

Detection of IgG-Associated Determinants in  
Reduced and Alkylated Preparations of Human IgG3 by  
Monoclonal Antibodies

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## TABLE OF CONTENTS

Abstract	3
Introduction	4
Materials and Methods	10
Results	15
Discussion	20
Conclusions	27
Literature Cited	28
Table I	38
Figures	39

## ABSTRACT

Using classical typing antisera, previous experiments have failed to demonstrate IgG3 in partially reduced and alkylated preparations of human IgG intended for intravenous application (IGIV). To establish that IgG3 is actually present in such preparations and to investigate the effects of reduction and alkylation on IgG3-associated epitopes, an enzyme-linked immunosorbent assay (ELISA) was designed using monoclonal antibodies as solid phase reagents and protein A-purified IgG3 as antigen. Three different samples of reduced and alkylated antigen were used: 1) IgG3 isolated from a ready-for-infusion IGIV; 2) IgG3 which was purified from an intramuscular (Cohn Fraction II) IgG solution before being subjected to a mild reduction and alkylation procedure; and 3) completely reduced and alkylated IgG3. The reduction and alkylation procedure did not affect the solubility of IgG3, indicating that IGIV prepared in this manner should contain normal quantities of IgG3. In the ELISA, solid-phase monoclonals which were cross-reactive with multiple IgG subclasses clearly reacted with reduced and alkylated IgG3. Furthermore, there was no substantial difference between the quantities of modified and native antigen required for 50% maximal ELISA signal. In contrast, solid-phase monoclonals with IgG3-restricted specificity did not recognize reduced and alkylated material. These results indicate that IGIV prepared by reduction and alkylation has a normal IgG3 content and confirm that some IgG3-specific determinants are altered by the modification procedure.



## INTRODUCTION

The IgG molecule, upon reduction, yields four polypeptide chains, two heavy chains ( $\gamma$ ) and two light chains, either  $\lambda$  or  $\kappa$ . Each light chain and  $\gamma$  heavy chain is made up of a series of folded regions called domains (1,2). The light chain has a variable region ( $V_L$ ) and one constant region ( $C_L$ ). The gamma heavy chain has a variable region,  $V_H$  and three constant domains,  $C\gamma_1$ ,  $C\gamma_2$ , and  $C\gamma_3$ . The variable regions of the gamma chain and the light chain contain hypervariable regions which are responsible for the antigen-binding specificity of the antibody (3). Each domain contains an intrachain disulfide bond. The interchain disulfide bonds occur 1) between the light chain and the heavy chain, and 2) between the heavy chains, in the hinge region of the IgG molecule (fig. 1). The IgG1, IgG2, and IgG4 heavy chains are connected by two to four hinge region disulfide bonds, while IgG3 molecules have approximately 15 hinge region disulfide bonds (4). Enzyme treatment of IgG cleaves the molecule into fragments at the inter-domain regions. Digestion with papain yields the Fab and the Fc fragments (5) and treatment with pepsin yields the  $F(ab)_2$  (6). The Fab portion of the Ig molecule has the important role of binding antigen at the combining site in the V region (5).

The four IgG subclasses are identified by antigenic and biochemical differences in the gamma heavy chain (7,8). The following

table shows the approximate percentages of subclasses in normal serum:

<u>IgG1</u>	<u>IgG2</u>	<u>IgG3</u>	<u>IgG4</u>	<u>reference</u>
65	23	8	4	9
60	29	6.5	4.1	44
70	20	8	2	10

IgG3 is heavier than the other subclasses with a molecular weight of 165,000 because of its extended hinge region (11,12). The IgG3 hinge is four times larger than the hinge of the other three subclasses and consists of a 17 amino acid sequence followed by a 15 amino acid sequence which is repeated three times (13). The larger IgG3 hinge also has a large radius of gyration in aqueous solutions (12). IgG3 is more susceptible to proteolysis than IgG1, 2 and 4 (14,15), has the fastest in vivo catabolism of all the subclasses (16,17), and is the only subclass that does not bind Staphylococcus aureus protein A (18,19).

Among other functions, the Fc portion of the IgG molecule binds Clq, the first complement protein involved in the classical pathway of complement activation. IgG3 is the most efficient subclass binding Clq, and IgG1, IgG2 and IgG4 bind in that order of decreasing effectiveness (20,21). The Fc portion also binds to the Fc receptor (FcR) on a variety of human effector cells. Human macrophages and monocytes possess Fc receptors, which have been shown to bind

subclasses IgG1 and IgG3, but not IgG2 and IgG4 (22,23). The recognition site for the macrophage FcR is thought to be located in the C $\gamma$ 2 domain with an indirect role being played by the C $\gamma$ 3 region (24,25). Similar observations have been made concerning the Fc receptor on human neutrophils; subclasses IgG1 and IgG3 appear to bind at a portion of the molecule involving the C $\gamma$ 2 and C $\gamma$ 3 domains (23,26).

A series of studies has demonstrated deleterious effects of reduction and alkylation on IgG binding to Clq and to effector cell Fc receptors. Binding to Cl is lost after reduction and alkylation of human IgG1, although Fc fragments from IgG1 retain Clq binding activity (28). IgG1 binding to human peripheral blood monocytes (24) and human granulocytes (27) is substantially diminished following reduction and alkylation. In addition, binding to B lymphocytes and to human neutrophils is lost by reduced and alkylated IgG1 and by IgG1 myeloma proteins with hinge region deletions (29). These results can be contrasted with those indicating that IgG and Fc binding to protein A is not lost following reduction and alkylation and is retained by the hinge-deletion IgG1 variants (29).

Human immune serum globulin (ISG), processed from Cohn Fraction II of pooled human plasma (30,31), is utilized for prophylaxis and therapy of immunologically compromised individuals. ISG is useful for immunosuppressed patients and certain normal persons for protection against hepatitis A, non-A non-B hepatitis, hepatitis B, rubella, varicella zoster, ruboela, rabies, CMV, and tetanus (32). It is used in treatment of sepsis in meningitis patients and neonates, and for



prophylaxis of transplant recipients (33-35). IgG replacement therapy helps patients with antibody deficiency syndromes such as X-linked agammaglobulinemia, common variable agammaglobulinemia, Ig deficiencies with increased IgM, immunodeficiency lacking IgM, immunodeficiency with thymoma, severe combined immunodeficiency, and deficiency of one or more classes or subclasses of Ig (36-38). It has also been beneficial for certain patients with idiopathic thrombocytopenic purpura, multiple myeloma or chronic lymphatic leukemia (32). Adjunct treatment for patients with primary and secondary T cell deficiencies is useful, as well (32).

Human immunoglobulin administered intravenously (IGIV) has advantages over IgG given intramuscularly, especially when large volumes must be applied. IGIV immediately achieves maximal IgG levels in the blood in a single dose, volumes are not restricted due to available muscle mass, material is not lost due to proteolysis before it reaches the bloodstream, and intravenous injections are less painful (39). However, ISG administered parenterally can elicit dangerous side reactions which have been closely correlated with anticomplementary (AC) activity (non-specific activation of complement) (39,40).

Although the causes of adverse reactions are not fully defined it is likely that AC activity results from IgG aggregates which form during the plasma fractionation procedure (39). Several modifications of ISG intended to reduce AC activity have been investigated, including low pH treatment, polyethylene glycol precipitation, enzymatic

treatment with plasmin or pepsin and chemical modification by reduction and alkylation, S-sulfonation, or  $\beta$ -propiolactone (41).

A mild reduction and alkylation procedure has been developed to produce a gamma globulin preparation suitable for intravenous administration with little anticomplementary activity. ISG is reduced by dithiothreitol (DTT) and alkylated by iodoacetamide so that, by regulating the ratio of DTT to ISG, four to five of the interheavy chain disulfide bonds are reduced. The physical and functional properties of this preparation have been reported (42-45). It is free from hazardous contaminants and 99% of the total protein content is gammaglobulin which consists of 89% whole (not-cleaved or split) IgG monomer. The process does not involve enzymatic treatment and 70-75% of the disulfide bonds remain intact.

Several in vitro and in vivo studies have demonstrated that IGIV produced by reduction and alkylation maintains important biological properties. Reduced and alkylated IgG has a mean plasma half-life comparable to that of ISG in normal subjects (46). Bing (47) and Römer et al. (45), have demonstrated significant Clq binding capacity for heat-aggregated preparations. Furthermore, Bing observed that, following heat aggregation, reduced and alkylated IGIV had near-normal C3-activating activity when assayed in both classical and alternative pathways. Both studies confirmed an earlier report (43) showing that ready-for-infusion preparations have low intrinsic anti-complementary activity. To ascertain the potential efficacy of IGIV compared to ISG, specific antibody titers have been determined against viral and



bacterial pathogens (44,48). In vitro tests using reduced and alkylated IGIV have demonstrated opsonophagocytic function against group B streptococci (GBS), Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli and Serratia marcescens (49-52). In in vivo studies utilizing rat and mouse models, IGIV protected against lethal challenge doses of GBS (21-23) and P. aeruginosa (53,54). These experimental results are in agreement with clinical observations of antibody-mediated protection against infection. Furthermore, IGIV prepared by reduction and alkylation has been utilized with success to replace intramuscular preparations for prophylaxis of individuals with a range of immune deficiencies (33-38).

In an analysis of commercial products for purity and antibody content, Römer et al. (44) described the IgG subclass distribution for various immunoglobulin preparations. By using classical immunodiffusion techniques, IgG1, IgG2, and IgG4 were readily demonstrated in IGIV prepared by mild reduction and alkylation. IgG3, however, was not found although it was preliminarily noted that the allotypic markers G3m(b) and G3m(g) were detectable using the passive hemagglutination inhibition technique. This apparent discrepancy could be explained by assuming that IgG3 subclass-specific epitopes are sensitive to reduction and alkylation while the allotypic determinants were not. In the experiments described here, monoclonal antibody-defined IgG3-associated determinants were studied and the effect of reduction and alkylation was compared on both IgG3-restricted epitopes and epitopes which exhibit a broader subclass distribution.

## MATERIALS AND METHODS

### Solid phase monoclonal antibodies

Eleven monoclonal antibodies specific for human IgG3 were screened for use as solid phase reagents in the ELISA and eight were found to be appropriate. We used ascites derived from clone ZG4 (Seward Laboratories, London, U.K.), clone SJ-33 (Miles Scientific, Naperville, IL), clone H0292 (Hybritech-Boehringer-Mannheim Biochemicals, Indianapolis, IN) and five clones produced at the Center for Disease Control, Atlanta, GA. which were generously supplied by Dr. Charles B. Reimer. The latter clones, designated in this manuscript as CBI-AH7, CBI-AG12, CBI-BG12, CC4-DC10, and CC4-AH11, are designated by Reimer et al. as HP6048, HP6047, HP6049, HP6050 and HP6051, respectively (73). Other subclass restricted monoclonals used as solid phase reagents were from clones SL-13 (anti-IgG 1+3) and SL-16 (anti-IgG1), and were purchased from Miles Scientific. Using myeloma reference proteins supplied by the WHO/IUIS Immunoglobulin Subcommittee, antibody SL-13 reacted with all IgG1 and IgG3 proteins tested, but did not recognize IgG2, IgG4, IgM, IgA or light-chain isolates (H. Langbeheim, pers. comm.). Clone SL-16 has been demonstrated to produce antibody specific for the G1m(f) allotype (73). IgG-specific monoclonal antibodies used were GG-4, GG-5 and GG7 (Miles Scientific) and these were reactive with the Fc portion of the  $\gamma$ -heavy chain. The other IgG-specific antibodies, BD6-CH3 and BC5-BB6 (C. Reimer), react with the Fd fragment (73) which consists of the  $V_{H1}$  and  $C_{H1}$  domains of IgG. The optimal concentration was determined for

each monoclonal antibody for use in ELISA; SJ-33, SL-13, SL-16, GG-5, H0292 and GG-7 were used at 1:100, GG-4 was used at 1:83, BC5-BB6 was used at 1:1000 and all other antibodies were used at 1:500.

#### Antigen preparations

Native IgG3 and reduced and alkylated IgG3, used as antigen preparations in the ELISA, were prepared from two independent lots of ISG. Lot 2886 was divided into two fractions, a fraction processed at low pH (native), and a fraction (IGIV) which was partially reduced and alkylated for intravenous administration (Gamimune®, Cutter Laboratories, Berkeley, CA). IgG3 was then isolated from each of the lot 2886 fractions subsequent to processing. Alternatively, IgG3 was isolated from ISG lot 2727 before processing. A fraction of this purified material was then partially reduced and alkylated under conditions similar to those used to produce IGIV. An IgG standard solution (Tri-Partigen, Calbiochem - Behring, La Jolla, CA.) was used as a positive control.

#### Isolation of IgG3

Purified IgG3 was generously provided by Michael Dumas, Cutter Laboratories. The two step purification process employed modified methods of Weinstein et al. (55) and Hjelm (56). The ISG used as a source of IgG3 was Cohn Fraction II as prepared from pooled human plasma at Cutter Laboratories. The ISG material was equilibrated with Tris-NaCl buffer (0.05 M Tris, 0.85% NaCl,  $10^{-3}$  M  $MnCl_2$ ,  $10^{-3}$  M  $MgCl_2$ ,  $10^{-3}$  M  $CaCl_2$ , 0.01%  $NaN_3$ , pH 7.5), and 1 gram was passed through a



similarly equilibrated 3.0 x 25 cm. concanavalin A-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ). The bound material containing IgG3 was eluted with 0.2 M 1-0-methyl- $\alpha$ -D-glucopyranoside, 0.5 M NaCl, 0.01% NaN<sub>3</sub>, pH 7.5 buffer containing 10<sup>-3</sup> M MnCl<sub>2</sub> 10<sup>-3</sup> M MgCl<sub>2</sub> and 10<sup>-3</sup> M CaCl<sub>2</sub>.

The fractions containing IgG3 were pooled and concentrated by ultrafiltration on a PM10 membrane (Amicon, Danvers, MA) and equilibrated by diafiltration with 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M glycine, 0.01% NaN<sub>3</sub>, pH 7.0. The samples ( $\leq$  120 mg) were applied to a 1.0 x 6.0 cm protein A-Sepharose 4B column (Pharmacia), with an IgG binding capacity of approximately 25 mg of IgG/ml. The column had been equilibrated with the same phosphate-glycine buffer, pH 7.0. The unbound IgG3 fraction was assayed for trace IgG1, IgG2, IgG4, IgM and IgA by double diffusion in agarose gel using specific antisera (Miles Scientific).

#### Reduction and alkylation of IgG3

The purified IgG3 was partially reduced and alkylated under relatively mild conditions as previously described (42). Under these conditions, an average of 4.75 interchain disulfide bonds per molecule are dissociated while intrachain disulfides are left intact. The number of dissociated disulfide bonds was quantitated by S-carboxymethylcysteine amino acid analysis (57). Completely reduced and alkylated IgG3 was prepared from native material by reduction with 50 mM dithiothreitol (15 min. at room temperature) followed by alkylation with 110 mM iodoacetamide (1 hour at room temperature).

This material was dialyzed and stored in PBS with sodium azide.

#### ELISA

The test was a modified sandwich enzyme-linked immunosorbent assay (58). Round bottom polystyrene 96 well plates (Immulon 2, Dynatech Laboratories, Alexandria, VA) were coated with 50  $\mu$ l/well of solid phase antibody diluted in phosphate buffered saline pH 7.2 (PBS) and incubated for three hours. All incubations were performed at room temperature. The excess solid phase antibody was then removed, the plates were washed with PBS, and protein binding capacity was blocked by filling the wells with PBS-1% bovine serum albumin (BSA). After one hour, the BSA was removed and the plates were washed in PBS with 0.05% Tween-20 (PBS-T). Fifty microliters of IgG3 or IgG control diluted in PBS-T were then added to appropriate wells and incubated for one hour. All samples were tested in duplicate. After one hour, the plates were washed three times with PBS-T and incubated for one hour with 50  $\mu$ l/well alkaline phosphatase-conjugated, affinity purified goat anti-human IgG ( $\gamma$ chain, Hyclone, Logan, Utah). The enzyme conjugate was used at a dilution of 1:2000 in PBS-T. In one experiment, an alkaline phosphatase conjugate of the anti-IgG monoclonal GG-5 (Miles Scientific) was used at a dilution of 1:500. The plates were subsequently washed three times with PBS-T and substrate was added at 100  $\mu$ l/well. The substrate was a 1 mg/ml solution of p-nitrophenyl phosphate disodium (Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine-HCl buffer pH 9.8 containing 0.5 mM  $MgCl_2$ . After 30



minutes, the plates were read with an automated ELISA reader (Dynatech Instruments, Inc. Torrance, CA) at wavelength 405nm.

#### SDS-Polyacrylamide gel electrophoresis

The procedure of Weber and Osborn (59) was used with the exception that IgG samples were prepared by incubation for one hour at 60°C in gel buffer containing 0.3% sodium dodecyl sulfate. Briefly, an 0.2% SDS, 10% polyacrylamide disc gels and the electrophoresis apparatus were prepared. Ten to fifty microliters of the protein samples were then applied. Electrophoresis was performed at constant current of 8ma per gel. The gels were stained with Coomassie Brilliant blue.

## RESULTS

### Solid-phase anti-IgG3

An ELISA was developed to detect reduced and alkylated IgG3 using monoclonal antibodies as a solid-phase immunoadsorbant, purified IgG3 as antigen, and alkaline phosphatase labeled anti-human IgG as the enzyme conjugate. It was necessary to determine the optimum concentration for each solid-phase monoclonal, as some antibodies exhibited a distinct prozone. The ascites were used at dilutions of between 1:83 and 1:500 which provided low background and reproducible results. Purified IgG3 from the unbound fraction of the protein A-sepharose column was found to contain trace amounts of IgG2 and IgA but no detectable IgG1, IgG4, or IgM by immunodiffusion. In addition, SDS-PAGE of completely reduced IgG3 revealed only a  $\gamma 3$  heavy chain and none of the lower molecular weight IgG1, IgG2, or IgG4 heavy chains (fig. 3). The use of an affinity purified, gamma chain-specific enzyme conjugate contributed to low backgrounds. The sensitivity of the assay was demonstrated by absorbances well above background at 24 ng/ml protein using control IgG or purified IgG3 preparations. In experiments where absorbance readings at higher antigen concentrations reached a plateau, those concentrations which resulted in 50% of maximum absorbance were used as a unit of comparison between various preparations.

The ELISA data using solid phase monoclonals specific for human IgG3 is shown in figure 2. Four human immunoglobulin preparations were

tested against antibody derived from clone SJ-33 (fig. 2A): a control IgG standard, native IgG3, partially reduced and alkylated IgG3, and IgG3 that had been completely reduced and alkylated. Optical density readings for the IgG standard, native IgG3, and partially reduced and alkylated IgG3 samples reached a plateau at higher protein concentrations, and dropped in adsorbance as the protein was diluted. Approximately forty times more partially reduced and alkylated material than native IgG3 was required to yield 50% maximum absorbance. However, completely reduced and alkylated IgG3 was not bound by SJ-33 over the range of concentrations tested. It appears that the determinant recognized by antibody SJ-33 is intact on 2-3 percent of IgG3 molecules following mild reduction and alkylation.

Four immunoglobulin preparations were tested on a second IgG3-specific solid-phase monoclonal derived from clone ZG4 (fig 2B): native IgG3 from lot 2886, partially reduced and alkylated IgG3, completely reduced and alkylated IgG3, and native IgG3 from lot 2727. The native preparations produced similar absorbance readings, while the mildly reduced and alkylated sample again demonstrated reduced binding relative to native IgG3. As with solid phase SJ-33, completely reduced and alkylated IgG3 was not bound by antibody ZG4. The three preparations tested on IgG3-specific antibody from clone H0292 (data not shown) were: native pH 4 IgG3, partially reduced and alkylated IgG3, and completely reduced and alkylated IgG3. Again, completely reduced and alkylated IgG3 was not bound and mildly reduced and alkylated IgG3 demonstrated limited binding relative to native



material. The limited binding of the partially reduced and alkylated sample was consistent with less than 3 percent residual, monoclonal-reactive determinants.

SDS-PAGE of IgG that has been reduced and alkylated for intravenous administration revealed residual, intact IgG (H<sub>2</sub>L<sub>2</sub>) while completely reduced and alkylated IgG3 preparations contained only heavy (H) and light (L) chains (fig. 3). It is possible, therefore, that the residual intact molecule is responsible for the detectable ELISA signal using partially reduced and alkylated IgG3. Five additional IgG3-specific monoclonals were tested against high concentrations (18 µg/ml) of IgG3 in the ELISA. Again, none of the IgG3-specific reagents bound significant amounts of completely reduced and alkylated material (table I). Competitive inhibition assays have not been performed to determine if these antibodies recognize distinct determinants, but it is apparent that at least one IgG3-restricted epitope is altered by reduction and alkylation. Overall, these results are consistent with the reported inability to detect IgG3 in reduced and alkylated IGIV with classical IgG3-specific antisera (10).

#### Solid-phase anti-IgG1+3

Although solid-phase anti-IgG3 reagents did not bind fully reduced and alkylated IgG3 at high concentrations, anti-IgG1+3 and anti-IgG reagents did (table I). In an attempt to determine if normal levels of a determinant common to both IgG1 and IgG3 remain intact following reduction and alkylation, antibody derived from clone SL-13

was utilized as a solid-phase reagent. Purified IgG3 preparations were known to be free from significant IgG1 contamination because SDS-PAGE showed only  $\gamma 3$  heavy chain after complete reduction and alkylation (fig. 3) and gel diffusion results were negative using anti-IgG1 antisera. However, solid-phase anti-human IgG1 from clone SL-16 was included in each experiment as a control. Figure 4 shows results using twofold dilutions of control IgG, native IgG3 and mildly reduced and alkylated IgG3 tested against solid phase anti-IgG1+3 (fig. 4A) and the anti-IgG1 control (fig. 4B). Absorbance readings using solid phase SL-13 reached a plateau at higher protein concentrations, with native IgG3 and partially reduced and alkylated IgG3 differing approximately 4-fold in their concentrations required for 50% maximal absorbance. Native and modified IgG3 were not bound by solid phase anti-IgG1 in significant amounts relative to the IgG control.

Whereas the experiment described in figure 4 utilized IgG3 which was partially reduced and alkylated after its isolation, we also tested IgG3 isolated from an IgG preparation which had been previously reduced and alkylated for intravenous administration (fig. 5). The native IgG3 used in this experiment was purified from the same IgG preparation before reduction and alkylation and was processed at low pH. Solid phase anti-IgG1+3 bound significant amounts of both IgG3 samples and the results were similar to those obtained using material which was purified before reduction and alkylation. Again, both preparations were bound in insignificant amounts by solid-phase anti-IgG1. These results indicate that reduced and alkylated IgG1 maintains affinity for



protein A and that IgG3 can be isolated from reduced and alkylated IgG with negligible IgG1 contamination. It has been previously demonstrated that reduced and alkylated IgG1, IgG2, and IgG4 maintain affinity for protein A after reduction and alkylation (19,44).

To show that the ELISA activity of the partially reduced and alkylated IgG3 was not due solely to residual intact molecules, completely reduced and alkylated material was tested on solid phase anti-IgG1+3 and anti-IgG1 control (fig. 6). The absorbance readings using this material on anti-IgG1+3 were indistinguishable from those using native IgG3. As expected, neither preparation was bound by anti-IgG1.

#### Solid-phase anti-IgG

Other solid-phase reagents were screened to determine the extent of change in the IgG3 molecule following reduction and alkylation. Figures 7 and 8 show five IgG-specific monoclonals; three of these react with determinants on the Fc fragment of the  $\gamma$ -heavy chain (fig. 7) and two react with determinants on the Fd portion of the molecule (fig. 8). All of the IgG-associated determinants recognized by these monoclonals appear to be intact on partially or completely reduced and alkylated IgG3. Furthermore, a comparison of protein concentrations yielding 50 percent maximal absorbance (where a plateau was achieved) does not reveal a significant difference between native IgG3 and reduced and alkylated preparations.

## DISCUSSION

A recent notice, issued by the Clinical Immunology Committee of the International Union of Immunological Societies in collaboration with the World Health Organization, described the desired characteristics of human immunoglobulin and the current indications for its use (32). It was recommended that human ISG should be free from aggregates and contaminants and that it should be as unmodified as possible while retaining biological properties.

Until more data are collected concerning the subclass distribution of protective antibody, modification of ISG for intravenous use should involve procedures allowing the retention of all subclasses. Few studies have attempted to correlate antibody activity against a given pathogen with a specific IgG subclass, but Beck has reported that significant activity against rubella, polio and herpes viruses is associated with an IgG3 fraction (60).

The results presented here show that none of the solid phase IgG3-specific monoclonals studied bound completely reduced and alkylated IgG3. It appears that the IgG3-specific determinants investigated are modified by reduction and alkylation; this confirms earlier observations by Römer et al., indicating that IgG3-specific antisera did not detect IgG3 in IGIV prepared by reduction and alkylation using immunodiffusion techniques (44). In contrast partially or completely reduced and alkylated IgG3 are bound by a monoclonal anti-IgG1 and 3 at levels similar to native IgG3, and five

anti-IgG monoclonals bound native and partially or completely reduced and alkylated IgG3. These five antibodies were GG-4, GG-5, GG-7, BD6-CH3, and BC5-BB6.

The heavy chains of IgG1, IgG2, IgG3, and IgG4 share greater than 90% homology, while the hinge regions of these subclasses share approximately 40% sequence homology (10). It is probable, therefore, that several of the most immunogenic IgG3-specific determinants are located in the extended hinge region peculiar to this molecule (fig. 1), and that this region is particularly sensitive to alteration by mild reduction and alkylation. Indeed, antibodies CBI-AG12, CC4-DC10, ZG4 and SJ-33 have been shown to have hinge region specificity (73). However, the results shown here using other solid-phase reagents demonstrates that several determinants recognized by monoclonal antibodies with broader subclass specificity are intact. Therefore, alterations may be restricted to a small portion of the IgG3 molecule following reduction and alkylation.

In several of the experiments described here, the ELISA absorbance readings for reduced and alkylated fractions plateaued at lower levels than those obtained using native material. One explanation for this difference could be that several determinants recognized by the goat anti-IgG enzyme conjugate were altered by reduction and alkylation. To test this, one experiment was performed using monoclonal GG-5, both as the solid-phase reagent and as the alkaline phosphatase conjugate. From the data shown in figure 7C, it



was known that the GG-5-reactive epitope was intact following reduction and alkylation. The results of this experiment were similar to those obtained using the polyclonal conjugate; absorbance values using native IgG3 were greater than those observed using reduced and alkylated IgG3 (data not shown). Therefore, the observed differences in maximal absorbance values can be attributed to variations between the IgG3 preparations which are independent of epitope integrity. Although little or no conformational change is noted for IgG upon reduction of interchain disulfides (61), the loss of these bonds could allow for increased flexibility of the molecule and lead to steric blocking of antibody binding sites. Alternatively the native IgG3 preparations may contain more aggregates which could amplify binding of enzyme conjugate after binding to the solid phase reagents.

Functional characterizations of IGIV routinely include antibody binding to viral and bacterial pathogens (44,48), while other parameters used to assess biological function have not been extensively studied. Additional activities of the immunoglobulin molecule can be analyzed by testing complement fixing ability, by measuring binding to Fc receptors on effector cell populations and by assaying opsonophagocytic activity. The opsonophagocytic assay measures the ability of IgG to bind to specific bacteria and enhance their subsequent phagocytosis by polymorphonuclear leukocytes. Activity can be expressed as the percent survival of viable cells in the clarified supernatant or by quantitating ingested organisms by microscopy or by radioactive label. The bacteria, phagocytic cells and antibody are

incubated with and without complement because phagocytosis is not always complement dependent. Reduced and alkylated intravenous gammaglobulin has in vitro opsonic activity against S. aureus, GBS types I, II and III, P. aeruginosa and E. coli (49,50). Several studies have focused on a comparison of native IgG with reduced and alkylated IGIV from the same plasma pool in opsonophagocytic assays. IGIV and ISG were shown to have comparable opsonic ability against GBS types Ia, II and III, although activity against type Ia was variable (52,62). However, IGIV had more opsonic activity than ISG to two strains of GBS type III (62). Hetherington and Giebink, using two strains of S. aureus and three types of Streptococcus pneumoniae, reported the presence of opsonophagocytic activity in IGIV, although this activity was reduced relative to companion lots of ISG (63). Recently, in a study comparing various commercial products, reduced and alkylated IGIV had lower opsonic activity than unmodified preparations (64). These latter experiments were done without the addition of complement and necessarily compared materials derived from different plasma pools with different levels of specific antibody. In vitro opsonophagocytic assays need to be developed and standardized so that meaningful comparisons of their results can be made. In vitro assays will then be more valuable indicators of protective antibody activity.

The most useful assays to date for predicting the clinical efficacy of IGIV preparations are in vivo protection assays using animal models. In vivo protection models use either normal or compromised rodents which are treated with intravenous IgG and



challenged with lethal doses of bacteria. Comparisons are made of the number of surviving mice in test groups and control groups. It has been shown that the combination of opsonic IgG and phagocytes provides the major protective mechanism in an in vivo murine protection assay against P. aeruginosa infection (54). Several studies indicate that IGIV is protective in murine models of P. aeruginosa (53, 54) and GBS (65) infection. In vivo protection assays have been utilized in comparisons of ISG and reduced and alkylated IGIV. Native IgG and reduced and alkylated IGIV from the same plasma pool were compared in burned mice challenged with isolates representing each of the seven P. aeruginosa Fisher-Devlin-Gnabasik immunotypes, and ISG and IVIG were shown to provide similar protective activity (65). Reduced and alkylated IGIV also protects at levels comparable to native IgG processed at pH 4.25 (66). Using several strains of GBS in a neonatal rat model, IGIV offered equivalent protection when compared to the ISG from which it was processed (52,62). In summary, studies utilizing experimental animal models have shown increased survival with application of reduced and alkylated IGIV, and the efficacy of such preparations appears to be comparable to that of native ISG.

Using protein A-linked Sepharose, only IgG3 can be isolated free from substantial contamination with other subclasses, and there is no technique currently in use which allows the preparation of clean IgG1, IgG2 or IgG4 from polyclonal IgG sources. Furthermore, it may be difficult to assess the contribution of trace IgG1, IgG2 or IgG4 contaminants using in vivo functional assays to determine the efficacy

of protein-A purified IgG3. The measurement of in vivo efficacy of purified IgG restricted to a given subclass will therefore require the application of a new technology. This technology should allow the investigator to isolate 1) pure, pathogen-specific antibody, and 2) the large quantities of subclass-restricted antibody which are required for in vivo efficacy testing.

Pure IgG subclasses can be obtained in the form of monoclonal antibodies produced by EBV-transformed human B lymphoblastoid cell lines (67). The ELISA described here has been used to detect human IgG and IgG3 in cell culture supernatants from EBV-transformed human peripheral blood B lymphocytes (data not shown). Furthermore, antigen-specific antibodies against pathogenic organisms have been isolated from clonal human cell lines (68-71). The selection and establishment of cell lines producing monoclonal antibodies with opsonic/protective capabilities will provide a large, continuous supply of uncontaminated material. This will make possible the comparison, in in vivo assays, of IgG subclasses before and after treatment by reduction and alkylation.

One of the effects of reduction and alkylation on the IgG3 molecule appears to be modification of determinants recognized by IgG3-specific antibodies. A body of evidence exists indicating that hinge region modifications, such as those induced by reduction and alkylation, also effect the functions of immunoglobulin G1, even though the relevant Fc binding sites are located in the C $\gamma$ 2 and C $\gamma$ 3 domains.

It might therefore be predicted that, when compared, native, partially reduced and alkylated and completely reduced and alkylated preparations will provide different levels of functional activity. This prediction is not supported by studies which have shown comparable in vivo protection from bacterial pathogens after administration of ISG or reduced and alkylated IGIV. Further experiments using refined immunoglobulin preparations are required to resolve this apparent discrepancy.

## CONCLUSION

Reduced and alkylated IgG3 from human immunoglobulin intended for intravenous administration was detected using a sensitive immunoassay. The effects of reduction and alkylation on IgG3-restricted epitopes and IgG-associated determinants with broader subclass distribution were investigated. IgG3-specific, solid-phase monoclonal antibodies did not recognize reduced and alkylated IgG3. This confirms previous studies which failed to demonstrate IgG3 in reduced and alkylated IgG and indicates that the IgG3 subclass-restricted epitopes that were studied are modified by this treatment. Monoclonal antibodies cross-reactive with more than one IgG subclass readily reacted with the modified IgG3 preparations, indicating that the IgG-associated determinants identified by these monoclonals remain intact after reduction and alkylation.



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Table I. ELISA activity of IgG3 preparations on solid phase monoclonal antibody

Solid phase antibody <sup>2</sup>	Specificity	Antigen <sup>1</sup> (O.D. <sub>405</sub> )							
		Experiment 1				Experiment 2			
		IgG standard	Native IgG3	IGIV IgG3	cR+A IgG3	IgG standard	Native IgG3	IGIV IgG3	cR+A IgG3
None		0.000	0.003	0.003	0.002	0.001	0.004	0.008	0.004
-38- CB1-AH7	IgG3	0.362	0.472	0.362	0.024	0.429	0.513	0.389	0.021
CB1-AG12	IgG3	0.200	0.253	0.202	0.028	0.321	0.331	0.222	0.028
CB1-BG12	IgG3	0.467	0.467	0.482	0.024	0.550	0.536	0.509	0.025
CC4-DC10	IgG3	0.252	0.424	0.278	0.039	0.231	0.343	0.224	0.025
CC4-AH11	IgG3	0.204	0.351	0.227	0.028	0.254	0.364	0.235	0.028
ZG4	IgG3	0.323	0.437	0.313	0.026	0.386	0.497	0.398	0.024
SL-13	IgG1+3	0.255	0.348	0.294	0.390	0.365	0.460	0.382	0.415
GG-5	IgG	0.599	0.741	0.372	0.670	0.824	0.969	0.454	0.710

<sup>1</sup> All antigen preparations were used at 18 µg/ml. All IgG3 preparations were from ISG lot 2886. IGIV IgG3 = partially reduced and alkylated IgG3; cR+A IgG3 = completely reduced and alkylated IgG3.

<sup>2</sup> SL-13 and GG-5 were used at an ascites dilution of 1:100. All other antibodies were used at 1:500.

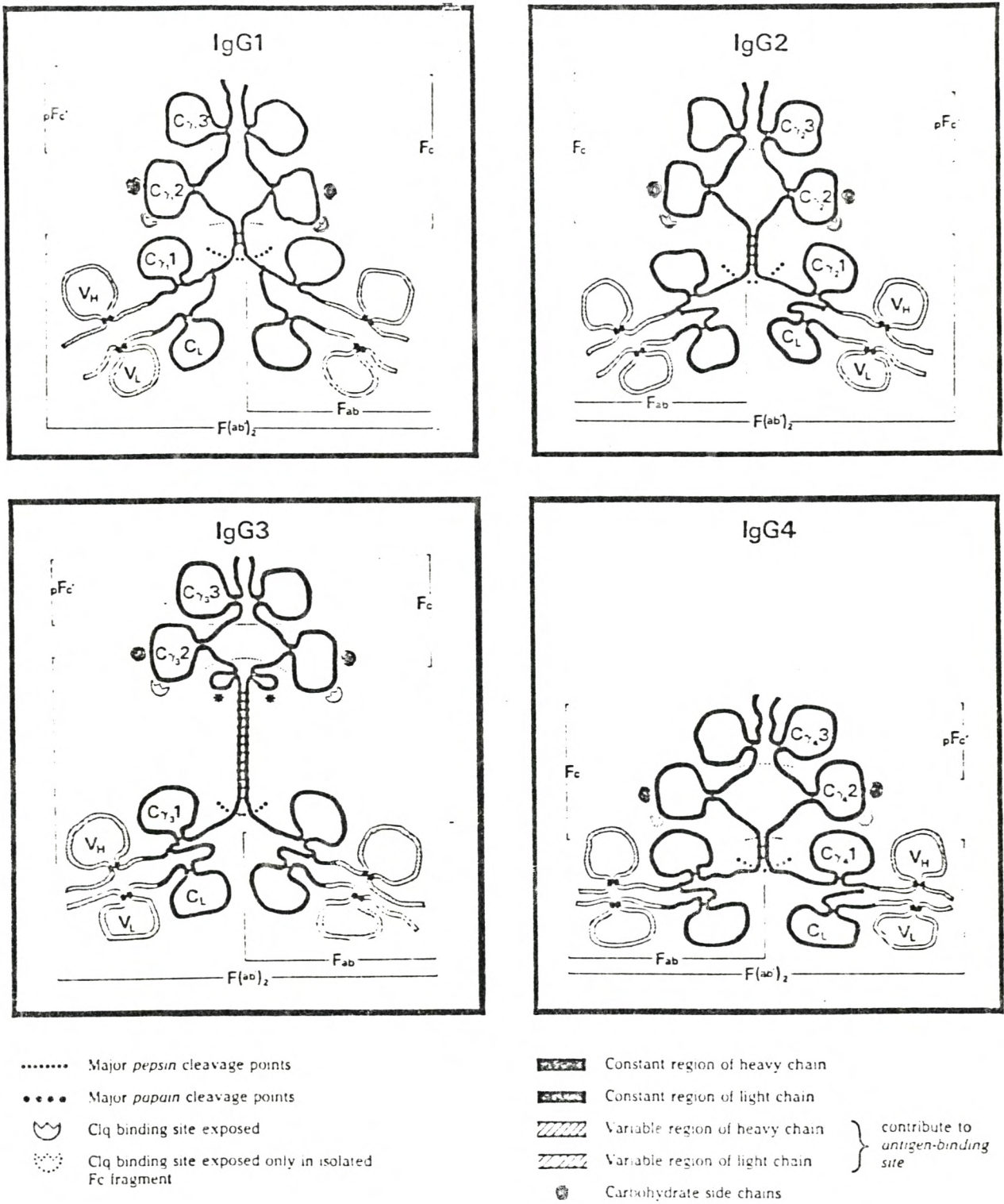
## FIGURE LEGENDS

### Figure 1

Figure 1 shows the structures of the four immunoglobulin subclasses.



Figure 1



\* The existence of this intra-chain loop is speculative

Figure 1 courtesy of Miles Laboratories, Inc.

Figure 2

O.D.<sub>405</sub> determined by ELISA using solid phase monoclonal anti-human IgG3 clone SJ-33 (A) and clone ZG4 (B). Antigen preparations were: (o) native IgG3, lot 2727; (●) completely reduced and alkylated IgG3, lot 2727; (Δ) pH 4 IgG3, lot 2886; (△) reduced and alkylated IgG3, lot 2886; (□) control IgG standard. Using solid phase clone ZG4 antibody, O.D.<sub>405</sub> was 0.31 using the control IgG standard at 18 μg/ml.

Figure 2

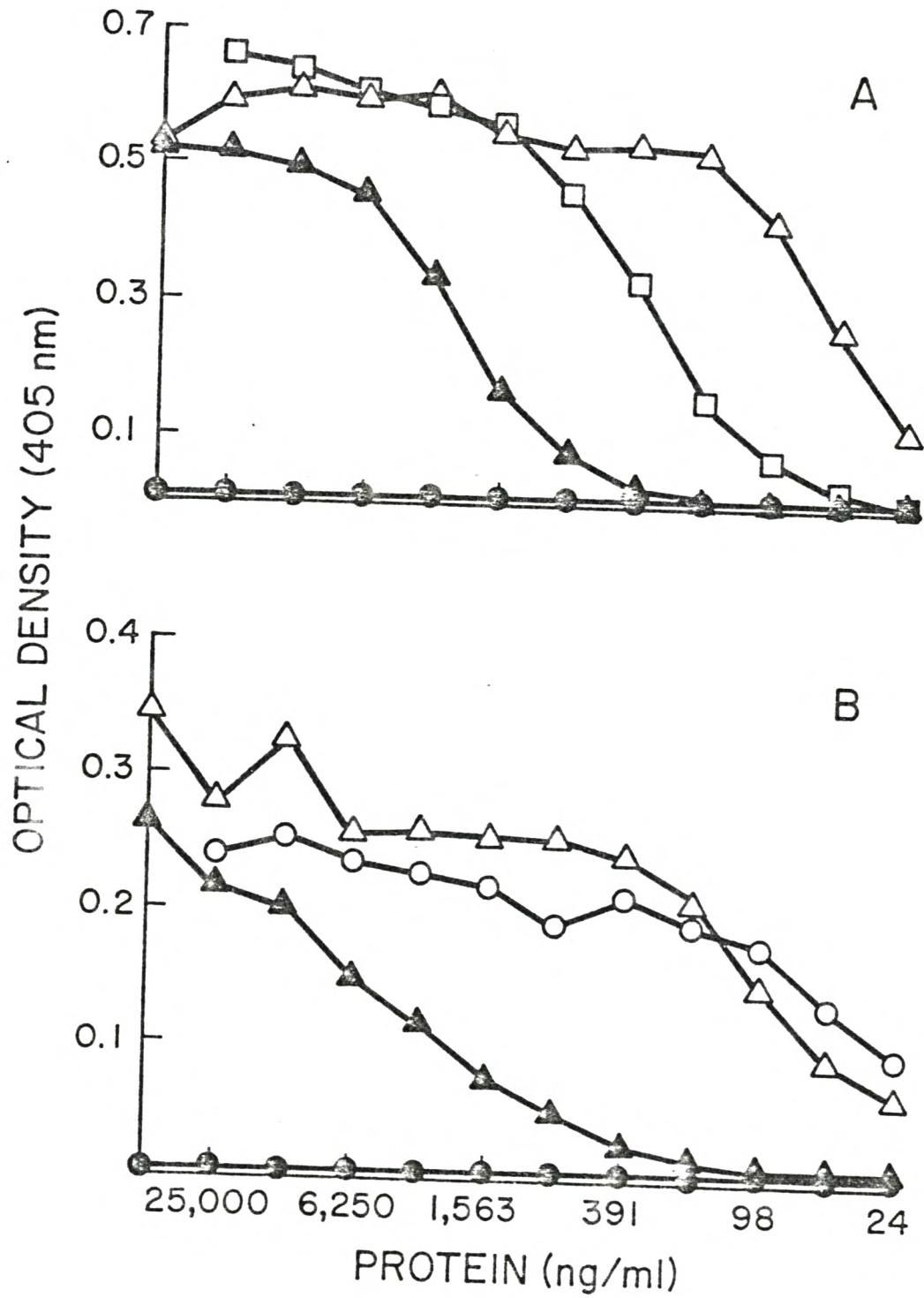




Figure 3

SDS-polyacrylamide gel electrophoresis of immunoglobulin preparations:

(A) ISG (Cohn Fraction II); (B) reduced and alkylated IGIV, lot 2886;

(C) completely reduced and alkylated IgG3, lot 2727.  $H_2L_2$  = intact

IgG, H=heavy chain, L=light chain. Bands X, Y and Z have not been

characterized but are presumed to represent  $H_2L$ ,  $H_2$  and HL complexes,

respectively (71).

Figure 3

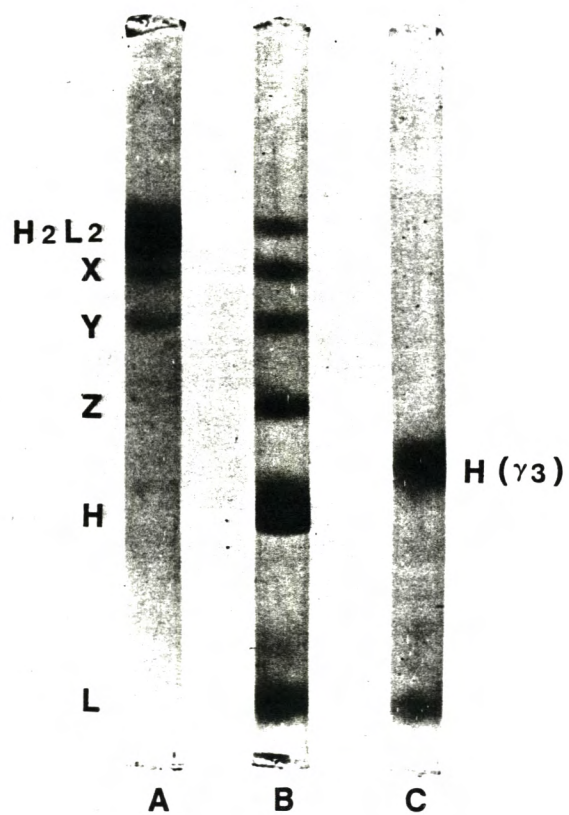


Figure 4

O.D.<sub>405</sub> determined by ELISA using solid phase monoclonal anti-human IgG1+3 (A) and anti-human IgG1 (B). Antigen preparations were: (o) native IgG3, lot 2727; (●) reduced and alkylated IgG3, lot 2727; and (□) control IgG standard.



Figure 4

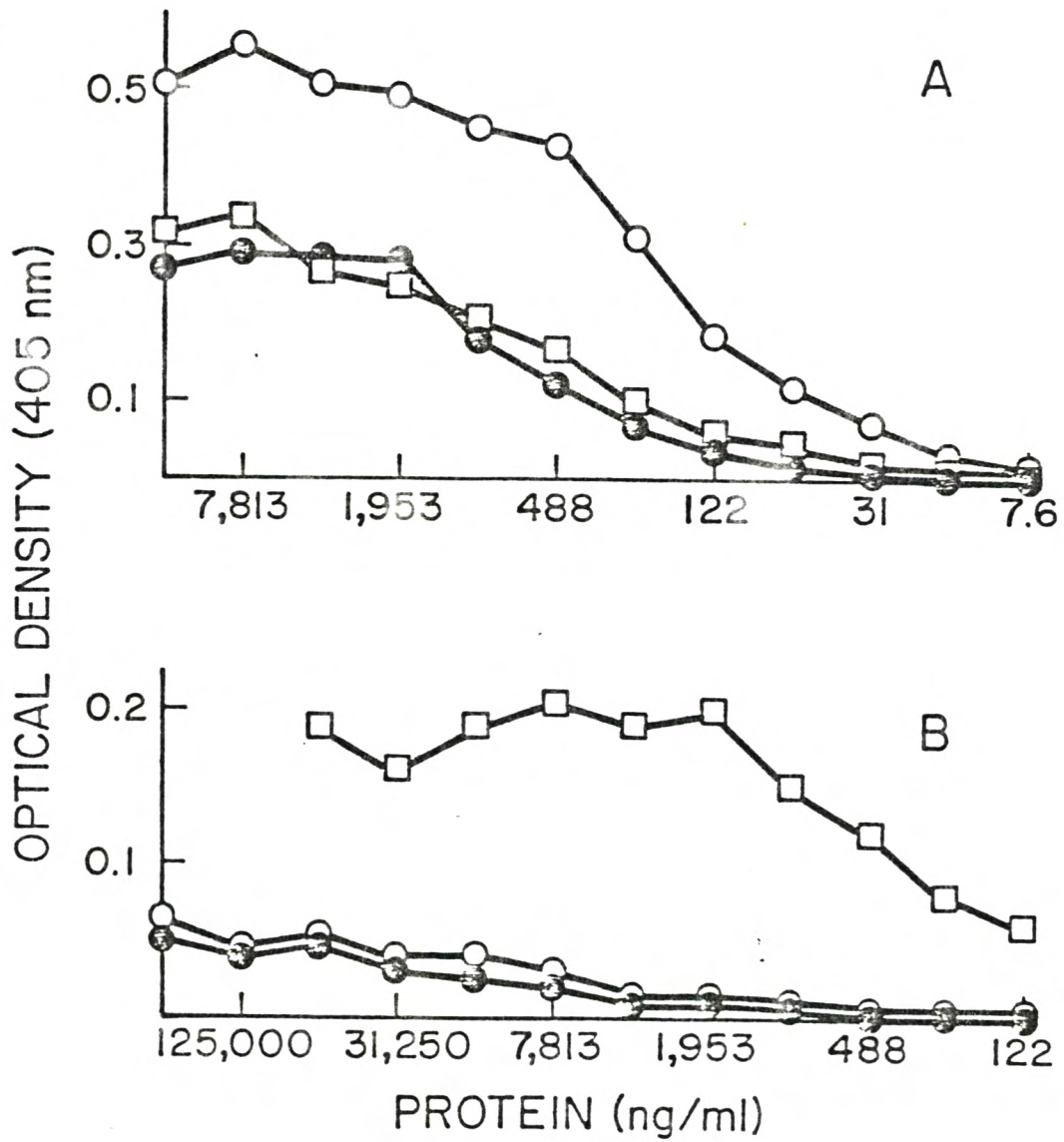


Figure 5

O.D.<sub>405</sub> determined by ELISA using solid phase monoclonal anti-human IgG1+3 (A) and anti-human IgG1 (B). Antigen preparations were:  
(o) pH 4 treated IgG3, lot 2886; (●) reduced and alkylated IgG3, lot 2886; and (□) control IgG standard.

Figure 5

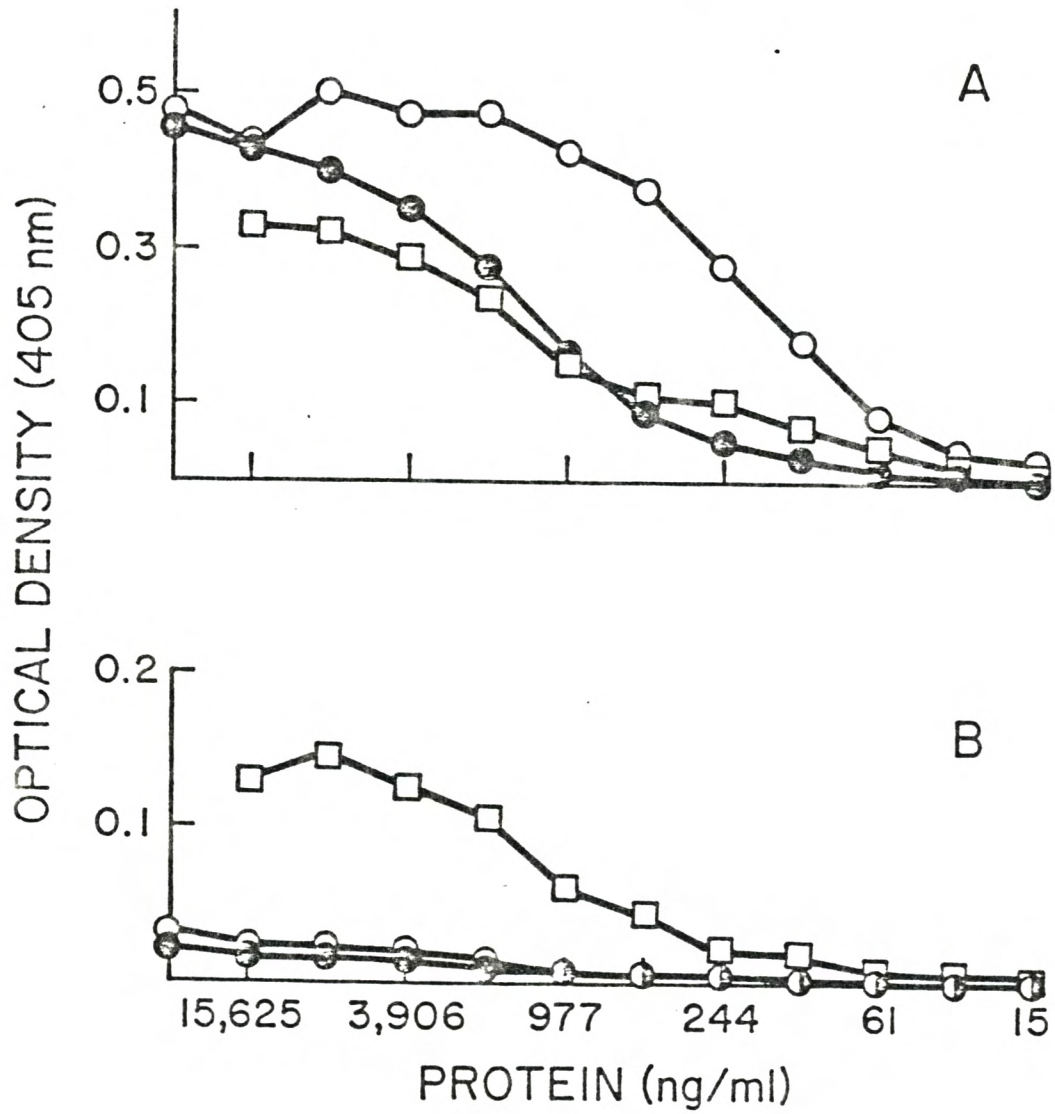




Figure 6

O.D.<sub>405</sub> determined by ELISA using solid phase monoclonal anti-human IgG1+3 (A) and anti-human IgG1 (B). Antigen preparations were: (o) native IgG3, lot 2727; (●) completely reduced and alkylated IgG3, lot 2727; and (□) control IgG standard.

Figure 6

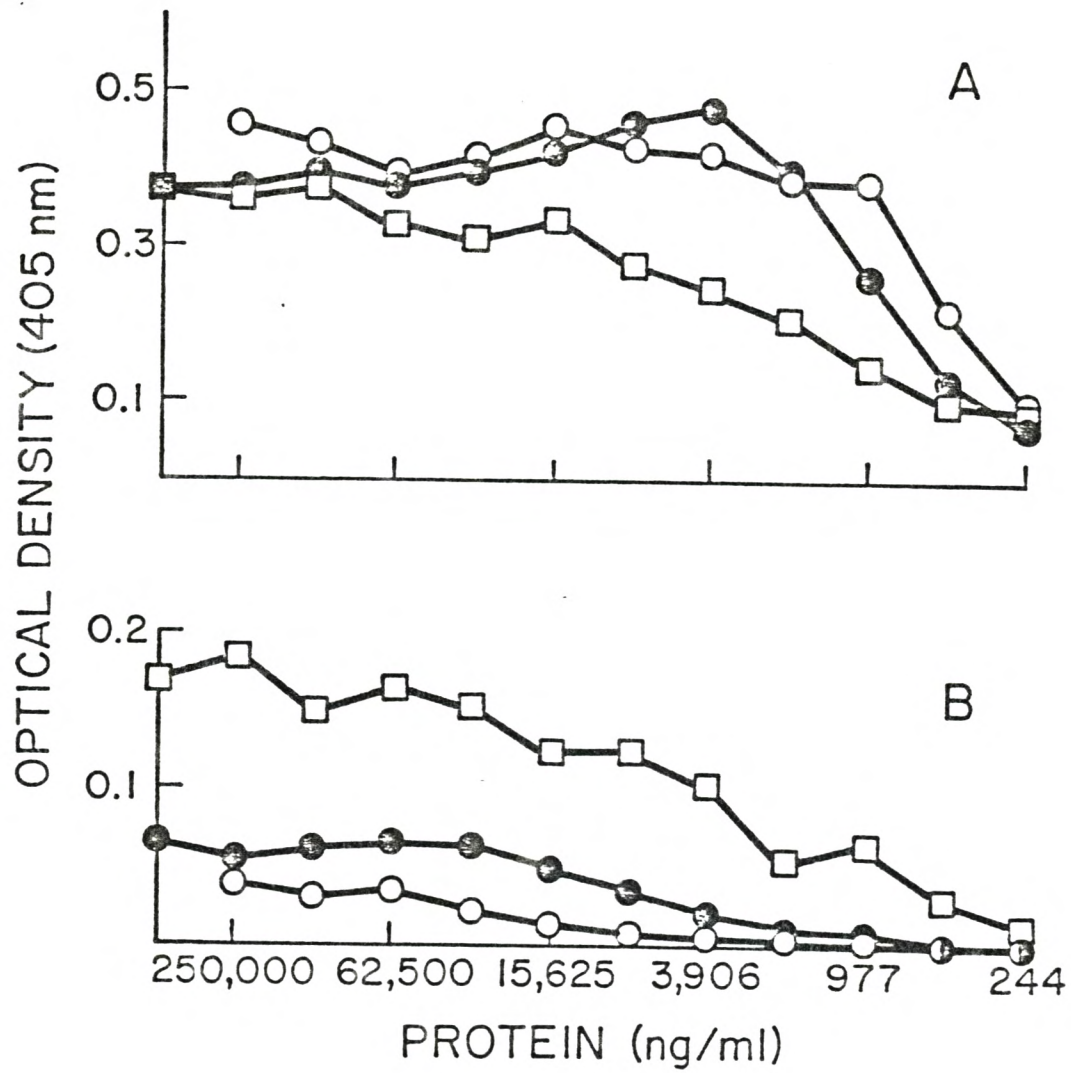


Figure 7

O.D.<sub>405</sub> determined by ELISA using as the solid phase monoclonal antibodies against human IgG: (A) clone GG-7 (Fc); (B) clone GG-4 (Fc) and (C) clone GG-5, (Fc). Antigen preparations were (o) native IgG3, lot 2727, (●) completely reduced and alkylated IgG3, lot 2727; (Δ) pH 4 treated IgG3, lot 2886; and (▲) reduced and alkylated IgG3, lot 2886. O.D.<sub>405</sub> of control IgG standard at 18 μg/ml was (A) 0.75, (B) 0.63, (C) 0.82.



Figure 7

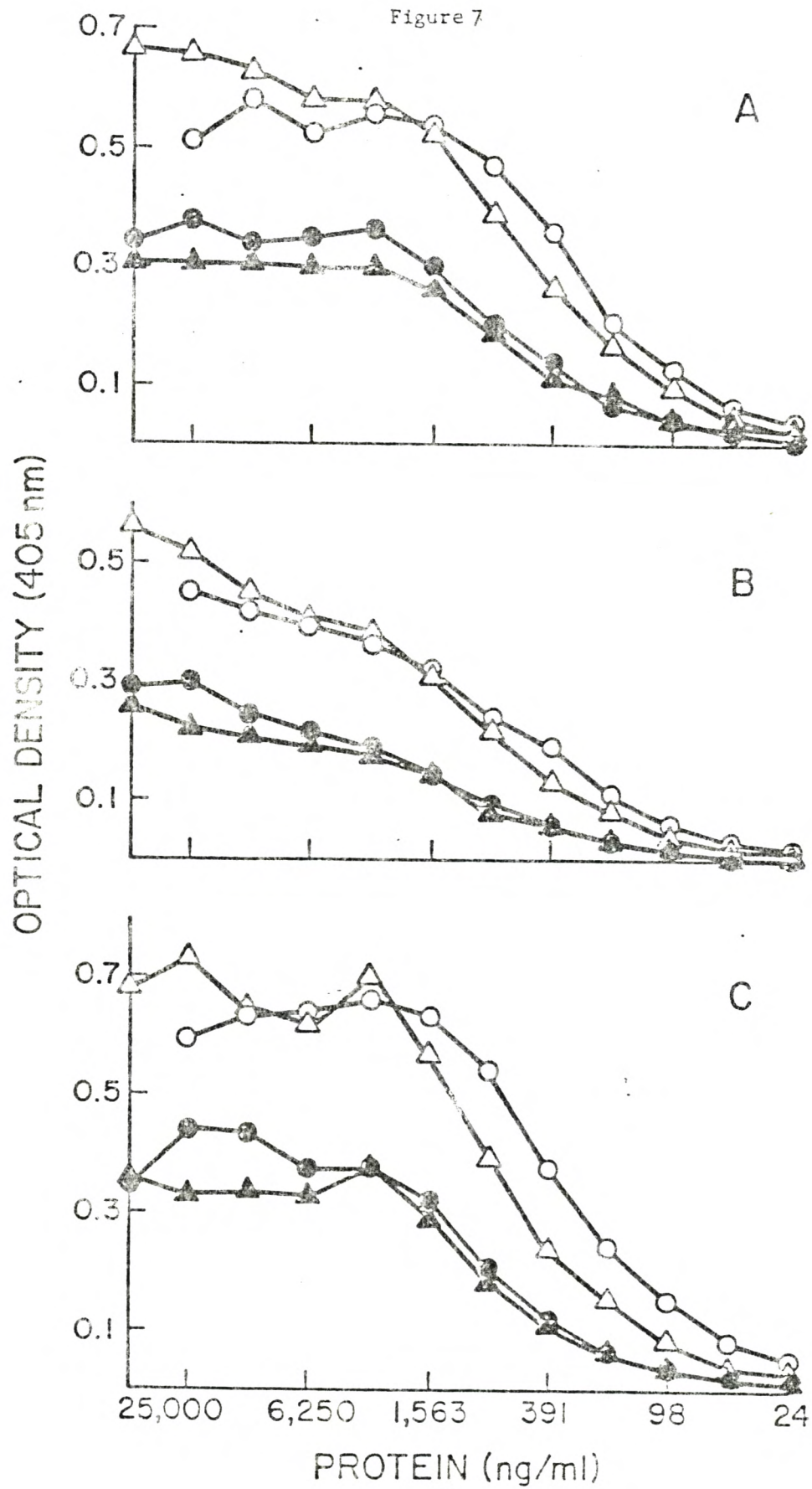


Figure 8

O.D.<sub>405</sub> determined by ELISA using solid phase monoclonal anti-human IgG (Fd): (A) clone BD6-CH3 and (B) clone BC5-BB6. Antigen preparations were: (o) native IgG3, lot 2886; (●) reduced and alkylated IgG3, lot 2886; (Δ) completely reduced and alkylated IgG3, lot 2727; and (□) control IgG standard.

Figure 8

