Useful Diagnostic Markers for Immunocompetence

Human IgG Subclasses

Answers for life.

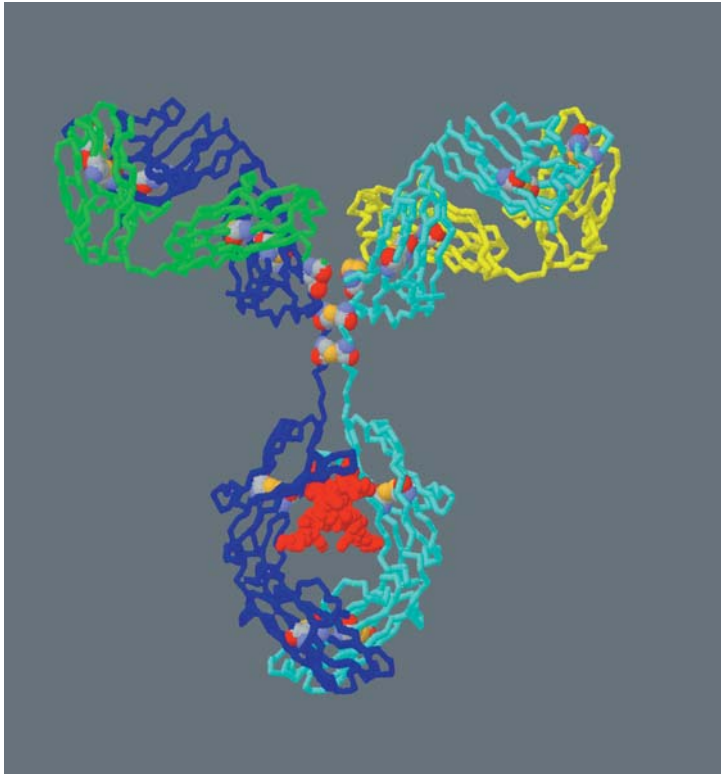**SIEMENS**

Computer-generated model of the human IgG1 glycoprotein (PDB-file IgG1-ALL.PDB, Eduardo A. Padlan).

One heavy chain is shown in blue and one in pale blue, the two light chains being depicted in green and yellow. Carbohydrate bound to the Fc portion of the molecule is shown in red. Disulfide bonds are in deep yellow.
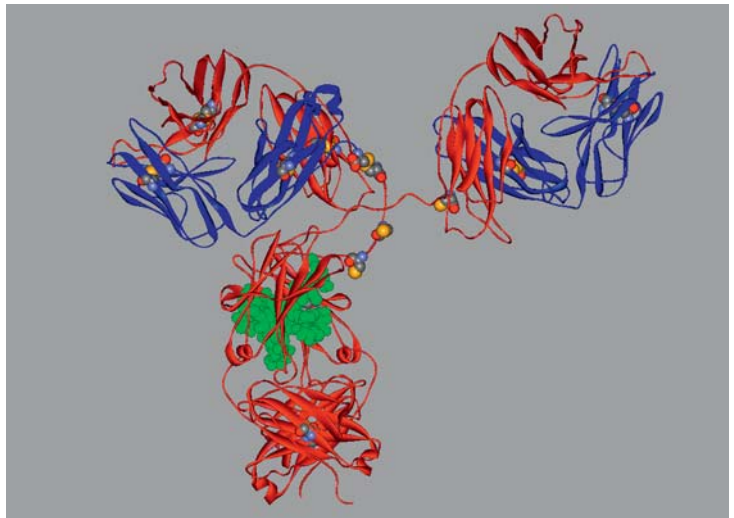
Crystal structure of
a neutralizing human IgG
molecule against HIV-1.
The two heavy chains are
shown in red, the two
light chains in blue.
(PDB-file IgG1-ALL.PDB,
Eduardo A. Padlan;
illustration by E. J.
Nieuwenhuys, Sanquin,
2003).

# Human IgG subclasses: useful diagnostic markers for immunocompetence

1 The immune system in a nutshell

The immune system consists of two functional components: the innate and the adaptive immune system (see figure 1). The *innate* immune system prevents penetration and spread of many infectious agents by means of a variety of physical, biochemical and cellular barriers (skin, mucosa, lysozymes, complement, phagocytes).

Apart from these first lines of defence, the *adaptive* immune system may be called upon to react against and clear the harmful agent. Furthermore, after the first attack, the adaptive immune system develops a specific immunological memory, leading to a stronger, faster and more effective response upon renewed contact with the same agent. The adaptive immune system consists of a variety of cells and molecules, among which lymphocytes and immunoglobulins are the key elements. There are two types of lymphocytes, T cells and B cells. T cells play a pivotal role in regulating the immune response and are also responsible for cell-mediated immunity, while B cells are essential in the effector phase of humoral immunity. After exposure to antigen and mostly with the help of T cells, B cells can differentiate into plasma cells which synthesize molecules (antibodies or immunoglobulins) that can react with the antigen[1,2].

Immunoglobulins are a group of closely related glycoproteins composed of 82 - 96% protein and 4 - 18% carbohydrate. The basic immunoglobulin molecule has a four-chain structure, comprising two identical heavy (H) chains and two identical light (L) chains, linked together by inter-chain disulfide bonds. Intra-chain disulfide bonds are responsible for the formation of loops, leading to the compact, domain-like structure of the molecule. The amino-terminal portions of the heavy and light chains, characterized by a highly variable amino-acid composition, are referred to as V_H and V_L, respectively. The constant parts of the light chain are designated as C_L, while the constant parts of the heavy chains are further divided into three distinct subunits: C_H1, C_H2 and C_H3 (figure 2). Functionally, the V regions are involved in antigen binding. The C regions interact to hold the molecule together and are involved in several biological activities, the so-called effector functions such as complement binding, passage through the placenta and binding to cell membranes.

Innate immune system: initial defense against infections

epithelial barriers   complement   phagocyte   Natural killer (NK) cell
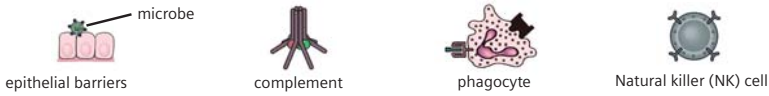
Adaptive immune system:
develops later and is mediated by lymphocytes and their products
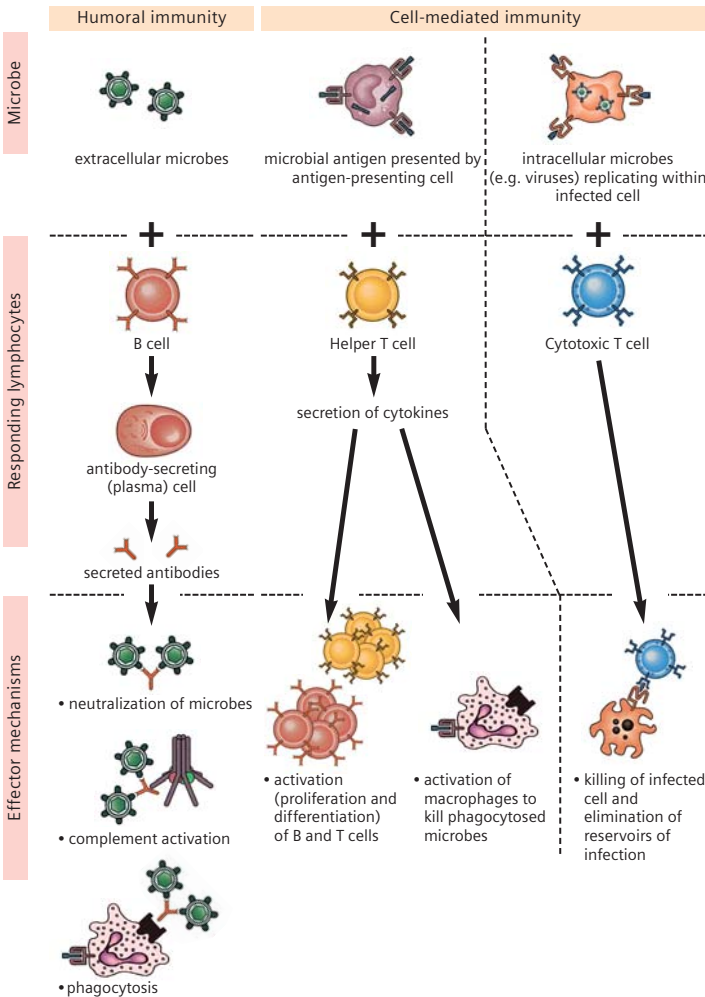
Figure 1

In humoral immunity, B cells recognize soluble or cell surface antigens (on extracellular microbes) and differentiate into antibody-secreting plasma cells. In cell-mediated immunity, helper T cells recognize antigens on the surfaces of antigen-presenting cells and secrete cytokines, which stimulate B cells and T cells; cytotoxic T cells recognize antigens on the infected cells and kill these cells.

5
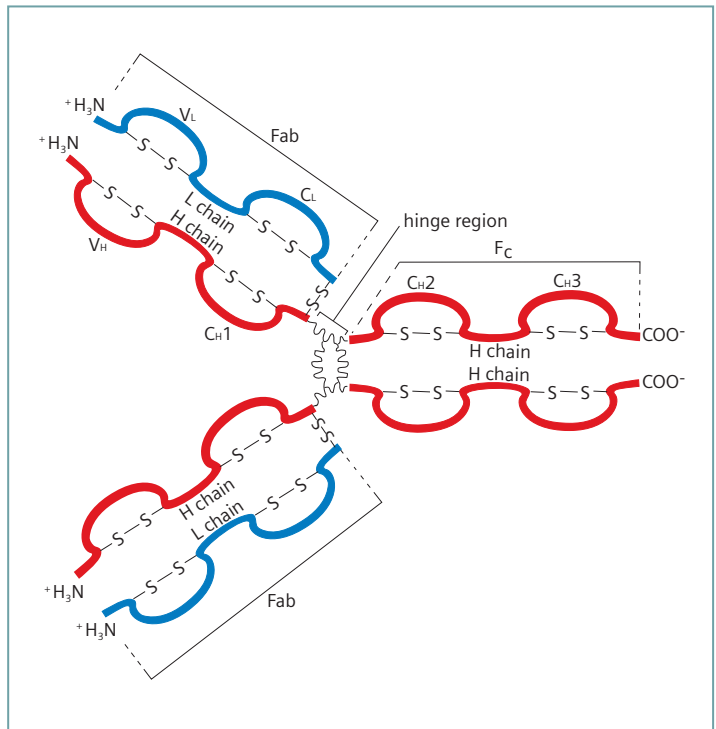
Figure 2

Schematic drawing of the basic structure of the human immunoglobulin molecule. The amino-terminal end is characterized by sequence variability (V) in both the heavy and light chains, referred to as the V_H and V_L regions respectively. The rest of the molecule has a relatively constant (C) structure. The constant portion of the light chain is termed the C_L region or domain. The constant portion of the heavy chain is further divided into three structurally discrete regions: C_H1, C_H2 and C_H3. The hinge region is a segment of the heavy chain, located between the C_H1 and C_H2 domains. Carbohydrate groups are attached to the C_H2 domains of the heavy chains (not shown). Fab: Fragment antigen binding; Fc: Fragment crystallizable.

2 IgG subclasses and humoral immunity

2.1 Immunoglobulins and humoral immunity

The glycoprotein immunoglobulin G (IgG), a major effector molecule of the humoral immune response in man, accounts for about 75% of the total of immunoglobulins in the plasma of healthy individuals. The immunoglobulins of the other four classes, IgM, IgA, IgD and IgE, each of which has characteristic properties and functions, constitute the other 25% of the immunoglobulins[3]. Antibodies of the IgG class are predominantly active during a secondary antibody response. Thus, the appearance of specific IgG antibodies generally corresponds with the 'maturation' of the antibody response, which is switched on upon repeated contact with an antigen. In comparison to antibodies of the IgM class, IgG antibodies have a relatively high affinity and persist in the circulation for a long time.

The five classes of human immunoglobulins can be distinguished on the basis of the amino acid composition. This is also the basis for antigenic differences between these molecules and for immunological recognition by specific antisera/antibodies[4].

2.2 Molecular basis of immunoglobulin synthesis

The polypeptide chains of immunoglobulins are encoded by three non-linked clusters of autosomal genes, one cluster coding for heavy chains of all classes and subclasses, a second one for kappa-light chains and a third one for lambda-light chains. These three gene clusters are called the H-, κ- and λ gene families respectively. In humans the H gene family is on chromosome 14, the κ gene family on chromosome 2 and the λ gene family on chromosome 22.

Molecular genetic studies have revealed the arrangement of gene segments within the heavy chain and light chain families. Each heavy chain is encoded by 4 distinct types of gene segments, designated V_H (variable), D (diversity), J_H (joining) and C_H (constant). The variable region of the heavy chain is encoded by the V_H, D and J_H segments.

The light chains are encoded by the 3 gene segments, V_L, J_L and C_L. The variable region of the light chains is encoded by the V_L and J_L segments.

The C gene segments of the heavy and light chains encode for the constant regions. Nine immunoglobulin heavy chain isotypes are found in humans: IgM, IgD, IgE, IgG (isotypically comprising four different subclasses IgG1, IgG2, IgG3 and IgG4) and IgA (with subclasses IgA1 and IgA2).

The C_H gene segments determine the class and/or subclass of the heavy chain, whereas the V_H, D and J_H regions determine the antigen-recognizing part of the immunoglobulin molecule. The heavy chain constant region genes lie 3' to the V_H, D and J_H genes. During the maturation of progenitor B cells to mature B cells an active heavy chain exon is formed by V_H, D, J_H integration (recombined $V_H DJ_H$ gene), followed by linkage to a certain C_H gene locus. This matrix is transcribed to mRNA and subsequently translated to an immunoglobulin heavy chain molecule. The C_H gene closest to the J_H locus, the $C\mu$ gene (IgM), is the first isotype gene to be expressed. The other C_H genes can subsequently be expressed by 'downstream' switching mechanisms with simultaneous deletion of the original isotypic C_H genes.

According to this model, the order of the C region genes on the chromosome largely determines the immuno-globulin isotype in an immune response. However, this mechanism may be influenced by isotype-specific switch factors such as IL-4 and IL-5. The relative localisation of the genes controlling the isotypes of the immunoglobulin classes and subclasses has the following 5' to 3'-oriented sequence on the DNA of chromosome 14 (figure 3):

$C\mu$ *(IgM)*-$C\delta$ *(IgD)*-$C\gamma$3 *(IgG3)*-$C\gamma$1 *(IgG1)*-pseudogene $C\epsilon$1-$C\alpha$1 *(IgA1)*-pseudogene $C\gamma$-$C\gamma$2 *(IgG2)*-$C\gamma$4 *(IgG4)*-$C\epsilon$ *(IgE)*-$C\alpha$2 (IgA2).

By comparing the four IgG subclass genes, it becomes clear that the genes for IgG3 and IgG1 are close together, as are those for IgG2 and IgG4[5]. The synthesis of each subclass is independently regulated. IgG1 and IgG3 levels generally rise more quickly than those of IgG2 and IgG4, possibly reflecting the occurrence of a successive 'downstream switch' in the immunoglobulin heavy chain constant region genes[6].

Figure 3

Schematic representation of the arrangement on chromosome 14 of the gene loci governing the constant region of the human immunoglobulin heavy chain. V_H denotes variable gene segments, D the diversity gene segments, J_H the joining gene segments.

$C\mu$ denotes IgM-C_H gene, $C\delta$ the IgD-C_H gene, $C\gamma$3 the IgG3-C_H gene, $C\gamma$1 the IgG1-C_H gene, $\psi\epsilon$1 a pseudogene (not expressed), $C\alpha$1 the IgA1-C_H gene, $\psi\gamma$ a pseudogene (not expressed), $C\gamma$2 the IgG2-C_H gene, $C\gamma$4 the IgG4-C_H gene, $C\epsilon$ the IgE-C_H gene and $C\alpha$2 the IgA2-C_H gene.

5'-|| ||---|| ||--|| ||--▢--▢--/ /--▢----▢----▢---▢--/ /-▢-/ /--▢---▢-----▢--▢-3'

V_H D J_H $C\mu$ $C\delta$ $C\gamma$3 $C\gamma$1 $\psi\epsilon$1 $C\alpha$1 $\psi\gamma$ $C\gamma$2 $C\gamma$4 $C\epsilon$ $C\alpha$2

Gene deletions resulting in a *complete* deficiency, a total lack, of IgG of one or more subclasses are very rare [7]. In fact, most abnormalities are based upon regulatory defects, resulting in a decreased level (deficiency) rather than a total lack of one or more immunoglobulin (sub)classes.

In some patients, IgG2 deficiency is associated with deficiency of IgG4, IgA1 and IgA2. Indeed, the genes encoding IgG2, IgG4 and the two IgA subclasses are closely linked and this combined deficiency is due to a regulation defect of the 'downstream switch' in the heavy chain genes. This may result in a maturation arrest in the immune response.

It is likely that the expression of the immunoglobulins whose genes are located downstream in the C_H region (especially IgG4 and IgE) requires more help from T helper 2 cells than upstream isotypes (IgG1 and IgG3) [8,9,10]. IgG4 and also IgG2 deficiencies are found in immunodeficiency states characterized by a predominant T cell defect, *e.g.*: in ataxia telangiectasia, AIDS and immune reconstitution after bone marrow transplantation (see section 4).

The observation that IgG subclass-limited responses occur suggests that the repertoire of V genes, as expressed in antibody diversity, differs between some subclasses.

2.3 Properties of human IgG subclasses

In the 1960's, extensive studies performed with specific polyclonal rabbit antisera against homogeneous human IgG myeloma proteins revealed the existence of four distinct subclasses of human IgG, which were designated IgG1, IgG2, IgG3 and IgG4, respectively [11,12,13]. Since early studies were performed with polyclonal antisera, rendered specific by absorption, the amounts of specific reagents were relatively limited [14]. In the 1980's, monoclonal antibodies against human IgG and its subclasses became available, which permitted more reproducible measurements and provided an improvement to studies dealing with IgG subclass levels in a variety of conditions [15,16,17].

Quantitatively, the relative serum concentrations of the human IgG sub-classes are as follows [18,19]: IgG1 > IgG2 > IgG3 ≈ IgG4. The four subclasses show more than 95% homology in the amino acid sequences of the constant domains of the γ-heavy chains.

The four IgG subclasses show their most conspicuous differences in the amino acid composition and structure of the 'hinge region', which is the part of the molecule which contains disulfide bonds between the γ-heavy chains (figure 2).

This region, between the Fab arms (**F**ragment **a**ntigen **b**inding) and the two carboxy-terminal domains (C_H2 and C_H3) of both heavy chains, determines the flexibility of the molecule [20,21]. In the schematic structure of IgG, shown in figure 4, it is seen that the molecule contains domain-like structures, in which the two identical antigen-binding Fab fragments and the single Fc fragment (**F**ragment **c**rystallizable) are quite mobile.

The upper hinge (towards the amino-terminal) segment allows variability of the angle between the Fab arms (Fab-Fab flexibility) as well as rotational flexibility of each individual Fab. The flexibility of the lower hinge region (towards the carboxy-terminal) directly determines the position of the Fab-arms relative to the Fc region (Fab-Fc flexibility). Hinge-dependent Fab-Fab and Fab-Fc flexibility may be important in triggering effector functions such as complement binding and Fc receptor binding.

The length and flexibility of the hinge region varies among the IgG sub-classes. The hinge region of IgG1 encompasses amino acids 216-231 and since it is freely flexible, the Fab fragments can rotate about their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide

Figure 4

Flexibility of the IgG molecule. Reproduced with permission from Immunology Today, February 1995 [22].

Fab arm waving

Fab elbow bend

Fab arm rotation

Fc wagging
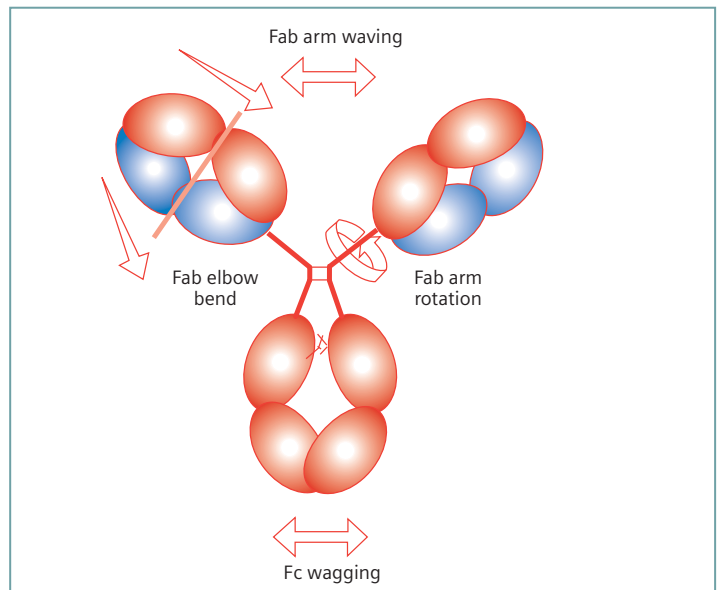
bridges [23]. IgG2 has a shorter hinge than IgG1, with 12 amino acid residues and four disulfide bridges. The hinge region of IgG2 lacks a glycine residue, it is relatively short and contains a rigid polyproline double helix, stabilized by extra inter-heavy chain disulfide bridges. These properties restrict the flexibility of the IgG2 molecule [24]. IgG3 differs from the other subclasses by its unique extended hinge region (about four times longer than the IgG1 hinge), containing 62 amino acids (including 21 prolines and 11 cysteines), forming an inflexible polyproline double helix [25,26]. In IgG3 the Fab fragments are relatively far away from the Fc fragment, giving the molecule a greater flexibility. The elongated hinge in IgG3 is also responsible for its higher molecular weight compared to the IgG of the other subclasses. The hinge region of IgG4 is shorter than that of IgG1 and its flexibility is intermediate between that of IgG1 and IgG2. The schematic structure of the four IgG subclasses is shown in figure 6.

As indicated in table I, the four IgG subclasses differ with respect to the number of inter-heavy chain disulfide bonds in the hinge region [26]. The structural differences between the IgG subclasses are also reflected in their susceptibility to proteolytic enzymes, such as papain [27], plasmin [28], trypsin [29] and pepsin [30].

IgG3 is very susceptible to cleavage by these enzymes, whereas IgG2 is relatively resistant. The sensitivity of IgG1 and IgG4 is intermediate, depending upon the enzyme used. Since these proteolytic enzymes all cleave IgG molecules near or within the hinge region, it is likely that the high sensitivity of IgG3 to enzyme digestion is related to the accessibility of its hinge.

Another structural difference between the human IgG subclasses is the linkage of the heavy and light chain by a disulfide bond. This bond links the carboxy-terminal of the light chain with the cysteine residue at position 220 (in IgG1) or at position 131 (in IgG2, IgG3 and IgG4) of the C_H1 sequence of the heavy chain. Since these positions are spatially close within the folded structure, they conserve the essential structure of the molecule.

There is a change in dogmas with respect to the composition of the IgG4 molecule. Whereas the classical dogma holds that each immunoglobulin molecule has two identical heavy chains and two identical light chains, it has been demonstrated that IgG4 may be built up of two different combinations of heavy and light chains. This implicates that one IgG4 molecule may have two different antigen specificities [31,32]. This suggested bivalency is illustrated in figure 5.

Figure 4

Cartoon illustrating the generation of bivalency by exchange of IgG4 half-molecules
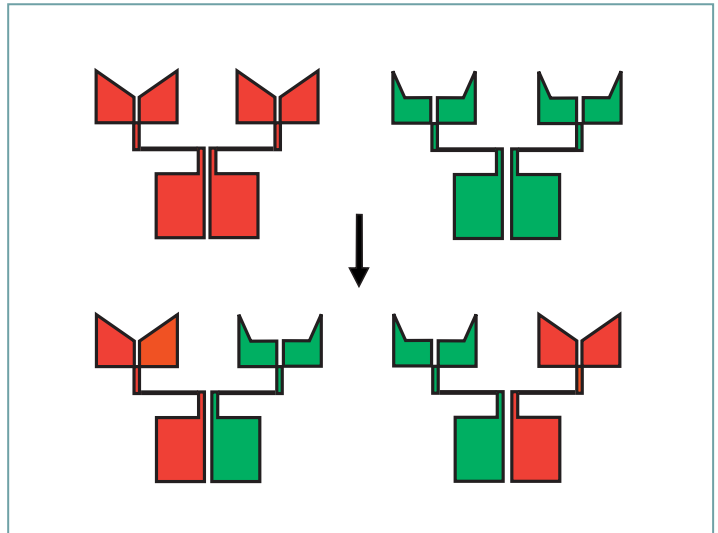
In addition to differences among genes encoding the IgG subclass proteins, each with different amino acid composition and derived properties, mutations *within* these genes have led to variations in the composition of IgG subclass proteins within the population. The latter mutations provide the basis for genetic markers (called Gm allotypes) and correspond with minor differences in the primary amino acid sequence of molecules of IgG of one subclass that occur in a species. These allotypic determinants are polymorphic epitopes, which are inherited in a Mendelian manner. Among individuals, different allelic forms are expressed. At present, immunoglobulin G can be typed for 18 different allotypes, located on the heavy chain. IgG3 is the most polymorphic, with thirteen G3m allotypes[33]. There are four IgG1 allotypes and one IgG2 allotype (the other allele being silent), whereas no allotypes have been detected on the heavy chains of IgG4.

Allotyping of immunoglobulins can be of diagnostic use in family and parenthood investigations[34,35].

As a consequence of the structural differences, the four IgG subclasses show differences in some of their physicochemical characteristics (table I) and biological properties (table II).

IgG1 **IgG2** **IgG3** **IgG4**
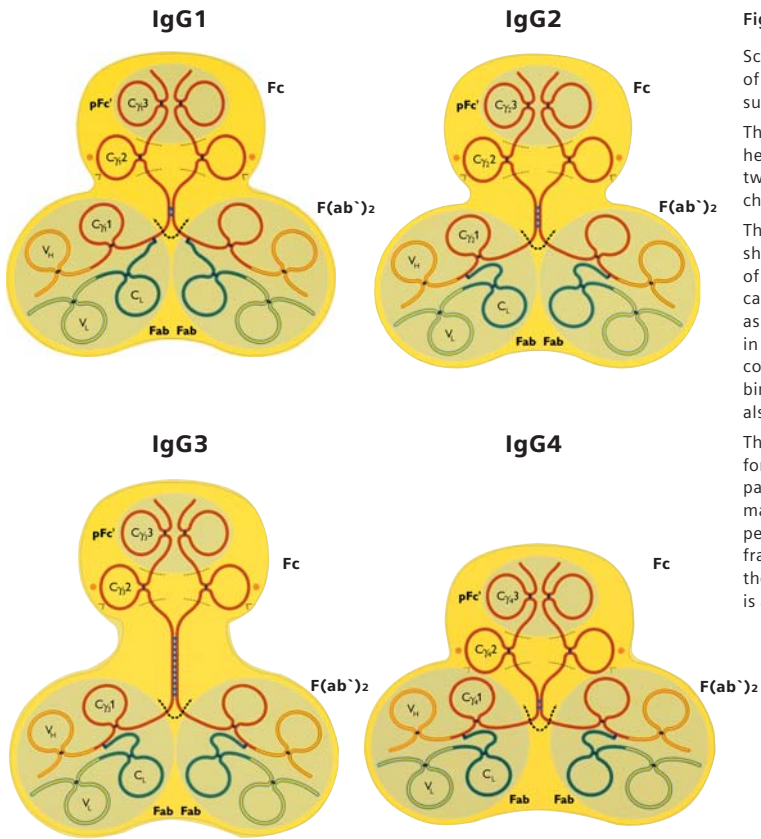
Figure 6

Schematic structure of the four human IgG subclasses.

The four domains of the heavy chains and the two domains of the light chains are shown.

The disulfide bonds are shown in blue. The site of attachment of a carbohydrate side chain (•) as well as the sites involved in the activation of complement, the C1q binding sites (↗ ↘), are also indicated.

The major cleavage points for pepsin (.......) and papain (•••••••) are marked. After cleavage with pepsin or papain, different fragments are generated, the nomenclature of which is also given.

13

Table I

Physicochemical properties
of human IgG subclasses

	IgG1	IgG2	IgG3	IgG4
Heavy chain type	$\gamma 1$	$\gamma 2$	$\gamma 3$	$\gamma 4$
Molecular mass (kD)	146	146	170	146
Amino acids in hinge region	15	12	62	12
Inter-heavy cha in disulfide bonds (in hinge region)	2	4	11	2
Susceptibility to proteolytic enzymes	++	+/-	+++	+
Number of allotypes	4	1	13	0

Table II Biological properties of human IgG subclasses

	IgG1	IgG2	IgG3	IgG4
Adult serum level range (g/l)	4.9-11.4	1.5-6.4	0.11-0.85	0.03-2.0
Proportion of total IgG (%)	43-75	16-48	1.7-7.5	0.8-11.7
Half-life (days)	21	21	7	21
Placental transfer	+	+	+	+
Antibody response to: proteins	++	+/-	++	+/-
polysaccharides	+	++	(-)	(-)
allergens	+	(-)	(-)	++
Complement activation				
C1q binding	++	+	+++	-
C1q binding, high epitope density	++	++	+++	-
enhancement alternative pathway	-	+	-	+/-
Binding to Fcγ receptors:				
FcγRI (CD64: monocytes, macrophages, neutrophils, dendritic cells)	++	-	+++	+
FcγRII (CD32: monocytes, macrophages, neutrophils, eosinophils, platelets, B cells, dendritic cells, endothelial cells)	++	(a)	+++	-
FcγRIIa-H131	++	+++	+++	-
FcγRIIa-R131	++	-	++	-
FcγRIII (CD16: neutrophils, eosinophils, macrophages, NK cells, subsets of T cells)	++	-	++	-
FcγRIIIb-NA1	+++	-	+++	-
FcγRIIIb-NA2	++	-	++	-
Binding to Staphylococcal protein A	++	++	(b)	++
Binding to Streptococcal protein G	++	++	++	++
Functional valency	2	2	2	1

(a): FcγRII allotype dependent; (b): IgG3 allotype dependent

2.4 Antibody activity of IgG subclasses

The IgG subclass distribution in specific antibody responses has been found to vary with the structure of the antigen (nature of carrier, number and nature of the epitopes, physicochemical properties), its dose and route of entry, as well as with the genetic constitution of the host. In contrast to T cell-independent (thymus-independent) antigens, T cell-dependent (thymus-dependent) antigens require interaction with helper T lymphocytes in order to stimulate B lymphocytes to antibody production.

Interestingly, stimulation of antibody responses towards certain antigens may result in a selective increase in IgG antibodies of certain subclasses [36,37,38]. Whereas antibodies against bacterial and viral *protein antigens* such as tetanus toxoid or outer-membrane components, which are T cell-dependent antigens, can be detected in all four IgG subclasses, IgG1 is the prevailing isotype, sometimes in combination with IgG3 [39]. Anti-protein antibodies of the IgG2 subclass generally provide only a marginal contribution. On the other hand, IgG antibodies against *polysaccharide antigens*, which are generally T cell-independent, generally show a much more restricted subclass distribution. Immunization with several encapsulated bacteria *(e.g.: Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae)* leads to an almost exclusive IgG2 anti-polysaccharide response [40]. An exception is seen in children under the age of 2-3 years, in whom anti-polysaccharide antibodies have been found to be of the IgG1 subclass [41]. *Repeated, long-term antigenic stimulation with* T cell-dependent antigens may lead to a marked IgG4 antibody response [42]. Some patients with haemophilia, who are regularly treated with clotting factor VIII or clotting factor IX, may develop antibodies against these administered proteins. A significant percentage of these antibodies is of the IgG4 subclass. In general, *anti-viral* IgG antibodies are highly restricted to IgG1 and IgG3, with IgG3 antibodies appearing first in the course of infection. The IgG subclass distribution in an *anti-bacterial* response will be more heterogeneous, since bacteria contain many different antigenic epitopes, with considerable variations in their protein and carbohydrate structures.

The IgG subclass determination of antigen-specific antibodies is still quite cumbersome, although many studies have been performed in this area. As a major drawback, no generally accepted international age-related reference values are

available. Moreover, different assay systems do not always provide consistent results. Antigen-specific antibodies are mostly determined by means of ELISA, the antigen being coated on a microtitre plate, followed by incubation with the antibodies to be characterized, and finally with enzyme-labelled monoclonal anti-IgG subclass-specific antibody.

2.5 Effector functions of IgG subclasses

The most important biological activities of antibodies are related to their effector functions, aimed at inactivation or removal of infectious agents and their products (*e.g.:* bacteria, viruses, toxins). Antibodies of the IgG class have two major effector functions: activation of complement and opsonisation (*i.e.:* the induction of phagocytosis). These effector functions, mediated via the (constant) Fc fragment, are induced secondary to the interaction of the antibody with its antigen via the (variable) Fab moiety (figure 7).
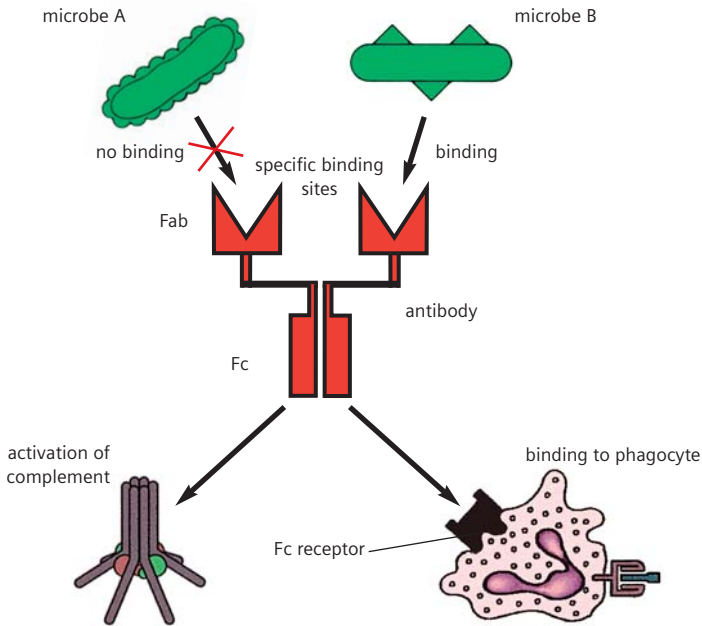
Figure 7

Antibodies act as flexible adapters, allowing various elements of the immune system to recognize specific microbes and their products. With the 'antigen-binding portion (Fab)' they bind to antigen; the Fc part interacts with other elements of the immune system, such as complement molecules or phagocytes. The antibody illustrated binds microbe B, but not microbe A. The Fc portion may activate complement or bind to Fc receptors on host cells, particularly phagocytes.

The four IgG subclasses differ from each other with respect to their effector functions (table II). This difference is related to differences in structure, notably with respect to the interaction between the variable, antigen-binding Fab fragments and the constant Fc fragment (figure 4). In particular, the length and flexibility of the hinge regions are different. The flexibility of the hinge region decreases in the order IgG3>IgG1>IgG4>IgG2. This probably relates to the higher activity of IgG3 in triggering effector functions, when compared to IgG of the other subclasses [21,43].

The influence of the IgG subclass structure on effector functions is summarized in figure 9.

2.5.1 Complement activation

Bactericidal activity is induced when the complete complement cascade (C1 to C9) is activated. The formation of the membrane attack complex (MAC) is the result of activation of complement via the classical pathway or the alternative pathway. Unlike the classical pathway, activation of the alternative pathway is not dependent upon antibodies, although many indications exist for an enhancing activity of antibodies in the alternative pathway. The MAC (C5b-9) is inserted into the membrane of target cells, after which these are killed by lysis. Furthermore, opsonisation of microorganisms by complement components (C3b, iC3b and C3dg) may result in phagocytosis. The structural diversity in the hinge region of IgG of different subclasses entails differences in their ability to activate complement. The initial event in the activation of the complement cascade by the classical pathway is the binding of C1q to sites present on the C_H2 domains of IgG. This binding occurs when the latter is aggregated, usually through formation of an antigen-antibody complex. Other components required for complement activation include a disulfide bond in the amino-terminal end of C_H2 (normally the hinge region), the presence of carbohydrates and paired C_H3 domains. The capacity of the four human IgG subclasses (in monomeric form) to bind C1q decreases in the order: IgG3>IgG1>IgG2>IgG4 [5,33]. IgG4 does not activate complement. Hinge-dependent Fab-Fab and Fab-Fc flexibility determines the accessibility of the complement binding site to C1q. IgG3, the human IgG subclass with the longest hinge, is the most effective complement activator. The inability of IgG4 to activate complement is due to the structure of the Fc fragment as well as steric hindrance of the complement binding sites by the Fab arms (figure 6).

Although IgG2 is a poor activator of the classical complement cascade, effective opsonisation and killing of certain bacterial strains and protection in bioassays by purified IgG2 have been reported. This may be explained by a combined effect of bacterial antigen and IgG2 on the activation of complement. Epitope density and accessibility of antigenic determinants on the bacteria and the density of IgG2 molecules bound in an immune complex determine the actual complement activation by IgG2. Alternatively, IgG2 may utilize the alternative pathway in complement activation in an Fc-independent way, bypassing C1q binding (table II) [44,45,46]. Complement activation via the alternative route appears to be especially important in the opsonisation and killing of bacteria.

2.5.2   Opsonisation and induction of phagocytosis

Most microorganisms are by themselves incapable to activate complement or to bind to phagocytes. Antibodies act as flexible adapters mediating the adherence of infectious agents to phagocytes. The antibody forms a bridge between the microbe and Fc receptors on the phagocyte membrane.

Interaction with Fc receptors

Phagocytosis is initiated by an interaction between the Fc fragment of the immunoglobulin and Fc receptors. Fc receptors for IgG (FcγR) are expressed primarily on effector cells of the immune system, in particular macrophages, monocytes, myeloid cells and dendritic cells.

Human IgG of the four subclasses shows differences in its interaction with FcγR's. Binding of the Fc part of IgG to an FcγR is instrumental in the induction of the cell's effector function [47,48]. In this way, FcγR's play a key role in connecting (IgG) antibody activity and cellular effector mechanisms. The latter comprise *e.g.*: phagocytosis, endocytosis, antibody-mediated cellular cytotoxicity, release of a range of inflammatory mediators, antigen presentation and clearance of immune complexes. Moreover, since several FcγR-bearing cell types (*e.g.*: macrophages, monocytes and dendritic cells) have the capacity to present antigens to T lymphocytes, FcγR-induced phagocytosis also plays a role in antigen presentation and amplification of the immune response. As a result, interaction between FcγR and IgG antibodies is pivotal in the immune response against infectious agents [49]. Since IgG subclasses differ in their affinity for FcγR, clinical manifestations of IgG subclass deficiencies will depend on IgG of which subclass is deficient.

Different types of human FcγR

It has been shown that on human leucocytes three distinct types of Fcγ-receptors occur, which are distinguishable by their structural and functional properties, as well as by antigenic structures detected by CD monoclonal antibodies. They are designated FcγRI, FcγRII and FcγRIII, respectively and are differentially expressed on (overlapping) subsets of leucocytes (table II)[50]. IgG1 and IgG3 bind to all three receptors. IgG2 and IgG4 bind to one receptor: IgG2 only binds to one variant of receptor FcγRII, while IgG4 only binds to FcγRI.

FcγRI (CD64), a high-affinity receptor expressed on monocytes, macrophages, neutrophils, myeloid precursors and dendritic cells, comprises isoforms Ia and Ib. FcγRI has a high affinity for monomeric human IgG1 and IgG3. Its affinity for IgG4 is about ten times lower, while it does not bind IgG2. There is no genetic polymorphism on FcγRI[50].

FcγRII (CD32), with its isoforms IIa, IIb1, IIb2, IIb3 and IIc, is the most widely distributed human FcγR type, being expressed on most types of blood leucocytes, as well as on Langerhans cells, dendritic cells and platelets. FcγRII is a low-affinity receptor, that only binds aggregated IgG. It is the only FcγR class able to bind IgG2.

A genetic polymorphism on FcγRIIa results in two distinct allotypes, FcγRIIa-H131 and FcγRIIa-R131, respectively[51,52]. This functional polymorphism is due to a single amino acid difference: a histidine (H) or an arginine (R) residue at position 131, which is critical for IgG binding. FcγRIIa readily binds human IgG1 and IgG3 and appears not to bind IgG4. The FcγRIIa-H131 has a much higher affinity for complexed IgG2 than the FcγRIIa-R131 allotype[52].

In Caucasian populations the heterozygous (H131/R131) form is found in about 50% of the population, the homozygous forms (H131/H131; R131/R131) each in about 25%.

FcγRIII (CD16) has two isoforms, both of which are able to bind IgG1 and IgG3. The FcγRIIIa, with an intermediate affinity for IgG, is expressed on macrophages, monocytes, natural killer (NK) cells and subsets of T cells. FcγRIIIb is a low-affinity receptor for IgG, selectively expressed on neutrophils. It is a highly mobile receptor with efficient collaboration with other membrane receptors. Studies with myeloma IgG dimers have shown that only IgG1 and IgG3 bind to FcγRIIIb (with low affinity), while no binding of IgG2 and IgG4 has been found[53,54]. The FcγRIIIb bears a co-dominant, bi-allelic polymorphism, the allotypes being designated NA1 (Neutrophil Antigen 1) and NA2[55].

FcγR genetic polymorphism, IgG subclasses and disease

In humans, IgG2 antibodies have been found to play a key role in immunity against infection with encapsulated bacteria. This defence does not only depend upon complement activation, since IgG2 has a poor complement-activating activity, at least when the epitope density is low. Indeed, it has been shown that phagocytosis by neutrophil granulocytes is the major underlying mechanism. Moreover, the FcγRIIa polymorphism is important, neutrophils from FcγRIIa-H131 homozygous individuals phagocytising better than those of FcγRIIa-R131 individuals [56]. This polymorphism will be more critical when IgG2 levels are low, as is the case in children. It has been seen that in children with meningococcal septic shock, the allotype FcγRIIa-R131 occurred twice as frequently as in normal donors [49]. Therefore children with the FcγRIIa-R131 phenotype will have many more problems in suppressing an infection with meningococci, than children homozygous for the FcγRIIa-H131 phenotype.

Figure 8 schematically illustrates the role of IgG2 in the defence against microorganisms and the different underlying routes. The first route involves the activation of complement, either by the classical pathway (C1, C4 and C2), or by the alternative pathway (which is potentiated by antibodies of the IgG2 subclass, whereas it should be kept in mind that IgG2 is a poor activator of the classical pathway). In activating the alternative pathway, bacterial components (indicated here as lipopolysaccharide, LPS) also play an essential role. Activation of the complement cascade, either by the classical or the alternative pathway, will lead to the activation of complement component C3. The binding of activated C3 to the bacterial membrane will lead either to direct killing (mediated by activation of the complement cascade C5 to C9), or to opsonisation, resulting in binding to complement receptors on polymorphonuclear cells (PMN), followed by phagocytosis. According to the second route indicated in figure 8, bacteria sensitized with IgG2 antibodies may also be phagocytosed when they bind to the FcγRIIa on the cell membrane of the PMN. This latter process will only be effective in individuals expressing an FcγRIIa of the allotype H131, since the other allele, FcγRIIa-R131, is unable to bind IgG2 effectively. With respect to the FcγRIIIb polymorphism, it has been reported that the combined FcγRIIa-R131 and FcγRIIIb-NA2 phenotype is associated with meningococcal infections [57].

Thus, in addition to a quantitative defect of IgG of various subclasses, effector functions of humoral immunity may also be negatively affected by defects in Fc receptors.
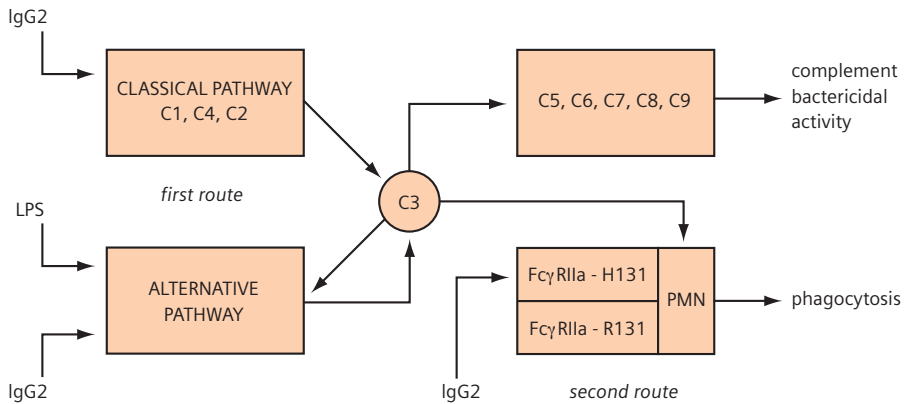
Figure 8

The role of IgG2 antibodies in two effector mechanisms resulting in complement bactericidal activity and/or phagocytosis.

LPS: lipopolysaccharide

PMN: polymorphonuclear cells

Four IgG subclasses;	
relative serum concentrations:	IgG1 > IgG2 > IgG3 ≈ IgG4

Differences in physicochemical properties
Differences in *structure* of the 'hinge region':
• length: number of amino acids
• number of inter-heavy chain disulfide bridges

Differences in the *flexibility* of the IgG molecule: IgG3 > IgG1 > IgG4 > IgG2

Differences in biological properties
'Fab-part' of the IgG molecule + antigen → triggering *effector functions* mediated via the 'Fc-part' of the IgG molecule.

• Activation of complement: IgG3 > IgG1 > IgG2 > IgG4

• Induction of phagocytosis (opsonisation)
  Binding to FcγR of effector cells:

FcγRI:	IgG3 > IgG1 >> IgG4
FcγRII:	IgG3 > IgG1 > IgG2
FcγRIII:	IgG3 ≈ IgG1

Figure 9

Summary of the influence of the structure of the IgG subclasses on their effector functions.

3 IgG subclasses in healthy children and adults

3.1 Reference values of IgG subclass levels

Children [54,58,61]

The levels of total IgG and each of its four subclasses follow a typical pattern during childhood. The IgG present at birth is mainly of maternal origin, because IgG of all four subclasses is transferred through the placenta.

In cord blood the concentration of IgG1 is 60% higher than in maternal serum, indicating an active transport from mother to child. The IgG2 serum concentration in the neonate is 10% lower than in maternal serum. The concentrations of IgG3 and IgG4 are the same in cord blood and in maternal blood.

After the first six months of life, during which IgG levels decrease, the infant's own IgG production sets in, eventually reaching adult levels. Each IgG subclass has an individual pattern of development, IgG1 and IgG3 attaining adult levels at an earlier age than IgG2 and IgG4. The serum levels of IgG1 and IgG3 reach about 50% of the adult serum levels at the age of one year and about 75% at the age of five, all with large interindividual variations. The IgG2 and IgG4 levels rise much slower. Their serum levels reach about 20% of the adult levels at the age of one year. At the age of five about 50% of the adult levels is reached, while at the age of fourteen it has increased to 70%. Again, all with large interindividual variations.

Thus, when analysing levels of IgG and its subclasses in paediatric patients, values should be compared with reference values for the same age group.

Adults [54,59,62]

There are numerous publications on IgG subclass levels in different populations. The levels of IgG subclasses do not have a normal distribution. Therefore the normal (reference) ranges of the IgG subclasses should be chosen between the 2,5 and 97,5 percentile ranges [60].

IgG subclass levels are associated with certain Gm allotypes: individuals homozygous for the G3m(b) allele have higher levels of IgG3 than homozygous G3m(g) individuals. Similarly, IgG2 subclass levels are slightly higher in individuals carrying the G2m(n) allotype compared to G2m(n)-negative individuals.

4   IgG subclasses in disease

Antibody deficiencies are the most frequently seen immunodeficiency in patients with an abnormally functioning immune system. Determination of the levels of the immunoglobulins is therefore highly important.

Deficiencies of IgG subclasses are an indication of a disturbed immune response, and usually lead to clinical symptoms in the patient, although decreased levels of IgG subclasses may also be asymptomatic. Several disease states are associated with decreased or increased levels of IgG subclasses.

The cause of decreased levels of IgG subclasses is mostly unknown. Gene deletions are extremely rare and reports on mutations of the genes are limited as well. Disturbances in the regulation of IgG subclass protein synthesis probably often underlie the decreased levels.

An antibody response may result in changes in the distribution of IgG subclasses in plasma, depending upon the nature of the antigen (*e.g.:* protein or polysaccharide) and the frequency and duration of the antigenic stimulation. This may result in increased or diminished levels of IgG of one or more subclasses.

4.1 IgG subclass immunodeficiencies, clinical relevance [63]

The most conspicuous consequence of a deficiency of one of the IgG subclasses is a defect of humoral immunity, although this does not necessarily lead to clinical manifestations. Over the last decades a large number of reports have appeared on deficiencies of IgG and its subclasses. A deficiency of total IgG will generally result in serious infectious problems. A decreased level of individual subclasses will have less dramatic consequences, although important infections may occur. Deficiencies can occur in IgG of one or more subclasses. Deficiencies of IgG subclasses can be subdivided in different groups. When the serum level of IgG of a subclass is below detection levels of the most sensitive techniques (ELISA/RIA), it is considered a *complete deficiency / absence* or a total lack. A complete deficiency of one or more subclasses, caused by deletions of genes on chromosome 14, is rare. Such a total lack of IgG of one or more subclasses, due to deletions of the immunoglobulin heavy chain constant region genes, is occasionally seen in healthy individuals. The fact that these individuals still produce protective antibody titres in the residual immunoglobulin classes or subclasses suggests that the deletion of the isotype(s) can be compensated adequately.

In *(relative)* deficiencies the level of IgG of one or more subclasses is below the normal (reference) range in healthy individuals. The association of decreased IgG subclasses with recurrent infections becomes more likely when the deficiency is an expression of immune dysregulation (*e.g.*: at the level of cytokine production).

Among the combined IgG subclass deficiencies, an IgG2 / IgG4 deficiency predominates.

Since a decreased level of IgG of one subclass may be accompanied by increased levels of IgG of one or more of the other subclasses, the total IgG level may well be normal. Consequently, the determination of IgG subclass levels is important, even when the total IgG level is within, or only slightly below, the reference range in healthy individuals.

An IgG subclass deficiency may result in a disturbed production of certain categories of antibodies. The most frequently identified selective antibody deficiency in children is an impaired response to polysaccharide antigens, such as those present in the capsule of pneumococci, meningococci and *Haemophilus influenzae* type B (HiB). Since IgG2 is the predominant antibody isotype produced in response to some polysaccharide antigens, it is not surprising that patients with decreased IgG2 levels may have an impaired response to infections with encapsulated bacteria.

The major clinical indication for measuring IgG subclasses is the occurrence of abnormally frequent and/or prolonged or severe infections that cannot be explained by the usual clinical and laboratory data. Especially in patients in whom the possibility of treatment with immunoglobulins is considered, it is advisable to determine IgG subclass levels[64]. In a proportion of these cases, decreased IgG subclass levels are found. The actual percentage will depend upon the kind of patients concerned (*e.g.*: children or adults), method of IgG subclass measurement and the normal reference values used.

The finding of a decreased level of one of the IgG subclasses can never provide a definite diagnosis, but should rather be considered an indication of a disturbance of the immune system, requiring further diagnostic investigation.

It appears that patients with IgG1 and/or IgG3 deficiency are more likely to have difficulty with chronic and recurrent infections of the lower respiratory tract. Patients with IgG2 and/or IgG4 deficiency are more likely to have recurrent infections of the upper respiratory tract (*e.g.*: sinusitis and otitis media[65]).

Deficiencies and complete deficiency / absence of individual IgG subclasses may have several consequences:

IgG1: IgG1 deficiencies often result in a decreased level of total IgG (hypogammaglobulinaemia). A deficiency of this quantitatively most important subclass is often associated with recurrent infections and might occur in combination with (individual) deficiencies of other subclasses, *e.g.*: IgG3 [38,66]. In a recent evaluation of IgG1 concentrations in adults (n=1175) with suspected IgG subclass abnormalities, decreased IgG1 levels were observed in 28% of the individuals (table III). Read et al. reported IgG1 subclass deficiencies in patients with chronic fatigue syndrome, whereas all other IgG subclasses and immunoglobulin isotypes were normal [67]. IgG1 deficiency is often associated with Common Variable Immunodeficiency.

IgG2: An IgG2 deficiency is the most often occurring IgG subclass deficiency in children (table III). An isolated IgG2 deficiency is associated with decreased responses to infections with encapsulated bacteria and after immunization with polysaccharide antigens [40, 68]. These patients show recurrent respiratory tract infections with pneumococci and/or *Haemophilus influenzae* type B [69, 70, 71]. IgG2 deficiency is associated with otitis media acuta and sinusitis. Association with ataxia telangiectasia and with Systemic Lupus Erythematosus (SLE) has also been reported. Low concentrations of IgG2 often occur in association with a deficiency of IgG4 and IgA.

IgG3: Along with IgG1, IgG3 antibodies are most frequently present in the antibody response to protein antigens. IgG3 deficiency has been associated with a history of recurrent infections, leading to chronic lung disease. Decreased IgG3 levels are frequently associated with IgG1 deficiency [65].

IgG4: An IgG4 deficiency is difficult to assess. In healthy children, IgG4 may occur in very low concentrations. Methods that are used to measure IgG4 levels have not always been sensitive enough to distinguish complete absence of IgG4 from low-normal IgG4 levels. Thus, in most studies the assessment of IgG4 deficiency is hampered by the high frequency of undetectable IgG4 levels, which is especially common in young children. Although several studies have shown that a large population of patients with recurrent respiratory tract infection have low IgG4 concentrations, the significance of this finding is not clear since a low concentration of IgG4 also occurs in a substantial percentage of healthy children [65, 72].

Sample origin	number of samples	IgG1	IgG2	IgG3	IgG4
Children* (mean age 5.8 years)	3854	4.9%	19.4%	6.3%	0.8%
Adults*	1175	28%	17%	13%	9%
Healthy individuals	162	8%	3%	1%	1%

*Individuals with suspected IgG subclass abnormalities
The samples were sent to Sanquin (The Netherlands) for diagnosis of suspected IgG subclass deficiency.

4.2 Deviations of IgG subclass serum levels in immunodeficiency syndromes

IgG subclass deficiency is a common feature in a number of primary as well as secondary immunodeficiency syndromes. Primary immunodeficiency diseases are the naturally occurring defects of the immune system. These primary defects may lead to recurrent protozoal, bacterial, fungal and viral infections of varying severity. The immune system can also be adversely affected secondarily by a variety of pathological conditions (including malignancy, metabolic diseases and malnutrition) and drugs; the results are secondary immunodeficiencies. Primary as well as secondary immunodeficiencies may result in recurrent or persistent infections.

A selective deficiency of IgG of one or more subclasses may as such present a primary immunodeficiency syndrome.

In table IV and V, a few examples are given of primary and secondary immunodeficiency diseases in which IgG subclass deficiencies may occur.

Primary immunodeficiency:	IgG subclass deficiencies:
IgA deficiency [73, 74]	frequently associated with IgG2 and IgG4 deficiencies, but IgG3 deficiencies may occur as well
Common Variable Immunodeficiency (CVID) [75, 76]	associated with decreased levels of IgG1, IgG2 and IgG4
Wiskott-Aldrich Syndrome (WAS) [77, 78]	IgG3 and IgG4 deficiencies are observed
Ataxia telangiectasia [79, 80]	IgG2 and IgG4 levels are usually very low, sometimes also associated with IgG3 deficiency
Chronic Mucocutaneous Candidiasis [81]	some patients have IgG2 and IgG4 deficiency, isolated IgG2 and IgG3 deficiencies are also observed

Secondary immunodeficiency:	IgG subclass deficiencies:
HIV-infection (stages III and IV) [82, 83, 84]	IgG2 and IgG4 levels are often decreased, while levels of IgG1 and IgG3 are increased
Radiation exposure, chemotherapy [85, 86] (Bone marrow transplantation)	often associated with low levels of IgG2 and IgG4

4.3 IgG subclasses and allergy [87, 88, 89]

Among allergen-specific IgG antibodies in allergic individuals, there is a preponderance of IgG1 and IgG4, while IgG2 and IgG3 responses are small.

Other findings in allergic patients include the following:

- Elevated IgG4 concentrations often occur in sera from patients with atopic eczema and dermatitis, probably as the result of prolonged antigenic stimulation [90].
- In allergy to many different allergens, allergen-specific IgG antibodies are predominantly of the IgG4 subclass and their levels increase during desensitization therapy. In the antibody response to desensitization/immunotherapy, initially mainly IgG1 is formed, whereas IgG4 becomes more prominent after 1-2 years.

Allergen-specific IgG4 has often been regarded as a two-headed pheno-menom: potentially harmful as well as potentially protective. However, when more had become known about IgG4

antibodies, it was hard to substantiate the harmful effects. The protective effects are still debated, but particularly from the field of parasitology the evidence is accumulating that IgG4 does, under certain conditions, effectively interfere with allergen-induced, IgE-mediated, effector cell triggering, *i.e.:* IgG4 acts as a blocking antibody. Recent data indicate a striking similarity with respect to the type of antigen that triggers the IgG4 and IgE immune responses. Since a marked difference in epitope specificity exists between the IgG4 and IgE antibodies, only a fraction of the allergen-specific IgG4 can effectively interfere with IgE binding. The use of IgG4 antibody assays to monitor immunotherapy is justifiable, but its value should not be overrated. However, if no IgG4 antibody is induced by conventional immunotherapy, the therapy is likely to have been ineffective. Immunotherapy may be considered to be immunologically effective if a substantial increase (10 to 100 fold) in allergen-specific IgG4 is induced [91].

4.4   IgG subclasses in other diseases

Decreased or increased levels of IgG subclasses in serum are associated with several other diseases, examples of which will be given here [92]:

4.4.1 Infectious diseases [6]

In most infections the first antibodies to appear will be of the IgM class, while those of the IgG class will be produced later. In general, microbial protein antigens will mainly evoke IgG1 and IgG3 antibody responses, with a minor contribution of IgG2 and IgG4 antibodies. On the other hand, polysaccharide antigens will predominantly induce IgG2 antibodies. Some disease-specific observations:

- Pneumococcal otitis media in children is associated with a decreased level of IgG2.

- Patients with recurrent infections by encapsulated bacteria often show decreased levels of IgG2 and IgG4.

- Recurrent respiratory infections with bronchiectasis are often associated with decreased levels of IgG2, IgG3 and IgG4.

- Cystic fibrosis with chronic *Pseudomonas aeruginosa* infection is associated with an increased level of IgG2 and IgG3, the latter of which seems to be of prognostic significance.

4.4.2 Autoimmunity

In autoimmune diseases the levels of IgG subclasses mostly do not differ from those in healthy individuals, but specific autoantibodies show variable subclass restrictions: frequently, autoantibodies are of the IgG1 and IgG3 subclasses [93, 94, 95].

- Human rheumatoid factors (RF) are defined as IgG, IgA or IgM antibodies against the Fc fragment of immunoglobulin. In most cases, RF have been found to bind to the Fc fragments of IgG.

  As for their specificity for a particular subclass, most RF have been shown to react with IgG1, followed by IgG2 and IgG4. Binding of RF to IgG3 is rare.

- Abnormal IgG1:IgG2 ratios have been reported in patients suffering from connective tissue diseases. In case of a selective polyclonal increase in IgG1, one should consider the possibility of Sjögren's syndrome. It has been suggested that this immunoglobulin abnormality is the product of a restricted oligoclonal B cell response and may thus be the consequence of a benign B cell lymphoma [96, 97, 98, 99].

- Autoantibodies to neutrophil cytoplasmic antigens (ANCA) are predominantly of the IgG1 and IgG4 subclass [100, 101]. Autoantibodies of the IgG3 subclass almost exclusively occur in patients with renal involvement [101].

4.4.3 Haemolytic disease of the newborn

Haemolytic disease of the newborn (HDN) is a disease in which newborn babies have anaemia caused by the breakdown of the erythrocytes. This haemolysis is triggered by maternal (IgG) antibodies, which have passed the placental barrier and bind to the fetal erythrocytes. During pregnancy, but mainly during childbirth, erythrocytes pass from the fetus to the mother. If the fetal erythrocytes express antigens which are not present on the maternal erythrocytes (e.g. Rhesus D antigen), antibodies directed against these blood groups may be produced by the mother.

Antibodies to blood group antigens show a more or less distinct IgG subclass profile. The influence of the IgG subclass of anti-Rhesus D antibodies on the severity of haemolytic disease of the newborn has been examined by many groups [102, 103, 104, 105]. In the majority of samples tested, both IgG1 and IgG3

antibodies were detected and the haemolytic disease was more severe than when only IgG1 was present. The significance of IgG3 antibodies is controversial. Some authors have suggested that IgG3 is always associated with overt haemolysis, but others could not confirm this. Blood group antibodies of the IgG4 subclass, in contrast to those of IgG1, IgG2 and IgG3 subclasses, are known not to cause clinical problems (haemolysis), which finding is mainly explained by the inability of such antibodies to activate complement [22, 106, 107, 108] and to bind to Fc receptors. The determination of IgG sub-classes is indicated especially when there is a clear discrepancy between serological findings and signs of increased red cell destruction *in vivo* [109].

4.5 Indications for measuring IgG subclass levels

As already mentioned in the previous sections, several studies indicate that (selective) IgG subclass deficiencies may be associated with disease. Specific examples are: bronchiectasis and severe, recurrent stages of otitis media, sinusitis, pneumonia and bronchitis. The possibility of an IgG subclass deficiency should be considered in all children with recurrent infections and chronic obstructive bronchitis. The association between IgG2 deficiency and severe recurrent infections of the respiratory tract in young children, caused by encapsulated bacteria, have led to an increasing demand for the determination of IgG subclasses in sera from children. However, it should be kept in mind that IgG subclass deficiencies in children may be transient. The level of IgG2 increases relatively late in childhood. Thus, when low IgG2 levels are found in children below the age of 4-5 years, it is advisable to monitor this level in the course of time, since it may be due to a temporary maturation block.

Obviously, IgG subclass deficiencies may also occur in all patients at risk for infections due to immunodeficiency, as occurs *e.g.:* in haematopoietic stem cell transplantation.

In general, IgG subclass levels should be measured whenever the total IgG level is decreased. An IgG subclass deficiency is, however, not excluded by a normal or even high total IgG concentration [64]. It is therefore essential to measure individual IgG subclass levels [110]. A correct diagnosis is essential in choosing the appropriate therapy [111].

There are several specific disease conditions in which measurement of IgG subclasses is recommended:

- Various specific infections, such as meningitis caused by pneumococci, *Haemophilus influenzae* (B) and meningococci or osteomyelitis and severe pneumonia;

- Recurrent purulent infections of the upper and lower respiratory tract (it is advisable to assess the actual causative agent(s) by means of serology and culture of the micro-organisms *in vitro*);

- Bronchiectasias and/or purulent infections of unclear etiology, such as cystic fibrosis, immotile cilia syndrome (Kartagener syndrome), or a history of earlier infections as *e.g.*: measles-pneumonia;

- IgA-deficiency associated with infectious disease (pneumonia, sinusitis, etc.);

- Diseases mentioned in chapters 4.2 and 4.4 .

4.5.1 Assessment of immune status

The determination of IgG subclass levels is included in the routine laboratory tests for the assessment of the immune status. They are used as a parameter of humoral immunity.

For diagnostic testing of paediatric and adult patients with recurrent infections in whom an immunodeficiency is suspected, the following protocol has been developed by the Dutch Working Party for Immunodeficiency [112].

A characteristic feature of this protocol for the assessment of immunological competence is a graduation in stages, in which tests with more or less increasing complexity are applied.

A. Patient's history

- Infections: localisation, frequency, duration, reaction to antibiotic therapy, nature of the infectious microorganism(s): viruses, bacteria, fungi, protozoa
- Allergy: asthma
eczema
diarrhoea
- Family: early deaths of other children occurrence of severe or unusual infections malignancies autoimmune diseases

B. Physical examination

- assessment of sites of infection (ear/nose/throat, lungs, abdomen, urogenital, skin)
- swelling or absence of lymphoid tissue (lymph nodes, adenoid, tonsils, spleen)

C. Non-specific immunity

- differential leucocyte count (basophils, eosinophils, neutrophils, lymphocytes, monocytes)
- haemolytic complement (classical and alternative pathway: CH_{50} and AP_{50})
- opsonic activity of patient's serum
- phagocytic activity of patient's granulocytes

D. Specific immunity

Humoral:
- serum levels of total IgG, IgM, IgA and IgE
- serum levels of IgG subclasses
- titres of allohaemagglutinins
- levels of IgA and IgM in saliva

Cellular:
Determination of lymphocyte subpopulations:
- T cells (CD3+)
- B cells (CD19+)
- T helper/inducer cells (CD3+, CD4+)
- T suppressor/cytotoxic cells (CD3+, CD8+)
- activated T cells (CD3+, HLA-DR+)
- NK cells (CD3-, CD16+, CD56+)

- Specific antibody responses before and after vaccination (Diphtheria/Tetanus/ Polio, Haemophilus influenzae B conjugate, Pneumovax, Meningovax type A/C). Since patients with IgG subclass deficiencies frequently suffer from infections with pneumococci and Haemophilus bacteria, it is important to measure specific antibodies against these microorganisms, before and after vaccination, using the polysaccharide- or conjugate vaccines now commercially available.

- Delayed type hypersensitivity skin tests (Tuberculin, Candida, Trichophyton, Proteus, Tetanus, Diphteria, Streptococcal antigens)

- T cell stimulation *in vitro* by mitogens and antigens.

The protocol is further specified and a multistage laboratory protocol for the diagnosis of immunodeficiency [113, 114, 115] is now available. By this protocol immunodeficient patients are identified in an efficient way. A low threshold for the performance of simple screening tests in the first stages allows early exclusion or identification of a potential immunodeficiency, whereas more elaborate tests leading to a diagnosis and a definitive classification are reserved for those patients in whom an immunodeficiency is more probable. The definitive classification is important for treatment strategies as well as for the identification of carriers and genetic counselling of the family.

Based upon the clinical presentation of the patient (symptoms and signs, as well as isolated microorganisms) one of the three schemes of the protocol will be followed:

scheme 1   for recurrent pulmonary and ENT
               (ear, nose and throat) infections,
scheme 2   for opportunistic infections,
               wasting or failure to thrive, or
scheme 3   for recurrent infections of surface areas
               or deeper organs.

In scheme 1 determination of IgG subclass levels is an important parameter. In this booklet only scheme 1 will be discussed

(scheme 1 is reproduced with permission from *Nederlands Tijdschrift voor Geneeskunde*, november, 2000 [114]).

Scheme 1 (see page 36) of the multistage laboratory protocol for the diagnosis of immunodeficiency offers a stepwise assessment of immunological competence in patients with recurrent respiratory infections. After elimination of common non-immunological underlying diseases in phase 1, severe antibody deficiency is ruled out in step 1 of phase 2. It is important to detect this rare entity early, before irreparable damage to the lungs has occurred.

More elaborate tests to detect milder forms of immuno-deficiency – like IgG subclass deficiencies – are reserved for those patients in whom symptoms persist. Some patients in whom results of initial laboratory screening are normal, develop the progressive hypogammaglobulinemia of common variable immunodeficiency in the course of time. Repeated testing can be necessary to detect this.

Scheme 1: Recurrent ENT and airway infections

Microorganisms: 1. Encapsulated extracellular bacteria, mainly *Haemophilus influenzae* type B, *Streptococcus pneumoniae, Neisseria meningitidis*

2. Sometimes enteroviruses (Echovirus, poliovirus), or *Giardia lamblia*

Phase 1

• Asthma/COPD* (do not forget Cystic Fibrosis)	→	treat
• Local ENT (ear, nose and throat) problems	→	treat
• Iron deficiency anemia	→	treat

Problem solved ? → **STOP**
If not, go to phase 2

COPD: Chronic obstructive pulmonary disease

Phase 2

Step 1	• blood count and differential
	• total IgG, IgA and IgM
	• total and specific IgE
Step 2	• IgG-subclasses
	• consider zone-electrophoresis for identification of homogeneous (monoclonal) immunoglobulin-components
	• CH_{50} and AP_{50}**
	• consider opsonic activity in serum against bacteria

Phase 3

Normal results in phase 2	*IgA/IgG-subclass deficiency*	*Agammaglobulinemia and severe hypogammaglobulinemia*	*Abnormal CH_{50}/AP_{50} ** *
wait and see for 3-6 months Problem **not** solved? Follow IgA/ IgG-subclass deficiency Worse clinical condition: perform all tests in phase 3	• specific antibody production 1 Vaccination status (Diphteria, Tetanus /*Haemophilus influenzae* type B / MMR: mumps, measles and rubella) 2 Booster response to pneumococcal **&** diphteria/tetanus vaccination	• lymphocyte subpopulations • booster responses if hypogammaglobulinemia is found • genetic determination of defect • exclude hyper-IgM syndrome	• determination of separate complement components • ANA (anti-nuclear antibodies)

Phase 4

Normal booster responses	*Abnormal booster responses*
If problems persist repeat after 2 years: • IgG, IgA and IgM, and IgG-subclasses • consider lymphocyte subpopulations (limited protocol) If problems persist, then repeat after 5 years: • Pneumococcal and diphteria/tetanus booster responses (seldom present: ciliary dyskinesia)	• lymphocyte subpopulations and lymphocyte proliferation tests • exclude hyper-IgM syndrome Diagnosis (after excluding others): CVID (common variable immunodeficiency)

**CH_{50} and AP_{50}:*
  measure for the activity of the classical pathway and the alternative pathway of complement activation, respectively.

IgG subclass deficiencies are frequently found. However, many individuals with IgG subclass deficiency do not have any symptoms. On the other hand, IgG subclass deficiencies may be associated with disease. Immunoglobulins opsonize microorganisms, which renders them more susceptible to clearance by phagocytosis. This process of opsonisation is especially important for the clearance of extracellular encapsulated bacteria which cause respiratory tract infections, such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*. Therefore, the determination of IgG subclass levels is mainly important in the work-up of patients with recurrent respiratory tract infections or unexplained bronchiectasis. Most patients with recurrent respiratory tract infections suffer from common underlying diseases such as asthma, allergy or COPD; clinically important antibody deficiencies are only occasionally encountered.

The quantitatively most important IgG1 subclass (roughly 60-70% of total serum IgG) contains important anti-protein antibodies. IgG1 deficiency generally results in hypogammaglobulinemia with increased susceptibility to bacterial infections. IgG2 (roughly 20-30% of total serum IgG) contains important antipolysaccharide antibodies. IgG2 deficiency itself is often asymptomatic, but it is associated with IgA and/or antipolysaccharide antibody deficiency in about 40% of cases. These combinations of deficiencies often lead to an increased frequency of respiratory infections with encapsulated bacteria. Therefore, the presence of IgG2 deficiency is first of all an indication for the determination of specific antibody responses. IgG3 (rougly 5-10% of total serum IgG) contains anti-protein antibodies. IgG3 deficiency generally leads to an increased susceptibility to infections, but it is uncommon. IgG4 deficiency is often seen in healthy individuals, and the solitary finding of decreased serum IgG4 (normally 1-5% of total serum IgG) is of no importance.

4.5.2 Therapeutic considerations

For individuals with IgG subclass deficiencies who also develop clinical symptoms (*e.g.*: recurrent infections), treatment should be considered. Treatment of such patients will generally consist of anti-microbial therapy, immunoglobulin substitution and vaccinations.

Conservative treatment, comprising early antibiotic treatment during infections, prophylactic antibiotic treatment in selected individuals and supportive symptomatic therapy should be the first line of treatment, sometimes supplemented with intravenous immunoglobulin. In patients with primary specific immunodeficiency who have significantly diminished serum IgG levels and/or demonstrate defects in antibody production, intravenous immunoglobulin substitution therapy is currently most often used, since administration of immunoglobulins will reduce the incidence of bacterial and viral infections.

Patients with selective IgG subclass deficiency may benefit from IgG re-placement (WHO-report)[116]. Especially in children with an IgG2 deficiency or an inappropriate response to pneumococcal antigens and a history of recurrent bacterial respiratory infections, substitution treatment with intraveneous immunoglobulin should be considered[117].

The maturation of an immune response can be enhanced by repeated vaccination. Vaccination with Diphtheria/Tetanus/Polio proteins (T cell-dependent antigens) from the age of three months is generally performed. Active immunization against polysaccharide antigens (T cell-independent antigens) is ineffective until about the age of 18 months, because of the slow ontogeny of the anti-polysaccharide immune response. By coupling the polysaccharide antigen to protein carriers (conjugate-vaccines), the T cell-independent response is changed into a T cell-dependent one, leading to an effective response against polysaccharide antigens in young children[118, 119, 120, 121].

In IgG2-deficient patients, only a marginal compensating mechanism exists, as illustrated by the impaired anti-polysaccharide response in all other immunoglobulin isotypes. This poor responsiveness can be partly bypassed by using conjugate-vaccines.

Nevertheless, it should be kept in mind that active vaccination procedures may fail, due to a deficient humoral immunity.

5 Assays for the determination of IgG subclass levels

Different methods are in use for the quantitative determination of IgG subclasses. The most important assays for the determination of IgG subclass levels are summarized in this chapter.

5.1 Radial Immunodiffusion (RID)

RID (Mancini) is a classical diagnostic method to determine the levels of IgG subclasses. This reliable assay is widely used and easy to perform. However, when large numbers of samples have to be processed, the relatively long incubation times is a disadvantage.

Principle of the RID

The relative concentrations of an antigen can be determined by a simple quantitative assay in which an antigen is placed in a well and allowed to diffuse into the agar containing a suitable dilution of an antiserum. As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation forms around the well. The diameter of this ring is proportional to the concentration of the antigen. By comparing the diameter of the precipitation ring with a standard curve (obtained by measuring the precipitation diameter of known concentrations of the antigen), the concentration of the antigen sample can be determined.

Antigen-excess, equivalence zone and antibody-excess

Excess of either antigen or antibody interferes with maximal precipitation, which occurs in the so-called equivalence zone. Here the ratio of antigen to antibody is optimal. A precipitation curve for radial immunodiffusion (RID) in which antibody-antigen immune complexes in relation to the antigen concentration is shown in figure 10. This plot of the amount of precipitated immune complexes versus decreasing antigen concentrations reveals three zones (starting from the centre of the agar plate to the edge of the plate). The first zone is a zone of antigen excess in which precipitation is inhibited and unreacted antigen can be detected along with small amounts of immune complexes of antibodies bound to antigen. The second zone is the equivalence zone. Here is the ration of antigen to antibody optimal: maximal precipitation in which antigen and antibody form large insoluble complexes and neither unbound antigen nor unbound antibody can be detected. In the zone of antibody excess, precipitation is inhibited and unreacted antibody can be detected and small complexes of antigen and antibody are observed.
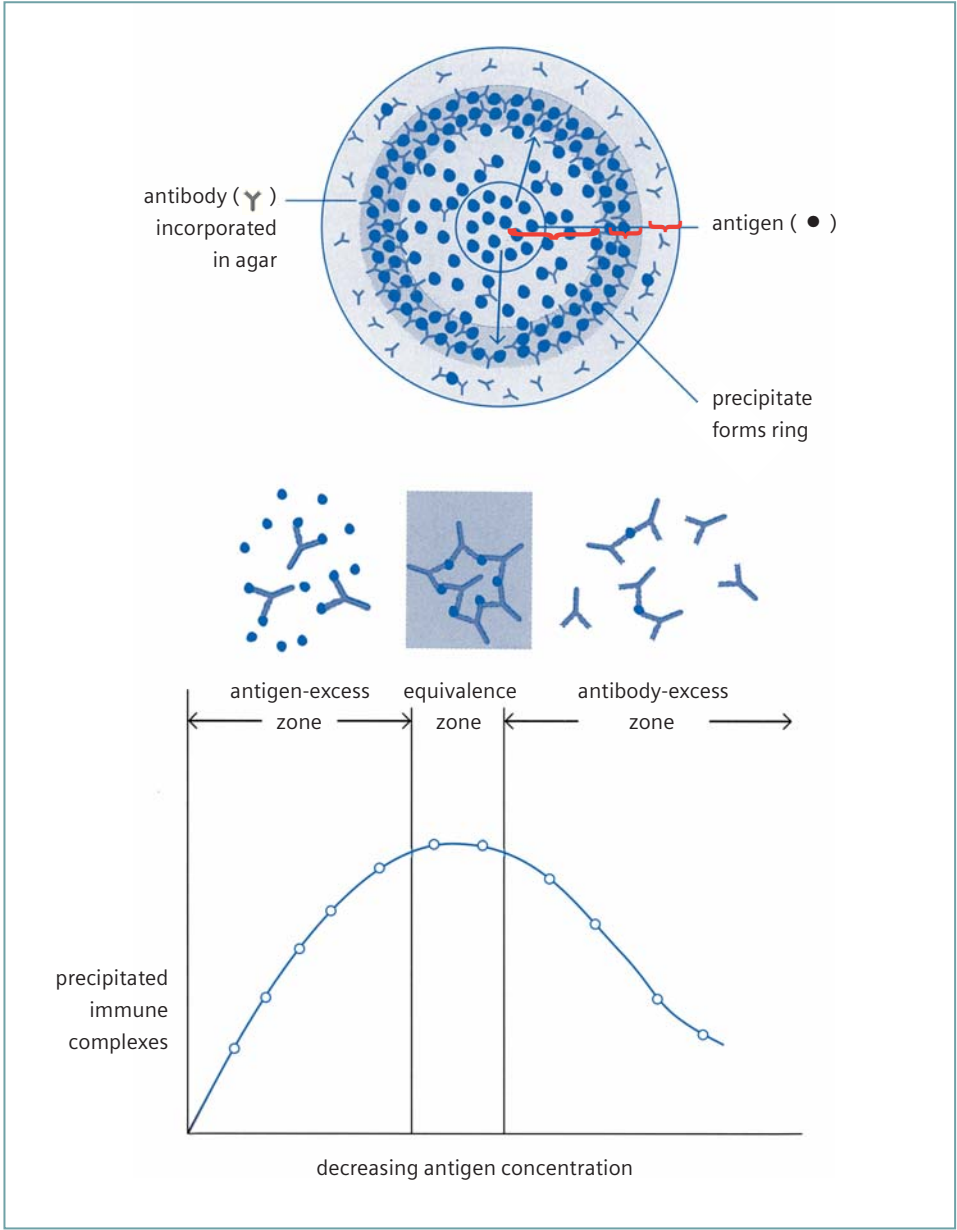
antibody (Y)
incorporated
in agar

antigen (●)

precipitate
forms ring

antigen-excess
zone

equivalence
zone

antibody-excess
zone

precipitated
immune
complexes

decreasing antigen concentration

Figure 10

Schematic drawing
of the principle of radial
immunodiffusion

Brief outline of the RID for determination of IgG subclass levels

The RID assay is performed in (ready-for-use) agar plates, containing the specific anti-IgG subclass antibodies (figure 11). Test samples, standard sera and control sera are prepared and added to the plates. After 48-64 hours incubation at room temperature the diameters of the immunoprecipitation rings are measured. The IgG subclass concentrations in the test samples may be quantified in two ways:

a) Calibration curve method: ring diameters and concentrations of the standards are plotted and the values of the test sample are determined by interpolation.

b) Tabular method: ring diameters of the calibration curve are listed and the values of the test sample are read from a table. It is not necessary to make a calibration curve.

The control serum is assayed to check the validity of the calibration curves and also the accuracy of the IgG subclass quantification, when using the table.
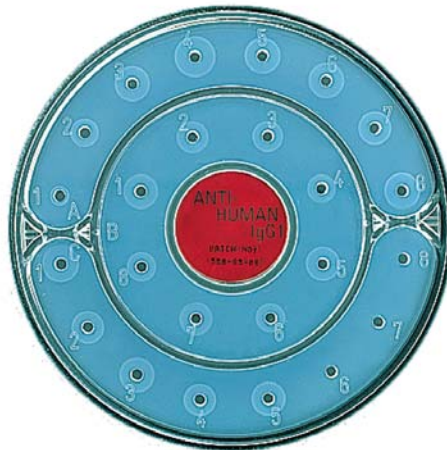

Figure 11

RID agar plate with precipitation rings.

Results are shown of a radial immunodiffusion assay for IgG1. The agar plate contains the anti-IgG1 antibody, and in the wells different samples, standards and controls are added. The diameter of the precipitation rings corresponds with the IgG1 concentration.

5.2 Nephelometry and turbidimetry

Nephelometric quantification is based upon the reaction of a monospecific anti-IgG subclass-specific antiserum with human IgG of the subclass to be determined. The generated immune complexes are quantified by measuring the side-scattered light. A distinct advantage of nephelometry is the relatively short incubation time. As another advantage, nephelometry assays may be readily automated and are therefore suitable for the routine measurement of IgG subclasses in large numbers of samples.

There are different measuring techniques in nephelometry. In the 'kinetic method' the light scatter is recorded at the highest reaction rate. In the 'fixed time' (end point) method light scatter is recorded after a fixed incubation time.

The developed latex-enhanced nephelometric reagents for the determination of IgG3 and IgG4 levels have significantly improved sensitivity, compared with conventional reagents. New assays, in which the latex technology of Siemens Healthcare Diagnostics is combined with the knowledge of IgG subclasses of Sanquin, are specific and highly sensitive.

Fixed time-nephelometry is illustrated in figure 12.
Brief outline of the method:

- Diluted samples (serum, plasma or other biological fluids), standard sera and control sera are introduced in the reaction tubes of the nephelometer;

- Appropriate anti-IgG subclass reagents and reaction buffer are added;

- Side-scattered light is recorded;

- IgG subclass concentrations in the test samples are calculated relative to the calibration curves, obtained with the nephelometric IgG subclass standard serum;

- A control serum is assayed to check the validity of the calibration curves and the accuracy of the IgG subclass determinations.
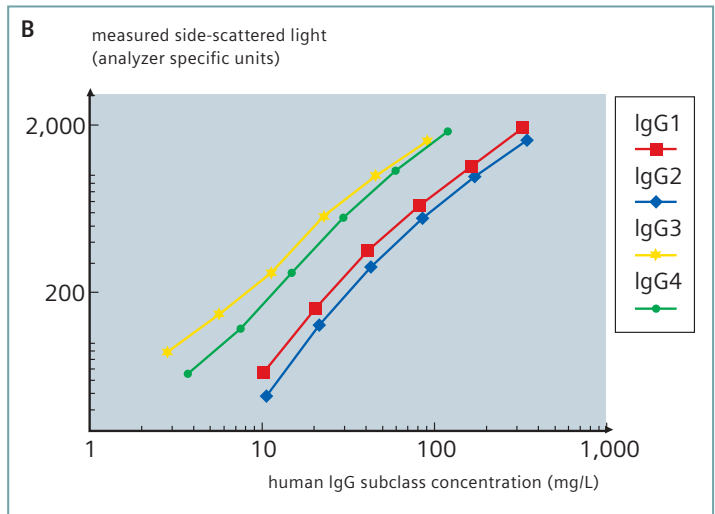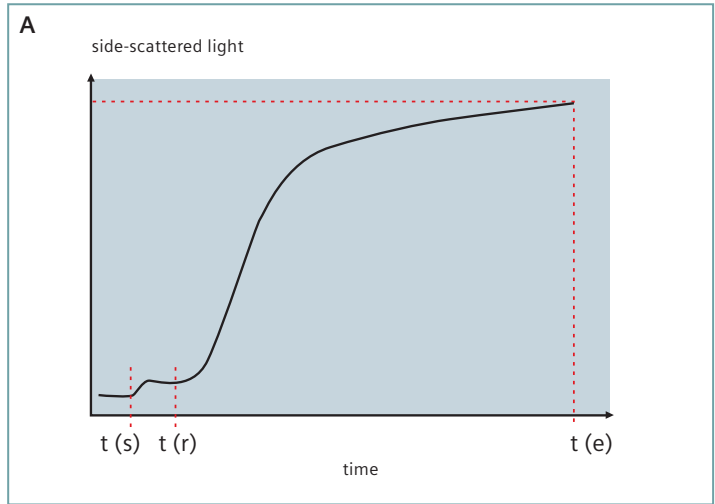
Besides nephelometry, the turbidimetry is another method based on the optical detection of antigen-antibody complexes in the fluid phase. However, in turbidimetry the decrease in *light transmission* is recorded, rather than the *side-scattered light*, which is measured in nephelometry.

Figure 12

Fixed time-nephelometry

A. The side-scattered light is plotted against time. At time t(s) the sample is added, followed by the reagent at time t(r). After a fixed incubation time t(e) the side-scattered light is measured.

B. The measured side-scattered light (in analyzer specific units) is plotted against the IgG subclass concentration (mg/L) of the diluted standard sera and the calibration curves are generated.

A

side-scattered light

t (s)   t (r)   t (e)

time

B

measured side-scattered light
(analyzer specific units)

2,000

200

1   10   100   1,000

human IgG subclass concentration (mg/L)

IgG1
IgG2
IgG3
IgG4

5.3 Radio Immuno Assay (RIA)

In the RIA, IgG of the various subclasses is quantified as immune complexes after binding of radioactively labelled specific antibody. In a 'direct' technique, a radioactively labelled anti-IgG subclass-specific antibody is used. In an 'indirect' technique, an anti-IgG subclass-specific antibody is first added, followed by a radioactively labelled secondary antibody directed against the first antibody.

Since working with radioactively labelled reagents requires special precautions and is relatively costly, radio immunoassays have been largely replaced by enzyme-linked immunoassays.

5.4 Enzyme-linked Immunosorbent Assay (ELISA)

In this technique, enzyme-labelled antibodies are used for detection. In the 'sandwich'-type of enzyme immunoassay, the IgG subclass of interest is captured by anti-human IgG subclass-specific antibody and quantified by an enzyme-labelled anti-IgG antibody. Because of its high sensitivity and specificity, this assay allows the accurate measurement of very low levels of IgG of the different subclasses. In figure 13, a schematic outline of the ELISA technique is shown. The sensitive ELISA comprises many incubation and washing steps. Because of the need for high dilutions when measuring IgG subclasses in sera, ELISA assays may be less reproducible in comparison with RID and nephelometry. For measurement of IgG and its subclasses in large numbers of samples, the ELISA is increasingly being replaced by nephelometry. The ELISA may be recommended for measuring IgG subclasses in body fluids other than serum/plasma, *e.g.*: saliva, cerebrospinal fluid and broncho-alveolar lavage fluid.

Brief outline of the method (figure 13):

An ELISA is generally performed in wells of microtitre plates.

- Wells of the plates are coated with unlabelled monoclonal anti-human IgG subclass-specific antibody and washed (figure 13A);

- Test samples, standard sera and control sera are introduced in the respective wells and incubated; The IgG subclass to be determined will bind to the solid phase and non-bound IgG is removed by washing (figure 13B);

- Enzyme-labelled anti-human IgG antibodies are added to each well and non-bound conjugate is removed by washing (figure 13C);

- Plates are incubated with substrate solution;

- After incubation, the coloured reaction product is measured photometrically (figure 13D);

- The concentration of the IgG subclasses in the test samples is calculated relative to the values of the calibration curve.
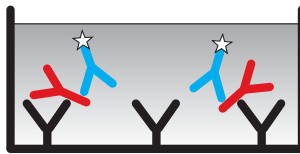
A. Monoclonal anti-human IgG subclass-specific antibody is coated on the well of the microtitre plate.

B. Test-, control- and standard human sera are pipetted into their respective wells. The IgG subclass to be determined is bound to the plate. Non-bound IgG is removed by washing.
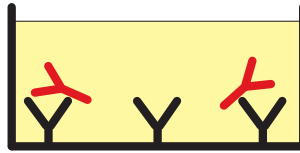
C. Enzyme-conjugated antibodies to human IgG are added.

D. After incubation with substrate the coloured reaction product is measured and the concentration of the bound IgG subclass of interest can be determined.
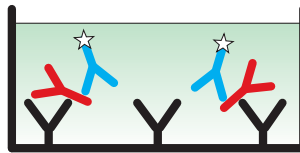

Figure 13

Different steps in IgG subclass ELISA.

Explanation of the symbols:

Y = Precoated monoclonal anti-human IgG subclass-specific antibody.

Y = IgG subclass to be determined, present in test sample.

Y = Enzyme-conjugated anti-human IgG antibody.

5.5 Immuno-affinity chromatography

In immuno-affinity chromatography IgG of each subclass is measured by monoclonal anti-IgG subclass-specific antibodies, bound to a solid phase in an open column. The captured IgG molecules are labelled with a polyclonal, fluorescently-labelled anti-IgG reagent. The amount of bound conjugate is proportional to the amount of captured IgG. The conjugate is eluted from the column and subsequently measured in a fluorescence reader. The IgG subclass concentrations of the test samples are calculated using the calibration curves.

5.6 Agglutination techniques, Direct Antiglobulin Test (DAT)

Haemolytic disease in adults and newborns may be due to the presence of anti-erythrocyte auto- or allo antibodies. Such antibodies, bound to the red cell membrane *in vivo*, can be detected in the direct antiglobulin test (DAT). In the DAT the erythrocytes are incubated with anti-human IgG antibodies. If red blood cells are sensitized (have IgG antibodies bound to their surface), the anti-human IgG causes the cells to agglutinate. Haemolytic Disease of the Newborn (HDN) and Auto Immune Haemolytic Anaemia (AIHA) are two diseases in which the DAT is positive. HDN is a disease in which erythrocytes of the newborn are destroyed by maternal antibodies directed against antigens on the baby's red blood cells. AIHA is an auto-immune disease in which auto-antibodies are produced against the body's own erythrocytes.

In case of a positive DAT due to IgG antibodies, it is important to determine the subclass of the IgG antibodies. Sequestration of sensitized red cells is dependent upon the IgG subclass of the antibody, because there are differences between IgG subclasses in their ability to activate complement and to bind to Fcg receptors on phagocytic cells (table II). In general, the haemolytic effect of the IgG subclasses ranges from high to low in the following order: **IgG3 > IgG1 > IgG2 > IgG4**.

Brief outline of the method:

In V-shaped wells of a microtitre plate, serial dilutions of anti-IgG subclass-specific antisera are introduced. Next, sensitized red cells are added. After overnight incubation at 4°C, the agglutination is read macroscopically. Agglutination may also be detected by running the red cell mixture over a gel column.

5.7 Comparison of the most frequently used assays

	Sensitivity	Intra assay variation	Inter assay variation	Automation	Assay time	Work load
Assay:						
RID	µg / ml	small	small	no	long (>48 hours)	moderate
Nephelometry	µg / ml	very small	very small	complete	very short (few minutes)	minimal
ELISA	ng / ml	medium	medium	partly	short (few hours)	high

Table VI

Comparison of the RID-, nephelometry- and ELISA assays for determination of human IgG subclass levels

5.8 Standardization and reference values

Officially recognized as an International Laboratory for Biological Standards since the 1970's, Sanquin is well aware of the value of reliable standardization. Thus, Sanquin's IgG subclass standard sera are accurately calibrated against the IgG subclass levels in the WHO-reference serum 67/97, recommended for human immunoglobulins. Measurements of the IgG subclass content of this preparation has been performed by a number of different research groups resulting in somewhat divergent results. The IgG subclass levels in the WHO-reference serum 67/97 were assessed at 5.0 g/l for IgG1, 2.6 g/l for IgG2, 0.4 g/l for IgG3 and 0.5 g/l for IgG4 by Klein and colleagues[122]. These values for the IgG subclass levels have been suggested by the International Union of Immunological Societies (IUIS) as the "correct" standard[123]. As soon as the IgG subclass levels are standardized in the new reference preparation for serum proteins, BCR/IFCC/CAP CRM470, Sanquin's IgG subclass standard sera will be calibrated against these levels.

A high specificity of the anti-IgG subclass antisera and accurate standardization are, however, not sufficient to ensure a high-quality assay for human IgG subclasses, if no reliable reference values are available. Sanquin has therefore conducted extensive studies by measuring IgG subclass levels in more than one thousand serum samples from healthy individuals. Likewise, more than five thousand serum samples from patients have been evaluated for their IgG subclass concentrations. In approximately 28 % of the latter sera one or more IgG subclass levels were decreased.

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