Immunology, 1974, **27**, 655.

The Production of Precipitating Antiglobulin Reagents Specific for the Subclasses of Human IgG

MARIJKE VAN DER GIESSEN, BETTY DE LANGE AND BEP VAN DER LEE

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands

(*Received* 12*th November* 1973; *accepted for publication* 21*st February* 1974)

Summary. Precipitating antisera against the four subclasses of human IgG were prepared by immunizing different species of animals (monkeys, rabbits, chickens, guinea-pigs and sheep) with whole IgG or Fc fragments. Freund's complete and incomplete adjuvant and $Al(OH)_3$ were used to enhance antibody formation.

In most of the experiments with rabbits, immunization against a particular subclass was accompanied by induction of unresponsiveness to the other subclasses.

Different animal species showed distinct preferences for production of certain IgG subclass antisera. Anti-IgG_3 could quite easily be produced in each of the animal species tested (monkey, rabbit and guinea-pig). Anti-IgG_2 and anti-IgG_4 could be raised in monkeys and, with greater efforts, in rabbits. Anti-IgG_1 could easily be raised in guinea-pigs and with difficulty in rabbits. In our hands monkeys did not react to IgG_1, possibly because only Gm(f+) paraproteins were used; experiments with rabbits and guinea-pigs showed that antibody formation against IgG_1 was promoted by the presence of the Gm(a) marker. The Gm(n) marker was found to influence anti-IgG_2 formation to an even greater extent: no subclass-specific antibodies were obtained when Gm(n−) IgG_2 paraproteins were used as antigen. Apart from IgG subclass-specific antibodies the following additional antibodies were often found: antibodies directed to a combination of a certain heavy chain and a kappa light chain in monkeys; antibodies specific for one or more antigenic determinants common to IgG_2 and IgG_3 in monkeys and rabbits immunized with IgG_2; antibodies specific for one or more antigenic determinants common to IgG_1 and IgG_2 in guinea-pigs and rabbits immunized with Gm(f+) IgG_1.

INTRODUCTION

For the past few years we have been involved in the preparation of precipitating antisera specific for the subclasses of human IgG (Dray, 1960; Ballieux, Bernier, Tominaga and Putnam, 1964; Grey and Kunkel, 1964; Terry and Fahey, 1964).

Animals of several species were used as other investigators used different animals with varying results (Kunkel, Yount and Litwin, 1966; Yount, Kunkel and Litwin, 1967; Lewis, Busch and Schur, 1970; Virella and Parkhouse, 1971; Morell, Skvaril, Steinberg, Loghem and Terry, 1972).

The animals were immunized with IgG or Fc fragments thereof. In some experiments,

Correspondence: M. van der Gèssen, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, Amsterdam, The Netherlands.

when immunizing against one subclass, we tried to induce unresponsiveness to the others according to the method of Spiegelberg and Weigle (1968). The results obtained will be discussed in this paper.

MATERIALS AND METHODS

Isolation of IgG paraproteins

IgG paraproteins were isolated by ammonium sulphate precipitation and ion exchange chromatography on DEAE–Sephadex equilibrated with phosphate buffer, 0·01 M, pH 7·4. After washing with this buffer further elution was subsequently performed with 0·05 M, pH 7·0 phosphate buffer to which increasing amounts of NaCl were added. In this way paraproteins are separated from other proteins and most of the remaining IgG as could be shown in immunoelectrophoresis against anti-total human serum and in double diffusion experiments against anti-subclass antisera and anti-kappa and anti-lambda.

Gm typing of the paraproteins was kindly done by Dr Erna van Loghem.

Isolation of IgG fragments

Purified IgG was hydrolysed with papain (Porter, 1959). A 1–1·5 per cent solution of IgG in phosphate-buffered saline (PBS) containing 0·002 M EDTA and 0·01 M cysteine was incubated for 4 hours at 37° with 1 per cent (weight) of papain (Merck). After the addition of a slight excess of iodoacetamide (Calbiochem) the mixture was dialysed against PBS and gel filtrated through Sephadex G-150 (Pharmacia) to separate unsplit IgG from the fragments. The fragments were separated on DEAE–cellulose (Kodak) equilibrated with 0·035 M Tris phosphate buffer, pH 8·5, with a continuous gradient to 0·5 M Tris + 0·59 M H_3PO_4, pH 4·0, according to the system used by Yagi, Maier and Pressman (1962) to separate guinea-pig IgG subclasses. Fab fragment, eluted in the first peak, was freed from a small amount of contaminating Fc fragment by DEAE–Sephadex chromatography at pH 7·5. Fc fragment was further purified on CM–Sephadex at pH 6·5. In later experiments contaminating Fab fragment was removed by absorption on anti-Fab–Sepharose immunosorbent.

Production of antisera

The following animals were used for the preparation of subclass antisera: rabbits (our own breed, Chinchilla and F_1 Alaska × Witte Wener), monkeys (cynomolgous *Macaca irus*), guinea-pigs (Renda bred), sheep (Tisselver), chickens (Shavers Star cross 288).

Immunization was carried out as follows: 0·1–1·0 mg antigen in 0·5–1·0 ml of saline was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into different sites. In some experiments antigen adsorbed onto $Al(OH)_3$ particles was used instead. After a rest period of at least 4 weeks a second injection consisting of the same amount of antigen was given with Freund's incomplete adjuvant or with $Al(OH)_3$. Rabbits received booster injections of 1 mg with adjuvant or 2–5 mg in saline every 4 weeks. To the other animals booster injections were given less frequently.

In most experiments performed with rabbits, unresponsiveness to three of the four subclasses was induced by intravenous administration of 10–15 mg of a centrifuged (1 hour at 45,000 ***g***) aqueous solution of a mixture of isolated paraproteins simultaneously with the immunizing injection as described by Spiegelberg and Weigle (1968).

In the first experiments the intravenous administration was repeated every time an

immunizing injection was given. In later courses it was only given if no precipitating activity against IgG could be detected in the last test sample.

Three weeks after the first and 1 week after each of the following injections blood was taken from each animal and checked for antibody activity and specificity in immunoelectrophoresis against normal human serum and in double diffusion experiments against 0·1 per cent solutions of selected paraproteins. Depending on the results obtained a large amount of blood was collected or further booster injections were given.

Absorption of antisera

Absorption was carried out by incubation at 37° for half an hour and at 4° overnight. After the precipitate formed was removed by centrifugation, the absorbed antisera were tested in immunoelectrophoresis and double diffusion experiments.

Antisera were rendered specific for IgG by absorption with serum fractions or isolated serum proteins if necessary. Subclass specificity was obtained by absorption with 1 per cent solutions of isolated paraproteins, including all heavy–light chain combinations and as many different Gm markers as possible. Fab fragment was also used if necessary.

For the absorption of anti-IgG_3 and anti-IgG_4 a patient's serum selected for deficiency of the subclass concerned, and containing sufficient amounts of IgG of other subclasses and serum proteins required, was often used. This procedure was particularly useful for anti-IgG_4 antisera, which often contained contaminating antibodies directed against β-globulins which were present in the isolated paraproteins used as antigen due to the high electrophoretic mobility of IgG_4 paraproteins.

A panel of approximately sixty isolated paraproteins (twenty IgG_1; twenty IgG_2; ten IgG_3; ten IgG_4) was regularly used to check the specificity. If additional specificity tests were necessary, other but less pure paraproteins were applied.

RESULTS

MONKEYS

On theoretical grounds monkeys should recognize subclass-specific antigenic determinants of human IgG more easily than other animals which are phylogenetically farther removed and therefore recognize common species-specific determinants. So we first tried to raise subclass antisera in monkeys. Three monkeys were immunized with 0·5–1·0 mg of each of the four subclasses. After two injections positive results were obtained with IgG_2, IgG_3 and IgG_4 as can be seen from Table 1. Immunization with IgG_1, however, did not result in subclass-specific antibodies, although antigen injection was repeated every 2 months for about a year.

One of the monkeys reacting positively to immunization with a Gm(n+) IgG_2 paraprotein produced, next to subclass-specific antibodies, a peculiar anti-Gm(n) antibody which is described by Giessen, Freyee, Rossouw and Loghem (1973).

Further immunization of six monkeys with other IgG_2 paraproteins and six others with a mixture of three paraproteins with different Gm markers resulted in only one specific antiserum.

It became evident from these and further experiments with IgG_2 that the monkeys reacted quite well to antigenic determinants dependent on the combination of a certain heavy and a certain light chain; surprisingly always a κ chain. To prevent the formation

of these antibodies Fc fragments were prepared for use in further immunization. In one case even the small amount of contaminating Fab fragment was enough to again give rise to an anti-($\gamma_2+\kappa$).

In two cases anti-Gm specificity was obtained, i.e. anti-Gm(a) and the above mentioned anti-Gm(n).

TABLE 1

RESULTS OBTAINED BY IMMUNIZING MONKEYS WITH DIFFERENT IgG SUBCLASSES

Subclass	Number of animals immunized	Antigen	Adjuvant*	Number producing subclass specific antibodies	Additional specific antibodies†
IgG_1	9	κ,Gm(f)	Fr.	–	$\gamma 1+\kappa$ (1)
	6	Mix	Fr.	1	$\gamma 1+\kappa$ (2) Gm(a) (1)
	3	Fc(Gmf)	Fr.	–	—
IgG_2	3	λ,Gm(n+)	Fr.	2	Gm(n) (1)
	3	κ,Gm(n−)	Fr.	–	$\gamma 2+\kappa$ (1)
	3	Mix	Fr.	1	$\gamma 2+\kappa$ (2)
	3	Fc(Gmn+)	Fr.	1	$\gamma 2+\kappa$ (1)
	2	Fc(Gmn−)	Fr.	–	—
IgG_3	3	κ,Gm(b)	Fr.	2	—
IgG_4	3	κ	Fr.	3	—

* Fr. = Freund's complete adjuvant.
† Other than subclass specificity, dependent on the antigen used.

RABBITS

Besides monkeys, rabbits were also immunized. The results are shown in Table 2. When rabbits were immunized with 1 mg of protein without inducing unresponsiveness no subclass-specific antisera, except anti-IgG_3, were obtained. This is consistent with data from the literature reporting that IgG_3 is highly immunogenic for rabbits and other animals (Lichter and Dray, 1964; Grossberg, 1967; Yount *et al.*, 1967; Virella and Parkhouse, 1971).

Against IgG_4 only weak antibodies were obtained. However, when inducing unresponsiveness to the other three subclasses by intravenous administration of an aggregate-free solution antibodies specific for IgG_4 could be raised quite easily.

Immunization with IgG_1 and IgG_2 under the same conditions gave rather poor results. Only three out of eight animals (two immunized with IgG_1 and one with IgG_2) produced subclass-specific antisera, which were weak besides. In contrast to the monkeys which formed antibodies against combined antigens of heavy and light chains the rabbits produced only anti-light chain-specific antibodies, either anti-κ or anti-λ.

More than once strong individual-specific antibodies were formed. This confirms our earlier observation that the Fab part of IgG is highly immunogenic for rabbits. Therefore, we subsequently immunized with Fc fragments obtained in a high state of purity. Out of ten rabbits injected with 1 mg of Fc fragment obtained from a Gm(f) positive IgG_1 paraprotein two produced anti-γ_1 antibodies and a third rabbit antibodies against an antigenic determinant common to γ_1 and γ_2 chains. This antigenic determinant apparently is rather immunogenic in rabbits as the serum of the two rabbits producing specific anti-γ_1 also

gave strong reactions with IgG_2 paraproteins before absorption. A further ten rabbits were immunized with an Fc fragment from a Gm(a) positive IgG_1 paraprotein; two of these produced anti-γ_1 antibodies. Cross-reactivity with IgG_2 paraproteins in this case was less pronounced.

With regard to the production of anti-γ_2 antiserum: of five rabbits immunized with Fc fragment of a Gm(n+) IgG_2 paraprotein two started to produce antibodies specific for antigenic determinants of this subclass after two and four injections of 1 mg respectively. A third one produced antibodies which did not distinguish between IgG_2 and IgG_3.

TABLE 2

RESULTS OBTAINED BY IMMUNIZING RABBITS WITH DIFFERENT IgG SUBCLASSES

Subclass	Number of animals immunized	Antigen	Adjuvant	Tolerance induced	Number producing subclass-specific antibodies	Additional specific antibodies*
IgG_1	3	Mix	Fr.	—	—	—
	4	Mix	$Al(OH)_3$	+	—	—
	4	Mix	Fr.	+	2 (weak)	—
	10	Fc(Gmf)	Fr.	+	2 (weak)	$\gamma 1+\gamma 2(1)$
	10	Fc(Gma)	Fr.	+	2	—
IgG_2	3	λ,Gm(n+)	Fr.	—	—	$\lambda(2)$
	4	Mix	$Al(OH)_3$	+	—	—
	4	Mix	Fr.	+	1 (weak)	—
	5	Fc(Gmn+)	Fr.	+	2	$\gamma 2+\gamma 3(1)$
IgG_3	6	κ,Gm(b)	Fr.	—	4	$\kappa(1)$
	3	κ,Gm(g)	Fr.	—	3	—
IgG_4	7	κ	Fr.	—	2 (weak)	κ (2)
	3	λ	Fr.	—	1 (weak)	—
	3	κ	Fr.	+	1	κ (1)
	3	λ	Fr.	+	3	—

* Other than subclass specificity, dependent on the antigen used.

OTHER ANIMALS

As the production of antibodies specific for IgG_1 was so far not very satisfactory, we immunized some animals of other species for which the IgG_1 subclass might be more immunogenic. We injected chickens, a goat, sheep and guinea-pigs with either whole IgG or Fc fragment thereof (Table 3).

The serum of some sheep which were immunized with total IgG was also checked for anti-γ_1 specificity as this was found before by other investigators (Morell, personal communication; Virella and Parkhouse, 1971). The chickens, goat and sheep (immunized with IgG) did not show any subclass specificity. The antibodies produced reacted with antigenic determinants common to all four subclasses. One of the sheep immunized with Fc fragment of a Gm(a)-positive IgG_1 paraprotein, however, started to make anti-IgG_1 after one injection with 0·1 mg of protein.

In contrast to the poor results obtained with the other animals immunized with IgG_1 the reaction of the guinea-pigs was quite striking. Seven out of ten produced quite strong anti-γ_1-specific antibodies after one injection with 0·5 mg of a Gm(a)-positive IgG_1 paraprotein. A further ten immunized with 0·1 mg reacted in the same way (eight of ten positive).

Immunization with a Gm(f)-positive IgG_1 paraprotein gave less good results. Here again some of the immunized animals recognized a common antigen of the γ_1 and γ_2 chain, as did the rabbit.

Further immunization of guinea-pigs with the other subclasses (see Table 3) confirmed that IgG_3 is highly immunogenic for all kinds of animals. Hardly any of the guinea-pigs produced antibodies specific for either IgG_2 or IgG_4.

TABLE 3

RESULTS OBTAINED BY IMMUNIZING OTHER ANIMALS WITH IgG SUBCLASSES

Animal	Subclass	Numbers of animals immunized	Antigen	Adjuvant	Number producing subclass-specific antibodies
Chicken	IgG_1	5	Mix	Fr.	—
		5	Fc(Gmf)	Fr.	—
Goat		1	Fc(Gmf)	Fr.	—
Sheep		3	IgG	Fr.	—
		2	Fc(Gma)	Fr.	1
Guinea-pig		20	κ,Gm(a)	Fr.	15
		10	κ,Gm(f)	Fr.	6 (weak)
Guinea-pig	IgG_2	15	λ,Gm(n−)	Fr.	1
		15	λ,Gm (n+)	Fr.	1
Guinea-pig	IgG_3	10	κ,Gm(b)	Fr.	7
Guinea-pig	IgG_4	20	κ	Fr.	2 (weak)

DISCUSSION

Different animal species showed distinct preference for the production of IgG subclass antisera.

In order to prepare anti-γ_3 antiserum almost any kind of laboratory animal is suitable. This subclass apparently possesses specific antigenic determinants which are highly immunogenic. This is stressed by the finding that sometimes animals immunized with whole IgG form antibodies specific for the IgG_3 subclass apart from those against common antigens of all subclasses. This was found by Terry and Fahey (1964) using monkeys and by Lichter and Dray (1964) after immunization of monkeys and a horse. We also observed this phenomenon after immunization of a horse and several rabbits.

For the preparation of anti-γ_2 antiserum, monkeys are the animals of choice (this paper; Kunkel *et al.*, 1966; Terry and Fahey, 1964; Lichter and Dray, 1964). However, some rabbits may also produce γ_2-subclass-specific antibodies if properly immunized (Kunkel *et al.*, 1966; Spiegelberg and Weigle, 1968). In our experiments this meant immunization with γ_2 Fc fragment and simultaneous induction of unresponsiveness to the other three subclasses. (From the first experiments it appeared that tolerance could be quite easily induced, as the rabbits that were immunized with $Al(OH)_3$ as adjuvant produced hardly any precipitating antibodies. A stronger adjuvant, e.g. Freund's complete adjuvant, was needed to break through the tolerance.)

In the above publications it was mentioned that in both rabbits and monkeys antibodies directed against antigenic determinants common to γ_2 and γ_3 heavy chains were often present. This is confirmed by our results. Extra absorption with IgG_3 was almost always necessary to render antisera of both monkey and rabbit origin γ_2-subclass-specific.

For the preparation of anti-γ_4 antiserum the same holds as for anti-γ_2. Monkeys react quite well to γ_4-specific antigenic determinants as do rabbits if they are made unresponsive to the other three subclasses. However, rabbits appear to react slightly better to γ_4- than to γ_2 determinants (see also Grey and Kunkel, 1964; Spiegelberg and Weigle, 1968). In our experiments the number of animals reacting positively to γ_4 was higher than that of those producing anti-γ_2 antibodies after the proper immunizations. Furthermore, it was necessary to immunize with γ_2 Fc fragment for the production of anti-γ_2 antibody, while anti-γ_4 antibody could be produced by immunization with whole IgG_4. It is concluded that the competition between common antigenic determinants on the Fab part, and γ_4-subclass-specific determinants on the Fc part of the IgG molecule was in favour of the latter.

Concerning the production of anti-γ_1 antibody: in contrast to what other investigators found (Lichter and Dray, 1964; Terry and Fahey, 1964; Skvaril and Morell, 1970) we had no success when immunizing monkeys with this subclass. A possible explanation for our failure is that we utilized Gm(f+) IgG_1 paraproteins and Fc fragments thereof, which apparently are less immunogenic, at least in rabbit and guinea-pig, than IgG_1 bearing the Gm(a) marker. No more monkeys were available to investigate this. However, the fact that the only monkey of the whole series that reacted positively when immunized with this subclass, was injected with a mixture containing a Gm(a+) and a Gm(ax+) protein besides a Gm(f+) one, makes it a likely explanation.

The first results obtained with rabbits were rather unsuccessful. After several attempts with different antigens, however, we could ascertain that immunization with γ_1 Fc fragment, preferably obtained from a Gm(a+) paraprotein, with simultaneous induction of unresponsiveness to the other subclasses, results in positive reactions in about 20 per cent of the cases.

In contrast to the low immunogenicity of IgG_1 in rabbits, in guinea-pigs this subclass appears to be highly immunogenic, especially if it bears the Gm(a) marker. The IgG_1 class has a unique position in this respect, because immunization of guinea-pigs with IgG_2 and IgG_4 was rather unsuccessful. The finding of Inchley, Grey and Uhr (1970) that human IgG_1 has greater cytophilic activity toward guinea-pig macrophages than the other immunoglobulin classes and subclasses might play a role in this respect.

In our experiments animals immunized with IgG_1 Gm(f+) proteins, both rabbits and guinea-pigs, frequently produced antibodies against (an) antigenic determinants(s) common to γ_1 and γ_2 heavy chains. This was also found by Spiegelberg and Weigle (1968) and Kunkel *et al.* (1966), although the results of the second group of investigators were obtained in rabbits immunized with IgG_2, instead of IgG_1. This antigenic determinant in our case was neither identical to 'non-b', as Gm(g+) IgG_3 globulins did not react, nor was it similar to 'non-a', as IgG_3 globulins did not react while Gm(a+) IgG_1 proteins did.

The only Gm specificities which were recognized and could be detected by precipitation techniques were anti-Gm(a) and anti-Gm(n); each specificity was detected once in a monkey. However, by haemagglutination inhibition tests, which were kindly done by Dr Erna van Loghem, the presence of antibodies against Gm markers and 'non-markers' was demonstrated in a number of animal sera, for instance anti-Gm(a), anti-Gm(f) and anti-Gm(b) in different rabbit antisera and anti-Gm(n) and anti-non-g in several of the monkeys and rabbits immunized with IgG_2. This was also found by Natvig, Kunkel, Yount and Nielsen (1968), and Natvig, Kunkel and Joslin (1969).

Concerning other animals suitable for preparation of anti-γ_1 antiserum, it appears that

sheep react quite well to the determinants of this subclass. Even when whole IgG was used γ_1-specific antibodies were sometimes obtained (Morell, personal communication; Virella and Parkhouse, 1971).

In our experience the antibodies recognizing common antigens present on all subclasses increased at the cost of subclass-specific antibodies as immunization was carried on. This was particularly the case if intravenous administration of IgG for tolerance induction was continued after the rabbit started to produce detectable precipitating antibodies. This can be expected as complexes formed by binding of intravenously administered antigen and antibodies present in the circulation would enhance production of more antibodies with specificity for that particular antigen. Therefore, the intravenous injection was omitted as soon as antibody activity could be demonstrated.

A further consequence of the increase of the general anti-IgG antibody titre with continued immunization is that if no subclass specificity can be detected after a limited number of injections it is no use to further continue the immunization. If class-specific antibodies are to be produced these will already show very early in the immunization course.

The immunization results further suggest that for immunogenicity (i.e. the capacity to elicit antibody formation) of a certain subclass the presence of a particular antigenic determinant is essential. For γ_2-specific antibody formation the presence of the Gm(n) marker appears to be a pre-requisite, while for the production of anti-γ_1 subclass antibodies the Gm(a) marker has an enhancing effect.

ACKNOWLEDGMENTS

The authors wish to thank the National Institute of Health at Bilthoven, Netherlands, for performing the immunization experiments on the monkeys. They are especially grateful to Miss M. van der Veer for her co-operation during the whole investigation.

The skilful technical assistance of Mrs B. ter Linden-Broekhof, Mrs A. A. Algra-van Veen and Mr A. van Droffelaar is gratefully acknowledged.

REFERENCES

Ballieux, R. E., Bernier, G. M., Tominaga, K. and Putnam, F. W. (1964). 'Gammaglobulin antigenic types defined by heavy chain determinants.' *Science*, **145**, 168.

Dray, S. (1960). 'Three γ-globulins in normal human serum revealed by monkey precipitins.' *Science*, **132**, 1313.

Giessen, M. van der, Freyee, W., Rossouw, E. and Loghem, E. van (1973). 'Qualitative and quantitative studies on IgG_2 globulins in individual human sera with an antiserum capable of differentiating between Gm(n+) and Gm(n−) proteins.' *Clin. exp. Immunol.*, **14**, 127.

Grey, H. M. and Kunkel, H. G. (1964). 'H-chain subgroups of myeloma proteins and normal 7S γ-globulin.' *J. exp. Med.*, **120**, 253.

Grossberg, A. L. (1967). 'Summary of the Japan-USA conference on recent developments in immunochemistry.' *Immunochemistry*, **4**, 485.

Inchley, C., Grey, H. M. and Uhr, J. W. (1970). 'The cytophilic activity of human immunoglobulins.' *J. Immunol.*, **105**, 362.

Kunkel, H. G., Yount, W. J. and Litwin, S. D. (1966). 'Genetically determined antigen of the Ne subgroup of gamma-globulin: detection by precipitin analysis.' *Science*, **154**, 1041.

Lewis, E. J., Busch, G. J. and Schur, P. H. (1970). 'Gamma G globulin subgroup composition of the glomerular deposits in human renal diseases.' *J. clin. Invest.*, **49**, 1103.

Lichter, E. A. and Dray, S. (1964). 'Immunoelectrophoretic characterization of human serum proteins with primate antisera.' *J. Immunol.*, **92**, 91.

Morell, A., Skvaril, F., Steinberg, A. G., Loghem, E. van and Terry, W. D. (1972). 'Correlations between the concentrations of the four subclasses of IgG and Gm allotypes in normal human sera.' *J. Immunol.*, **108**, 195.

Natvig, J. B., Kunkel, H. G. and Joslin, F. G. (1969). 'Delineation of two antigenic markers "non a" and "non g" related to the genetic antigens of human γ-globulin.' *J. Immunol.*, **102**, 611.

Natvig, J. B., Kunkel, H. G., Yount, W. J. and Nielsen, J. C. (1968). 'Further studies on the γG-

heavy chain gene complexes, with particular reference to the genetic markers Gm(g) and Gm(n).' *J. exp. Med.*, **128,** 763.

PORTER, R. R. (1959). 'The hydroysis of rabbit γ-globulin and antibodies with crystalline papain.' *Biochem. J.*, **73,** 119.

SKVARIL, F. and MORELL, A. (1970). 'The G 4 subclass in IgG fractions prepared by ion-exchange chromatography.' *J. Immunol.*, **104,** 1310.

SPIEGELBERG, H. L. and WEIGLE, W. O. (1968). 'The production of antisera to human γG subclasses in rabbits using immunologic unresponsiveness.' *J. Immunol.*, **101,** 377.

TERRY, W. D. and FAHEY, J. L. (1964). 'Subclasses of human γ_2-globulins based on differences in the heavy polypeptide chains.' *Science*, **146,** 400.

VIRELLA, G. and PARKHOUSE, R. M. E. (1971). 'Papain sensitivity of heavy chain subclasses in normal human IgG and localization of antigenic determinants for the subclasses.' *Immunochemistry*, **8,** 243.

YAGI, Y., MAIER, P. and PRESSMAN, D. (1962). 'Two different anti-insulin antibodies in guinea pig antisera.' *J. Immunol.*, **89,** 442.

YOUNT, W. J., KUNKEL, H. G. and LITWIN, S. D. (1967). 'Studies of the Vi (γ_2c) subgroup of γ-globulin. A relationship between concentration and genetic type among normal individuals.' *J. exp. Med.*, **125,** 177.