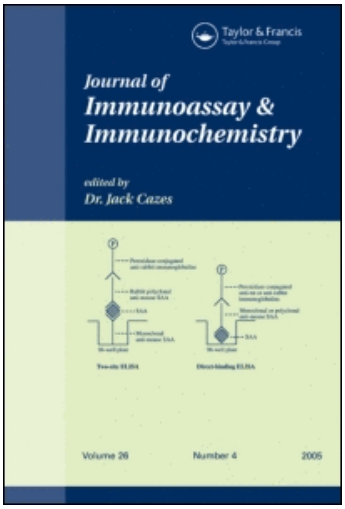

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MONOCLONAL ANTIBODY-BASED IMMUNOENZYMETRIC ASSAYS FOR QUANTIFICATION OF HUMAN IgG AND ITS FOUR SUBCLASSES

(KEY WORDS: Human IgG subclasses, immunoassay, quantification, monoclonal antibodies, quality control)

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ABSTRACT

A panel of 5 immunoenzymetric assays (IEMAs) has been developed for quantification of the total and four subclasses of immunoglobulin G (IgG) in human serum using IUIS/WHO documented monoclonal antibodies (MoAb). Human IgG specific MoAb was adsorbed to microtiter plates and used to capture IgG from serum. Peroxidase conjugated forms of polyclonal mouse anti-human IgG Fc or a mixture of 4 MoAb (anti-kappa, anti-lambda, anti-IgG Fc PAN and anti-IgG Fd PAN) were used as detection antibodies. Use of monoclonal antibody in chromatographically purified form was required for acceptable assay sensitivity (S) and working ranges (WR). All 5 IEMAs displayed good precision (intra-assay %CV < 5%, inter-assay %CV < 12%) and parallelism (inter-dilutional CV < 20%). Both HP6069 or HP6070 (anti-IgG1 Fc) worked well alone or together as capture antibodies in the IgG1 IEMA: WR = 20-1250 ng/ml, S = 15 ng/ml. HP6002 (anti-gG2 Fc) alone or in combination with HP6014 (anti-IgG2 Fd) produced an IgG2 IEMA with a WR of 5-200 ng/ml and S of 5 ng/ml. HP6047 (anti-IgG3 hinge) alone generated a sensitive IgG3 IEMA with a narrow working range: WR = 2-50 ng/ml, S = 1.6 ng/ml. Both HP6025 and HP6023 (anti-IgG4 Fc) worked equally well alone and together to produce a useful IgG4 IEMA: WR = 8-250 ng/ml, S = 7.8 ng/ml. HP6017 (anti-IgG PAN Fc) was combined in an equal molar ratio with HP6046 (anti-IgG PAN Fd) to produce a total IgG PAN IEMA with a WR of 5-530 ng/ml and a sensitivity of 5 ng/ml. All 5 IEMAs fulfilled requirements for robust clinical immunoassays that permit the quantitation of human IgG and its 4 subclasses.

INTRODUCTION

Immunoglobulins function in man to specifically bind foreign or non-self molecules, thereby inactivating and/or facilitating their clearance from the

body. In normal adults, immunoglobulin G (IgG) constitutes approximately 75% of the total serum immunoglobulins (1). It is the only class of immunoglobulin that crosses the placenta in humans to provide in vivo protection for the newborn during the first months of life. Along with IgM, certain subclasses of IgG fix serum complement, promoting the lysis of certain cells, bacteria and viruses and the clearance of immune complexes from serum. Binding of IgG onto macrophage Fc receptors "arms" the macrophages to function in a cytotoxic manner (2). Moreover, IgG may play a blocking or protective role in IgE-dependent immediate-hypersensitivity (3). Deficiencies in one or multiple subclasses of IgG, whether primary or acquired, have been associated with recurrent infections in children and adults (4).

Human immunoglobulin G (IgG) is distinguishable from IgM, IgA, IgD and IgE by structural differences in its heavy chain constant regions (1). Four subclasses of human IgG have been identified on the basis of their differences in structure (hinge size, inter-chain disulfide bond number, point of light chain attachment to heavy chain and kappa/lambda light chain ratio) and biological properties (serum concentration, cellular Fc receptor binding, fixation of complement, susceptibility to enzymatic digestion and biological half-life in serum) (2,5). While the heavy chain constant regions of the 4 IgG subclasses share greater than 95% sequence homology between any two subclasses, each IgG subclass has unique amino acid sequences, primarily in the hinge and Fc regions, that form distinct antigenic determinants.

In the 1960s, polyclonal antibodies were prepared to the individual human IgG subclasses by immunizing monkeys, rabbits, sheep and goats with IgG myeloma proteins. These antisera were exhaustively absorbed with heterologous IgG subclass myeloma proteins to restrict their binding to a single subclass. They have been useful in the identification of genetic variants (allotypes) of IgG and in the measurement the IgG subclasses in serum, primarily by gel immunodiffusion. Unfortunately, the polyclonal antisera have been invariably weak, not widely available and crossreactive between the IgG subclasses.

By the early 1980s, several laboratories had prepared murine monoclonal antibodies (MoAb) specific for the human IgG subclasses (6-9). Immunologists under the auspices of the International Union of Immunological Societies (IUIS)

and World Health Organization (WHO) performed a study of monoclonal antibodies with putative specificity for the human IgG subclasses using eight immunological techniques (10). Their report provided a world-wide consensus on the quality and performance of available monoclonal antibodies specific for the human IgG subclasses. A designation consisting of HP and a number (eg. HP6047) was assigned to each antibody in that study to allow traceability to its origin. These MoAbs have resolved many of the concerns expressed about the variable potency, limited availability and suboptimal specificity of earlier polyclonal antisera for the human IgG subclasses. While most of the HP-series monoclonal antibodies are now available from several commercial sources, there has been disagreement on which antibodies are most useful and in what form (purified, ascites, culture fluid) and assay configuration they can be successfully employed (11-15, see reviews 16,17).

In this study, we describe a general immunoassay methodology that employs a panel of documented HP-series monoclonal antibodies for quantitation of total IgG and the human IgG subclasses. Technical aspects of their use in the measurement of total human IgG subclass protein levels in human serum are discussed using precision, sensitivity and parallelism as criteria for optimization.

MATERIALS AND METHODS

Buffers.

Phosphate-buffered saline (PBS) contained 0.137 M NaCl (8 gm/L), 0.0027 M KCl (0.2 gm/L), 0.008 M Na_2HPO_4 (1.15 gm/L, anhydrous) and 0.0015 M KH_2PO_4 (0.2 gm/L, anhydrous), pH 7.4. Carbonate buffer contained 0.015 M Na_2CO_3 (1.59 gm/L, anhydrous), 0.035 M $NaHCO_3$ (2.93 gm/L, anhydrous), pH 9.6. PBS was combined with 0.05% Tween 20 (polyoxyethylene-sorbitan monolaurate) for washing or 0.01% thimerosal and 0.5% bovine serum albumin (BSA) for blocking microtiter plates and preparing serum dilutions. Citrate solution (0.1 M citric acid, pH 5.0) was used as a diluent for the 1 mg/ml o-phenylenediamine substrate solution used with the horseradish peroxidase (HRP) conjugated antibodies. p-Nitrophenylphosphate (PPNP) substrate for alkaline phosphatase (AP) was diluted to 1 mg/ml in substrate buffer containing 0.167M

$NaHCO_3$ (14 gm/L), 0.012M Na_2CO_3 (1.3 gm/L), 0.001M $MgCl_2$ (0.2 gm) and used immediately or stored frozen for up to 2 months. All chemicals unless otherwise stated were purchased from Sigma Chemical Company, St. Louis, MO.

Biologicals and Immunochemicals.

Chromatographically-purified human IgG myeloma protein of known subclass and light chain specificity were kindly provided by Dr. Hans Spiegelberg, Scripps Clinic and Research Foundation, La Jolla, CA. Human IgG Fc and Fab fragments were prepared from chromatographically-purified human IgG obtained by fractionation of a normal human serum pool with diethylaminoethyl (DEAE) cellulose and Sephadex G-200 chromatography (18). Biotin-hydrazide, biotin N-hydroxysuccinimide ester, avidin and avidin-HRP were obtained from Calbiochem Corporation, La Jolla, CA. Four immunoglobulin reference preparations (IRP) with previously reported IgG subclass levels as determined by immunodiffusion and/or immunoassay were used to document assay sensitivity and parallelism. These included the WHO IRP 67/97, British IRP 67/68 (19), Centers for Disease Control IRP (CDC, Atlanta, GA) (IP1644) (20) and the Netherlands IRP (H00-01) (21). Target values published for the WHO 67/97 IRP (22) were used to construct the dose response curve: IgG1 (5.0 G/L), IgG2 (2.6 G/L), IgG3 (0.4 G/L), IgG4 (0.5 G/L), and total IgG (8.5 G/L).

Mouse Monoclonal anti-human IgG Antibodies.

The HP-series murine monoclonal antibodies used in this study were isolated from ascites produced by the Hybridoma Reagent Laboratory, Houston, TX from documented hybridoma cells lines described in the IUIS/WHO report (10). Characteristics of the antibodies used in this study are summarized in Table 1. All MoAbs were purified from ascites or culture fluid by DEAE cellulose ion exchange chromatography followed in selected cases by hydroxylapatite or size exclusion (G-200) chromatography (23). Protein A affinity chromatography was not used to avoid immunoglobulin denaturation in the acid elution step and loss of specificity resulting from contamination by Protein A. Precipitation with ammonium sulfate was also avoided to minimize denaturation of the MoAb.

Quality Control of Monoclonal Antibodies.

Monoclonal antibody immunochemical purity was assessed by a combination of zone electrophoresis, cross-immunoelectrophoresis, SDS-polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF) and IEF affinity immunoblot

TABLE 1

ANTI-HUMAN IgG MONOCLONAL ANTIBODIES

Clone Number	Human IgG Specificity	Mouse Isotype	Mean pI*	ELISA Specificity Analysis** IgG1	IgG2	IgG3	IgG4	Ka *** L/M x 10^7
HP6017	PAN IgG Fc	IgG2a-k	7.6	100	100	99	100	NA
HP6045	PAN IgG Fd	IgG2a	6.5	100	99	100	100	7.8 G1,2,4 117 G3
HP6046	PAN IgG Fd	IgG1	6.3	100	100	100	99	NA
HP6001	IgG 1 Fc	IgG2b	7.9	100	0.25	<0.01	<0.01	1.0
HP6069	IgG 1 Fc	IgG1-k	6.5	100	0.2	0.1	0.02	15
HP6070	IgG 1 Fc	IgG1-k	6.2	100	0.1	<0.1	<0.01	NA
HP6002	IgG 2 Fc	IgG1	7.1	<0.01	100	<0.01	<0.01	13
HP6014	IgG 2 Fd	IgG1-k	6.6	0.08	100	0.8	0.06	3.6 Kappa 37 Lambda
HP6047	IgG 3 Hinge	IgG1	6.6	0.04	0.09	100	<0.01	78
HP6050	IgG 3 Hinge	IgG1-k	6.7	<0.01	<0.01	100	<0.01	10
HP6023	IgG 4 Fc	IgG3-k	7.7	0.08	<0.01	0.18	100	19
HP6025	IgG 4 Fc	IgG1-k	6.3	0.48	0.08	0.18	100	20
HP6054	Lambda	IgG3	6.5	Kappa = 0.2		Lambda = 100		120
HP6062	Kappa	IgG2a-k	7.0	Kappa = 100		Lambda = 0.03		NA

* mean isoelectric points (pI) were determined by IEF-affinity immunoblot analysis (see methods).

** Specificity is expressed as a % crossreactivity as determined by dilutional analysis of monoclonal antibodies in the ELISA.

*** Affinity constants (Ka) were obtained from Ref 33. NA = not available.

analysis using previously reported methods (23, 24). Routine quality control of all ascites and purified monoclonal antibody was performed using IEF, generally in combination with affinity immunoblotting (25). SDS-PAGE and IEF were performed with precast gels using the Phast System (Pharmacia, Piscataway, N.J.). Specificity of the MoAb in ascites, culture fluid or in purified form before and after enzyme conjugation was documented by dilutional analysis using two immunoassays: a direct binding ELISA and a capture IEMA.

In the direct binding ELISA, microtiter plates were coated with PAN (IgG1-4) human IgG, individual human IgG subclass 1, 2, 3, or 4 myeloma protein or human IgG Fc and Fab fragments. Monoclonal antibodies in ascites or purified form were analyzed in 8-10 dilutions. Bound murine antibody was detected with either horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG (H+L) (Jackson Immunoresearch, West Grove, PA) or goat anti-mouse IgG (H+L) (Caltag, San Franscisco, CA). Both conjugates were preabsorbed with human IgG to remove crossreacting antibodies. Optical density was measured in a Titertek Multiscan (Flow Laboratories, Alexandria, VA) connected to an IBM PC/XT personal computer. The data were analyzed using an ELISA template (26) prepared for Symphony (Lotus Corporation, Cambridge, MA) and 50% maximum binding levels were compared to determine percent cross-reactivity.

The capture IEMA tested MoAb specificity in an actual capture configuration. Purified MoAb was adsorbed to microtiter plates and dilutions of IgG myeloma proteins of known specificity were captured. Bound human IgG was then detected with one of two conjugated antibodies (HRP-conjugated goat antibody to human IgG, Caltag, San Franscisco, CA, absorbed against mouse IgG, code H17507, lot 0101; or HRP-conjugated polyclonal mouse anti-human IgG (Fc), Jackson Immunoresearch, West Grove, PA, code 209-035-103, lot 6618). Sigmoidal dilution curves were generated and non-specific binding to BSA-coated wells was subtracted to compute net optical density. Crossreactivity was computed as a ratio of reciprocal dilution of the MoAb binding to heterologous versus homologous myeloma protein which gave equivalent optical density in the assay.

Conjugated Monoclonal Antibodies.

Chromatographically purified murine monoclonal antibody was conjugated with horseradish peroxidase, alkaline phosphatase or biotin. The first enzyme-

conjugation approach involved the oxidation of the carbohydrate moieties of fluorodinitrobenezene-blocked enzyme with sodium periodate to form aldehyde groups. The enzyme-aldehyde was then bound to free amino groups on the antibody (27). The Schiff base formed by this reaction was finally stabilized with $NaBH_4$. A second enzyme conjugation method involved a heterobifunctional crosslinking procedure (28). Maleimide functional groups were introduced into the enzyme using the maleimidobutyryloxysuccinimide ester (GMBS) (eg. 1.2 micromoles of GMBS in 10 ul of dried tetrahydrofuran was added to 9.8 mg or 200 nM of HRP in 1 ml of PBS, pH 7.0, 30 min, 30°C). Murine IgG was thiolated using N-succinimidyl-S-acetylthioacetate (SATA) (eg. 5 mg IgG in PBS, pH 7.5, 1:13 molar ratio IgG:SATA in dried dimethylformide, 10 min, 23°C). Hydroxylamine (1 M, 0.1 ml/ml) was added to enzyme-GMBS just prior to dropwise addition of IgG-SATA. After 2 hrs at 37°C, the mixture was dialyzed against PBS and stored at -20°C in 10 mg BSA/ml and 40% glycerol. Biotinylation of murine IgG MoAb was performed using biotin N-hydroxysuccinimide ester and biotin-X-hydrazide according to a previous method (29). Chemicals for conjugation and biotinylation were obtained from Calbiochem Corporation, La Jolla, CA.

RESULTS

Immunoenzyametric Assay for Total IgG Subclass Protein.

The 5 IEMAs for IgG1, IgG2, IgG3, IgG4 and total IgG should be considered similar assays in terms of methodology but different in terms of specific details (reagents, performance, serum dilutions). Many variations of the non-competitive two-site immunoenzymetric assay were tested for use in measurement of the human IgG subclasses. All 5 IEMAs were routinely performed either in a single day or in a two day assay using an overnight serum incubation at room temperature (23°C). Each IgG subclass was analyzed in a separate microtiter plate containing its own WHO 67/97 8 dilution standard curve. Considerable preplanning was required to insure that sufficient volumes of each test, reference and control serum dilution were available since the actual dilutions required for the IEMAs varied from 1:500 to 1:2,048,000. The most reproducible immunoassay format as defined by intra- and inter-assay precision, sensitivity and parallelism was the non-competitive two-site immunoenzymetric assay outlined in Table 2. Unique aspects of each assay procedure are now discussed.

TABLE 2

IgG SUBCLASS IEMA PROTOCOL

1. Capture monoclonal antibody coating:

   A. Label 5 microtiter plates: IgG1, IgG2, IgG3, IgG4 and IgGPAN.
   B. Dilute purified monoclonal antibodies (MoAb) for each of the 5 IEMAs in PBS to 10 ug/ml. (minimize loss on plastic trays and tubes)
   C. Coat purified MoAb into its respective microtiter plate (0.1 ml/well).
   D. Incubate at 37°C for 2-6 hrs. (make note of MoAb lot numbers and dilution details).

2. Unreacted site blocking:

   A. Without washing, add 0.1 ml of PBS-0.5 gm% BSA-0.01 gm% Thimerosal.
   B. Incubate for 1-2 hrs at room temperature while making serum dilutions.
   C. Prepare dilutions of reference and test sera (see Table 3) in PBS-0.5% BSA-0.01 gm% Thimerosal-0.005 gm% Phenol Red. Phenol red produces a red tint which facilitates pipetting with the 50/50 addition method.

3. Biological Fluid (Serum) Incubation:

   A. Wash all wells (3x) with PBS-Tween.
   B. After last wash, pipette 50 microliters of PBS-0.5% BSA into all wells to prevent drying during serum addition step (50/50 addition method).
   C. Pipette 50 microliters of each dilution of reference and test sera into their respective wells in duplicate. Use an 8 point reference serum standard curve and analyze 3 appropriate dilutions of test sera in each IEMA (see Table 3).
   D. Incubate overnight (12-14 hrs) at room temperature or 2 hr at 37°C.

4. Conjugation Incubation:

   A. Wash all wells (3x) with PBS-Tween.
   B. Pipette 0.1 ml of conjugate (anti-human IgG PAN) into each well.
   C. Incubate 2 hrs at 37°C.

5. Substrate Development

   A. Wash all wells (3x) with PBS-Tween.
   B. Pipette 0.1 ml of substrate (1 mg/ml: OPD for HRP, PPNP for AP) into all wells. If using HRP, stop reaction with 0.05 ml of 4N H_2SO_4.
   C. Read all wells in a microtiter plate reader at 492 nm (HRP-OPD) or 405 nm (PPNP-AP).
   D. Interpolate unknown sera optical density from the dose-response curve generated with the calibrator-reference serum standardized against the WHO 67/97 reference human serum.

1. Capture Antibody Coating. The objective of this step is to maximally bind capture MoAb to the solid phase (plate) while maintaining its immunoreactivity. Adsorption of antibody to microtiter plates was used in this study because the technique was least expensive and available to all investigators. Methods for covalently coupling antibody to plastic have become available and in some cases increase the amount of MoAb bound to the wells in comparison to adsorption. However, covalent coupling methods were not used in this study because such methods were not found to enhance the assay's working range and sensitivity (data not shown).

Chemical purity of the monoclonal antibody was the single most important variable that influenced the amount of immunoreactive anti-human IgG MoAb that could be bound to the microtiter plate. Maximal coating was obtained between 3 to 10 ug/ml of chromatographically purified MoAb (Figure 1). Adsorption of MoAb in ascites or culture fluid after dilution 100 to 1000 fold in water and drying on the plate has been suggested as one method to coat MoAb onto plates (11, 15). In this study, the use of unpurified MoAb in ascites with the dilution/drying method produced large variation and low binding capacities when compared to direct coating of chromatographically purified antibody (data not shown). Standardization of the coating procedure between lots of ascites and culture fluids was especially difficult due to the variable amount of protein (albumin, transferrin) other than MoAb in the preparation.

Microtiter plates from four sources were compared in this study: Immulon II removalwells and polyvinyl chloride plates (Dynatech, Alexandria, VA); Falcon MicroTest III flexible plastic plates (Beckon-Dickinson, Oxnard, CA); Linbro EIA plate (Flow Laboratory, McLean, VA); and Serocluster U vinyl plates (Costar, Cambridge, MA). All these plates bound purified antibody maximally at 3-10 ug/ml and they produced equivalent final assay performance when directly compared (data not shown). Dilution of the purified MoAb in carbonate buffer and phosphate buffered saline produced similar results in terms of human IgG binding capacity and assay sensitivity using HP6002/6014 (anti-IgG2), HP6047 (anti-IgG3) and HP6023/6025 (anti-G4) (data not shown). We chose PBS for the majority of the assays because is was more physiologic than the carbonate buffer.

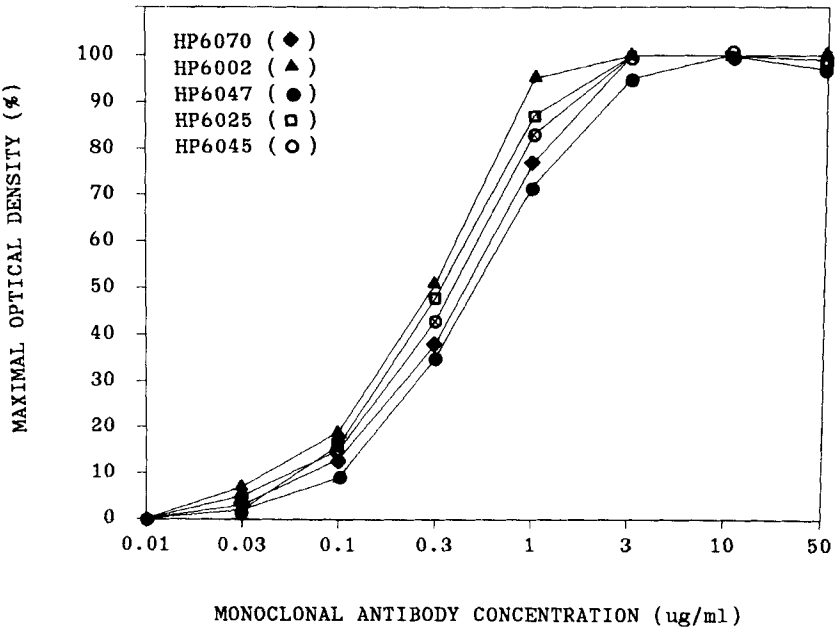

Figure 1. Determination of optimal concentrations of murine IgG monoclonal antibody for maximally coating microtiter plates. Chromatographically purified murine monoclonal antibodies (see Table 1) were diluted in PBS: HP6070 anti-IgG1, HP6002 anti-IgG2, HP6047 anti-IgG3, HP6023 anti-IgG4, HP6025 anti-IgG4, HP5045 anti-IgG PAN Fd. Each was pipetted into its respective wells in an Immulon II microtiter plate (0.1 ml/well, 0.01 to 50 ug/ml). Following a 2 hr incubation at 37°C, peroxidase conjugated sheep anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) was added (0.1 ml/well, 1:50,000 dilution). O-phenylenediamine (1 mg/ml) was added in citrate buffer with hydrogen peroxide (3 ul/22 ml). Following color development, the reactions were quenched with 4N H_2SO_4 and the optical density was measured at 492nm. Maximum adsorption of purified mouse IgG to Immulon II plastic wells occurred at 3-10 ug/ml (0.3-1 ug of MoAb per well).

Biotinylated monoclonal antibodies were bound to microtiter plates that had been previously coated with avidin. Figure 2 displays two pairs of dose-response curves that were produced using purified MoAb absorbed to uncoated plates or biotinylated MoAb in PBS-BSA bound to avidin-coated plates. Interpolated test serum results obtained from assays using both these capture antibody configurations were equivalent. This indicates that biotinylated MoAb can be effectively used for insolubilizing capture antibodies. The biotinylated MoAbs have the added advantage that they can be stored in a protein (eg. BSA) containing buffer. Their shelf-life is therefore longer than the same

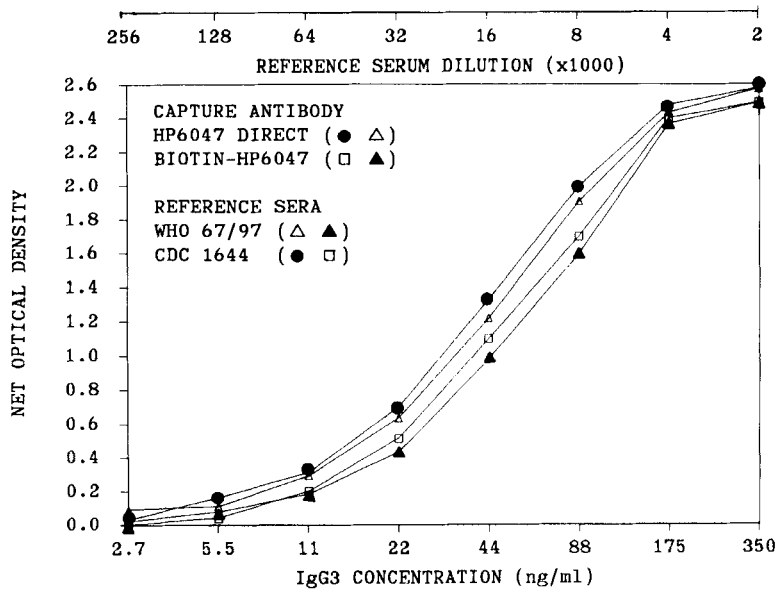

Figure 2. Comparison of the immunoreactivity of HP6047 (anti-human IgG3) MoAb after adsorption directly on uncoated microtiter plate wells (DIRECT) or after biotinylation and binding to avidin coated wells (BIOTIN-HP6047). Two human reference sera (WHO 67/97 and CDC 1644) were analyzed in both DIRECT and BIOTIN-HP6047 coated wells. OD values obtained with the CDC serum were interpolated from the corresponding WHO 67/97 reference serum standard curve. Interpolated values for the CDC 1644 serum were the same in the IEMAs using HP6047 (anti-IgG3) MoAb either adsorbed or biotinylated and bound to avidin: DIRECT COATING: 60.5 ± 6.7 mg%, 11% inter-dilutional (ID) CV, n = 5 dilutions; BIOTIN-HP6047: 62.3 ± 4.8 mg%, 8% ID-CV, n = 5 dilutions. Inter-dilutional %CVs < 10% demonstrate assay parallelism.

antibody in an unconjugated, purified state which cannot be stored in carrier protein if it is to be used for direct adsorption.

2. Blocking unreacted sites. It is common practice in microtiter plate based immunoassays to block unreacted sites after the primary coating step with an irrelevant protein (eg. 0.1-0.5% BSA or gelatin in PBS). Problems arise in the IgG subclass assays if human serum albumin is used as the carrier protein because it contains a small percentage (<1%) of immunoglobulins that produce a high background in the assay. Rather than suctioning the MoAb from the well before adding the blocking protein, the carrier protein can be pipetted into the filled well. Efficacy of blocking is not diminished because the concentration of the blocking protein is in excess (0.5 gm/100 ml) of the well's protein binding capacity (typically 1 ug/well).

3. Biological Fluid (Serum) Incubation. The assay step which contributes the most to assay imprecision and inaccuracy in the total IgG subclass IEMAs is the preparation of high dilutions of serum (1:500 to 1:2,048,000). Selection and preparation of all the dilutions for the reference and test sera is a tedious and yet indispensible aspect of the assay. While precision and accuracy can be improved by the use of automatic pipetter/diluters, all dilutions in this study were intentionally performed manually to examine the assay's worst case. Table 3 summarizes suggested serum dilutions that can be used for the reference (WHO 67/97 or a secondary reference serum pool) and test sera. Serum dilutions vary from a 1:1000 in the IgG4 assay to 1:2,048,000 in the total IgG assay when 8 reference serum dilutions are used to construct the dose-response curve.

Target values for the WHO 67/97 reference calibrator are noted in Table 3. The WHO 67/97 is considered the most reliable international reference preparation for the human IgG subclasses at present. Each laboratory must prepare its own working standard by combining aliquots of sera (eg. 50-100) from HIV negative, healthy adults and cross-standardizing it against the WHO 67/97. The HO-002 immunoglobulin reference preparation from the Netherlands Blood Transfusion Service or the U.S. National Immunoglobulin Reference Preparation (CDC IS-1644) can be used as independent internal controls during the standardization process (see Table 3).

We have observed significant variation in the net optical density when the identical serum dilution is pipetted into wells at the right, left, top and bottom of the microtiter plate (30). A gradual loss of antibody binding from right to left and top to bottom can result from (a) too short a serum incubation time (30 min to 1 hr) in relation to the time required to pipette a 96 well plate (10-15 minutes); (b) drying of the well which produces variable degrees of non-specific binding; (c) unequal rates of conjugate and substrate addition from left to right or top to bottom. One procedure which minimizes these trends is the 50/50 serum addition method which involves pipetting 50 ul of buffer to all wells after the last wash to keep them moist during the pipetting of 50 ul of serum dilutions into their respective wells. When this method is combined with a long serum incubation period (eg. 4 hr to overnight), left to right and top to bottom effects can generally be eliminated. The addition of trace amounts of phenol red to the serum diluent buffer (PBS-0.5% BSA, Table

TABLE 3

IMMUNOENZYMETRIC ASSAY PERFORMANCE

Parameter	IgG1 IEMA Ref* ng/ml	Test **	IgG2 IEMA Ref* ng/ml	Test **	IgG3 IEMA Ref* ng/ml	Test **	IgG4 IEMA Ref* ng/ml	Test **	Total IgG Ref* ng/ml	Test **
Serum Dilution										
1:100 (0.1K)	X	X	X	X	X	X	X	X	X	X
1:1000 (1K)							500			
1:2000 (2K)							250	T		
1:4000 (4K)	1250		650		100		125	T		
1:8000 (8K)	625		325		50	T	62.5	T		
1:16000 (16K)	312.5		162.5	T	25	T	31.3		531.5	
1:32000 (32K)	156.3	T	81.3	T	12.5	T	15.6		265.8	
1:64000 (64K)	78.1	T	40.6	T	6.3		7.8		132.9	
1:128000 (128K)	39.0	T	20.3		3.1		3.9		66.4	T
1:256000 (256K)	19.5		10.1		1.6				33.2	T
1:512000 (512K)	9.8		5.1		0.8				16.6	T
1:1024000 (1024K)									8.3	
1:2048000 (2048K)									4.2	
Capture Antibody	HP6070 ± HP6069		HP6002 ± HP6014		HP6047 ± HP6050		HP6025 ± HP6023		HP6017 + HP6046	
Working Range ng/ml	20-1250		5 - 200		2 - 50		8 - 250		5 - 530..	
Sensitivity ng/ml	15 (6069 or 6070)		5.0 (6002 + 6014)		1.6 (6047 ± 6050)		7.8 (6025 or 6023)		5.0 (6017 + 6046)	
Reference Sera ***	IgG1		IgG2		IgG3		IgG4		Total IgG	
WHO 67/97 (gm/L)	5.0		2.6		0.4		0.5		8.5	
H00-02 (gm/L)	5.9		3.2		0.5		0.5		10.1	
CDC IS1644 USNRP	6.95		3.15		0.7		0.61		11.4	
95 Percentile (11) (adults, n=63)	1.8-7.8		1.0-4.6		0.3-1.4		0.08-1.8		3.8-15.0	

* Ref = Reference serum refers to a calibrator. IgG subclasses concentrations in the WHO 67/97 reference are presented in nanograms per ml for dilutions to be analyzed.

** Standard recommended dilutions of test sera are denoted as "T". All dilutions were performed using no less than 10 ul of sample. "X" refers to the first dilution of 1:100 that was prepared for reference and test sera.

*** IgG subclass target values for the primary reference and control sera (WHO 67/97 and H00-02 and CDC IS1644) were extracted from Reference 11 and 22.

2) produces a red tint which facilitates accurate pipetting of each serum into its respective wells.

4. Conjugate Incubation. Selection of the optimal enzyme-conjugated anti-human IgG detection antibody is complicated by the fact that some of the HP-series murine monoclonal anti-human IgG subclass antibodies crossreact with goat or sheep immunoglobulin (31). Moreover, the use of a MoAb capture and a different MoAb as the enzyme conjugate for detection of bound IgG can result in unexpectedly low responses. This may reflect steric hinderance of capture and detection MoAbs that bind structurally similar, restricted epitopes (32). Thus, a polyclonal enzyme-conjugated mouse anti-human IgG Fc (Jackson Immuno-research, West Grove, PA) was selected as the routine conjugate for all five IEMAs. Its reactivity for all 4 subclasses of human IgG was demonstrated using solid phase IgG myelomas of known subclass type. A mixture of peroxidase-conjugated MoAbs (HP6017: anti-human IgG Fc PAN; HP6046 anti-human IgG Fd PAN; HP6062 anti-human kappa; and HP6054 anti-human lambda) has shown some promise as an alternative conjugate (low NSB, high signal to noise ratio) for the IgG subclass IEMAs. Care, however, must be exercised when cocktail of monoclonal antibodies specific for the human IgG is used because widely differing affinity constants of the antibodies (33) can bias the final immunoassay result in favor of one type of IgG (kappa vs lambda bias, eg HP6014; IgG subclass bias, eg HP6045; see Table 1).

5. Substrate Development. Multiple chromogenic and fluorogenic substrates can be used in these immunoassays. O-phenylenediamine and p-nitrophenylphosphate were selected in these studies for horseradish peroxidase and alkaline phos-phatase, respectively. Ideally, a development time should be set for stopping the color development and reading the plate. Practically, however, the assays' working range can be manipulated by allowing the development time to vary between 5 and 15 minutes. In this manner, the assay working range can be tailored to the test specimen levels. If the patient is deficient in IgG, the maximal assay sensitivity can be obtained by developing the plate until the buffer blank begins to increase. The objective is to minimize the buffer blank (non-specific binding) and maximize the signal in the desired region of the dose-response curve. It is, however, more common that the dilutions of serum

are too high and they exceed the working range of the assay. The total IgG subclass IEMAs are sensitive enough to detect down to 2-15 ng/ml of IgG protein. When measuring high levels of IgG subclass protein in serum, it can be advantageous to stop the plate earlier to salvage patient information when the selected serum dilutions were too high. The disadvantage of manipulating the working range of these IEMAs by varying the development time is that the standard curve and precision profile of the assay does not remain constant between assays. Doses at 25%, 50% or 75% maximum binding which are commonly used as quality control parameters for the standard curve can vary considerably which makes decisions about acceptance of assay data complicated.

General Performance of the IEMAs.

The minimal detectable dose and working range of the 5 IEMAs are presented in Table 3. The inter-assay reproducibility and intra-assay precision of all the assays were < 12% and <5% coefficient of variation (CV), respectively. Measurements with higher CVs were routinely repeated. All reference and test specimens were analyzed in duplicate at two or three dilutions. Inter-dilutional CVs < 20% were obtained in all 5 IEMAs with at least 2 serum dilutions which demonstrated parallelism between the dilutions of test sera and the reference serum in the assay. Criteria for defining the sensitivity and working range of the dose-response curve were based on the imprecision of the reference serum measurements. Sensitivity was defined as the dose that produced 3 SD above the buffer blank optical density. The lower working range limit was considered the dose at twice the buffer blank optical density. The upper working range limit was the dose at 10% less than the plateau optical density.

Biological versus Assay Variability.

Observed random error in the IgG subclass IEMAs ranged from 1-15% when sera were analyzed in duplicate at 3 dilutions and the mean IgG concentration of 2 or 3 dilutions was computed. A long serum incubation step was selected to minimize variation due to the time required to pipette serum into the 96 well microtiter plate. Assay (analytical and dilutional) variation, however, may limit the ability of the IEMA to detect small changes in the IgG subclass levels of a patient over time (17, 32). The imprecision (combined analyt-

TABLE 4

VARIABILITY IN IMMUNOGLOBULIN G MEASUREMENTS

Pt#	SPEC DATE	IgG1 HP6070 mg%	IgG1 HP6069 mg%	IgG1 M±SD mg% **	CV %	IgG2 HP6014+ HP6002 mg%	IgG3 HP6047 mg%	IgG4 HP6025 mg%	Total IgG HP6017+ HP6046 mg%	Total IgG SUM 1-4 mg%	IgG * Sum Measured
1	110487	623	690	657±47	7%	251	18	43	817 ± 74	969	119%
2	110487	545	560	553±11	2%	97	37	31	762 ± 103	718	94%
3	110487	563	583	573±14	2%	303	30	30	840 ± 37	936	111%
3	112387	522	528	525±4	1%	310	30	35	808 ± 84	900	111%
4	110487	330	408	369±55	15%	68	16	13	404 ± 55	466	115%
4	112387	485	610	548±88	16%	92	16	17	617 ± 30	673	109%
5	041585	410	452	431±30	7%	80	19	7	455 ± 89	537	118%
5	120186	480	408	444±51	11%	67	17	7	387 ± 54	535	138%
5	103087	483	445	464±27	6%	63	17	7	655 ± 77	551	83%
5	110487	438	366	402±51	13%	55	15	6	519 ± 26	478	92%
5	112487	520	537	529±12	2%	86	18	7	502 ± 60	640	127%

* Total sum IgG1-4 was computed using the mean IgG1 values of the measurements produced by separate IEMAs using HP6069 and HP6070 as capture MoAb.

** Mean ± 1 SD and inter-assay %CVs of IgG1 concentrations (mg%) produced in the IgG1 IEMA using HP6069 or HP6070 as a capture antibody.

ical intra-assay, inter-assay and inter-dilutional variation) of the IEMAs was generally less than 20%. Thus, a change in the IgG subclass protein level in an individual of less than 20% may not be detectable in these assays. To examine inherent variation in individuals over time, a small number of sera were collected from healthy individuals over short (1-2 weeks) and, in one case, long (1-2 years) intervals. Analysis of these sera in the IEMA indicated that the levels of total IgG1-4 can be very consistent within an individual over time periods of a week to as long as 2 years (Table 4). The greatest variation was observed in the IgG1 levels. For this reason, 2 individual MoAb capture antibodies (HP6069 and HP6070) were investigated for their ability to generate comparable estimates of IgG1 in the IEMA. Variation between the IgG1 levels in the IgG1 IEMA using both capture MoAbs separately was equivalent to the variation observed in the general IEMA method.

The IgG subclass IEMAs provide the investigator with a unique method of internal quality control. The sum of the individual IgG subclass levels as measured by the IgG1, IgG2, IgG3 and IgG4 IEMAs should equal the concentration of total IgG determined by the IgG PAN IEMA. Table 4 compares the sum of individual IgG subclass 1-4 with the total serum IgG measured in the IgG PAN IEMA. The sum/measured IgG ratio varied from 83 to 138% (111% $\pm$ 16%, n=11). While the IgG1-4 sum was generally 10% higher than the measured total IgG in most of the determinations, no systematic bias could be identified in the assays. Differences between the sum and measured total IgGs may reflect the cummulative error from the 4 individual IEMA measurements.

DISCUSSION

One major objective of a clinical laboratory investigator is the production of an accurate estimate of an analyte that can be compared to a "predetermined normal range". This involves the selection of an assay configuration that minimizes systematic bias and random error. The earliest assay of human IgG subclass protein used polyclonal antisera in gel radial immunodiffusion (RID) (16, 17). The RID was simple to perform, however, criticism of this method focused on the questionable specificity and inter-lot variability of the polyclonal antisera and the limited sensitivity of the RID for IgG3 and IgG4. The commercial availability of IUIS/WHO documented MoAbs has resolved many of the concerns of specificity and it has fostered the development of reproducible immunoassays that can detect ng/ml levels of IgG subclass protein.

Several immunoassay configurations have been proposed to quantify IgG subclasses in human serum. One employs a PAN IgG capture antibody to extract all 4 subclasses of IgG from serum and a conjugated anti-IgG subclass MoAb to detect only one IgG subclass. This assay is constrained by its inability to bind IgG3 and IgG4 in the presence of a predominance of IgG1 and IgG2 (>85% of the total serum IgG). When serum is diluted to a concentration where total human IgG is limiting, then the level of IgG3 and IgG4 is too low to be detected by conjugated anti-IgG3 and anti-IgG4 MoAbs in this assay configuration. Moreover, this assay requires "excess" capture antibody to extract IgG of all 4 subclasses from serum, which is difficult to obtain in a microtiter plate configuration that has a limited total protein binding capacity.

A second proposed assay employs a competitive reaction between enzyme-labeled human IgG myeloma protein of one subclass and serum IgG for limited anti-human IgG subclass MoAb binding sites. This assay has many appealing features including the conservation of anti-human IgG MoAb and good properties of sensitivity, precision and parallelism. Its major disadvantage stems from the limited availability of purified human IgG1, IgG2, IgG3 and IgG4 myeloma (or polyclonal) protein and the requirement for labeling the IgG with bulky enzymes that can mask restricted monoclonal antibody binding sites.

To date, the most widely used immunoassay configuration for total IgG subclass measurement has employed IgG subclass-specific monoclonal antibody to capture a single IgG subclass and an enzyme-conjugated PAN IgG specific antibody to detect bound IgG (11-17). Variations between the reported assays have included the actual MoAbs used as capture and detection antibodies, the purity of the coating MoAb (ascites vs chromatographically purified), incubation times, type of microtiter plates, recommended working dilutions of serum and the conjugated anti-human IgG (PAN) detection antibody. In this study, a panel of antibodies was selected from the IUIS/WHO report that displayed good specificity (10, 11, 13, 17) and high affinity (33) for their homologous IgG subclass protein. Variability in coating of microtiter plates was minimized by using chromatographically-purified antibody which is commercially available from the Hybridoma Reagent Laboratory (Houston, Texas). The serum incubation time was maximized (overnight) to minimize error resulting from the time required to pipette serum into 5 plates. Because, total IgG subclass measurements are rarely required immediately, an overnight serum incubation was considered acceptable. A wide variety of polystyrene and polyvinylchloride microtiter plates have been successfully used to adsorb purified monoclonal antibody directly or avidin for subsequent binding of biotinylated MoAb. Finally, considerable effort has been expended to define recommended reference and test serum dilutions for the IEMAs so all 5 assays could be simultaneously performed (Table 3).

IgG1 comprises approximately 70% of the total IgG in normal human serum. Inherent variation in the IgG1 IEMA would therefore be expected to contribute the most to poor agreement between the total IgG computed as the sum of the

levels from the four IgG subclass IEMAs and that measured by the total IgG IEMA. Initially, HP6001 (anti-human IgG1 Fc) was used as a capture antibody. However, due to its low affinity, HP6001 was discontinued because it produced an unsatisfactory working range and sensitivity. Instead, MoAb from 2 clones prepared in Finland (HP6069 and HP6070) were used separately and together as capture antibodies in the IgG1 IEMA. A useful working range (20 to 1250 ng/ml) and good assay performance were achieved using both MoAbs. Good agreement was obtained between the IgG1 levels produced using either HP6069 or HP6070 MoAb as a capture for serum IgG1 (inter-assay CVs $\leq$16%, Table 4). In routine assays, they are used together at 5 ug/ml each to obtain the widest assay working range.

IgG2 is generally considered the most difficult IgG subclass to detect primarily due to its compact structure and the limited number of IgG2 specific MoAbs. A combination of HP6002 (anti-human IgG2 Fc) with HP6014 (anti-human IgG2 Fd) was used with success to quantitate IgG2. When used together in equal concentrations (5 ug/ml each), these MoAbs produced an IEMA with a working range of 5-200 ng/ml of IgG2. HP6014 binds to an epitope located in the Fd (heavy chain of the Fab'_2) of human IgG2. Interestingly, it exhibits an IgG2 lambda to kappa light chain bias and thus, HP6014 should not be used alone as a capture antibody for IgG2. Rather, it can be effectively used in combination with HP6002 to avoid inaccuracies caused by differences in light chain distributions of IgG2 proteins between individuals and the reference serum pool.

The IgG3 and IgG4 IEMAs posed no major technical problems. HP6047 with or without HP6050 produced a sensitive and parallel IEMA for IgG3. Its working range was narrow and dose-response curve steep, primarily due to the high affinity ($Ka = 7.8 \times 10^8$ L/M) of HP6047 MoAb. The high affinity of HP6047 anti-IgG3 forces the analysis of serum at remarkably high dilutions of 8,000 to 32,000. Both HP6023 and HP6025 (anti-IgG4) MoAbs performed equally well in the IgG4 IEMA together and separately as capture antibodies. HP6025 binds to some goat immunoglobulins and therefore use of an enzyme-conjugated goat anti-human IgG detection antibody with HP6025 as a capture produces high buffer blanks. Use of a monoclonal or polyclonal mouse anti-human IgG detection antibody

eliminated any background problems and allowed effective use of HP6025 as an IgG4 capture antibody. Routinely, HP6025 and HP6023 anti-human IgG4 MoAbs are used together to maximize the assay's working range.

Most clinical immunology laboratories measure the total serum IgG with rate/endpoint nephelometry or radial immunodiffusion. We chose to measure the total serum IgG by IEMA using HP6017 (anti-IgG PAN Fc) in combination with HP6046 (anti-IgG PAN Fd). The IgG PAN IEMA has a useful working range of 5 to 530 ng/ml of human IgG. Use of the two antibodies HP6017 (anti-IgG Fc PAN) and HP6046 (anti-IgG Fd PAN) complement each other well and produce a robust assay.

The major clinical indication for the measurement of human IgG subclass protein is the identification of selective or total IgG subclass deficiencies in individuals (especially children) with a history of recurrent infections. Identification of such a deficiency using the IgG subclass IEMAs can aid in the diagnosis and management of the patient with an infection, possibly with immunoglobulin replacement therapy when the deficiency is severe. Use of the IgG subclass measurement to diagnose an IgG subclass deficiency presupposes that the normal range for each IgG subclass is well defined in terms of age, race and sex. Problems arise in the interpretation of IgG subclass results from the IEMA because the normal ranges are not generally well characterized. What may be an abnormal variation (rise or fall) in one individual may still be within the population norm. Thus, further study will be required to define the utility of the reported normal IgG subclass ranges or percentiles in relation to individual biological variation. This report documents monoclonal antibody-based immunoenzymetric assays that can be used to explore the relationship between the individual and population variation in the IgG subclasses. Each IEMA possesses a clinically useful working range, sensitivity, precision and parallelism. Quantification of the human IgG subclasses represents an achievable frontier in the characterization of human immunoglobulins.

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