounted for only 0.6% of the total IgG2b. The third population in rank was anti-TT IgG2a which comprised 7% of total anti-TT activity and accounted for 0.3% of total IgG2a. IgG2c, either specific to TT or non-specific, was hardly detectable (< 1% of anti-TT IgG, data not shown).

DISCUSSION

The quantitative ELISAs developed for the four rat IgG isotypes proved very good both in terms of sensitivity and reproducibility. The high correlation between total IgG assayed directly and that calculated by summing the four IgG subclasses also strongly supported the reliability of this assay system, as did the assay of mock mixtures of pure IgG subclasses. The assays were suitable for the analysis of natural (total) IgG as well as antigen-specific IgG, as demonstrated by studies with TT.

The plasma total IgG concentration is known to increase progressively during ontogeny. In children IgG reaches adult concentrations by about 6 years (22) while, in the rat, we previously showed plasma total IgG to increase significantly between 40 and 70 days of age (19). Although the precise mechanisms that control the concentrations of circulating immunoglobulins have not been discerned, this age-related increase is thought to be due partly to the maturation of B cells and partly to the enhancement of helper T cells function and/or changes in the balance of helper and suppressor functions (22). In the rats in this report, IgG concentrations were still increasing at 80 days of age. However, the distribution of the IgG subclasses remained unchanged as total IgG increased.

In the primary response to TT in the normal Lewis rat, IgG1 anti-TT comprised more than half of the total anti-TT activity, followed by IgG2b>IgG2a>>IgG2c. This pattern for the TT-specific IgG isotypes was quite different from that of total IgG (IgG2b>IgG2a>>IgG1, IgG2c). Thus, TT specifically activated the production of IgG1 antibody, which, based on its steady-state concentration, was produced only at a low level in the absence of antigen stimulation.

These immunoassays should prove very useful in studies of the ontogeny of antibody production, vaccine-

specific responses, nutritional influences, and the isotype-specific class switch as influenced by cytokines and other regulatory factors.

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