The University of Manitoba

Cross-Reactivity of Solubilized Fc Receptors of Rat Basophilic Leukemia Cells with Rat and Mouse IgG Subclasses and Other Immunoglobulins

by

Michael R. Kepron

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

Department of Immunology

Winnipeg, Manitoba

July, 1989

ISBN 0-315-54796-0

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Acknowledgements

I am indebted to Dr. Arnold Froese for his guidance and extreme patience throughout my years as a graduate student.

I offer my sincere thanks to my wife Ruth, both for the sacrifices that she has made over the years of my graduate career, and for her indispensable assistance in the actual production of the thesis.

I also wish to express my appreciation to the other members of the lab for all that I learned from them, and especially to Ms. Karol McNeill for her excellent technical assistance.

Finally, I would like to thank the members of my advisory committee for their patience and helpful feedback during the course of this project.

Abstract

Rat IgE-Sepharose binds two surface receptors from solubilized rat basophilic leukemia (RBL) cells. These glycoproteins are referred to as H and R, however current nomenclature refers to them as $Fc_\epsilon R_L$ and $Fc_\epsilon RI(\alpha)$ respectively. Results presented in these studies indicate that the same two proteins also bind specifically to normal rat IgG-Sepharose, and are capable of interaction in solution with IgG monomers from rat and a variety of heterologous species, although the affinities of the receptor/IgG interactions vary characteristically for each receptor between species and subclasses of IgG. The rat immunoglobulins can be ranked in order of their affinities for R as follows: IgE >> IgG_{2a} > IgG_1 > IgG_{2b}. For H, the affinities are of the order: IgE > IgG_{2b} > IgG_1 > IgG_{2a}. Although rat IgG_{2c} interacted with both H and R, a precise ranking of its affinity was not possible. All of the heterologous IgGs interacted with H considerably more strongly than with R. The results suggest that the IgG/receptor interaction may be important in itself and may also have an influence on the interaction of the receptors with IgE.

Abbreviations

BBS, borate buffered saline

BSA, bovine serum albumin

BSS, balanced salt solution

CPM, counts per minute

EDTA, ethylenediamine tetra-acetate

FPR, fluorescence photobleaching recovery

FCS, fetal calf serum

Ig, immunoglobulin

INA, 5-iodonaphthyl-1-azide

MPDC, Micro-ProDiCon

NP-40, Nonidet P-40

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate buffered saline

PBSG, phosphate buffered saline glycine

PCA, passive cutaneous anaphylaxis

PMSF, phenylmethylsulfonylfluoride

RBL, rat basophilic leukemia

rIgG, normal rat IgG

RMC, rat mast cell

SDS, sodium dodecylsulfate

TD, tracking dye

Tf, transferrin

TfR, transferrin receptor

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Chapter 1

Introduction

The system of interest basically consists of two parts: a receptor, which is a part of a cell membrane, and a ligand, which is bound by this receptor. In this particular case, the receptor selectively binds immunoglobulin proteins.

Historically, the immunoglobulins were the first part of the system to be discovered, and it only became apparent somewhat later that these proteins were capable of binding to a cellular receptor. As a result, at this point in time, our understanding of immunoglobulin structure and function is considerably better than our understanding of the receptor. This introductory discussion begins therefore with a general description of the immunoglobulin proteins.

Immunoglobulins

The immunoglobulins (antibodies) are a family of structurally related proteins which are capable of recognizing and binding to foreign or nonself molecules (antigens). This binding subsequently leads to the activation of a variety of secondary cytotoxic and phagocytic mechanisms which inactivate and/or eliminate the antigen from the body. The antibody molecule thus serves the dual purpose of: (a) identifying the antigen and (b) alerting various defense mechanisms.

The role of antigen identification requires that the antibody be capable of distinguishing self from nonself. The immune system's B cells therefore produce a vast array of structurally similar immunoglobulin molecules, each capable of recognizing a *specific* antigenic determinant. Each B cell produces antibodies of only *one* antigenic specificity, but the enormous diversity of B cells results in a repertoire of antibodies that covers virtually every conceivable antigen. An individual even possesses B cells capable of producing anti(self) antibodies, however these are (usually) suppressed. The mechanism of this suppression, and of immunoregulatory systems in general, is presently the focus of a great deal of attention.

The other major function of the immunoglobulin, that of inducing various biological activities, is the responsibility of a distinct portion of the molecule called the *Fc region*. It is this part of the molecule that is of primary concern in the work that follows in later chapters.

General Structure

Immunoglobulins in their monomeric form are four-chain macromolecules consisting of two identical heavy chains and two identical light chains. The two heavy chains are covalently bound together by interchain disulfide bonds, and each light chain is similarly bound to a heavy chain. Such a structure is represented schematically in Figure 1.1. On the basis of amino acid sequence data, both the heavy and light chains can be divided into stretches of approximately 110 amino acids which show strong homology from one species to another within the same immunoglobulin class (see below). Each of these stretches includes an *intrachain* disulfide bond which encloses a peptide loop of some 60 to 70 amino acids, and is folded into a compact globular structure called a *domain* (1). In all classes of immunoglobulin, the light chains consist of two such domains. The number of domains that make up the heavy chain varies between 4 and 5, depending on the class, but in the case of the G class immunoglobulin (IgG) shown, the heavy chains consist of four domains. Edelman suggested in 1970 that each of these domains has evolved to fulfil a specific function (2). For instance, the $-NH_2$ terminal domains of both the heavy and light chains are responsible for the antigen binding function of the molecule. The amino acid sequences of these domains contain hypervariable residues which determine the antigenic specificity of the binding site, and the domain is therefore referred to as the *variable region* of each chain.

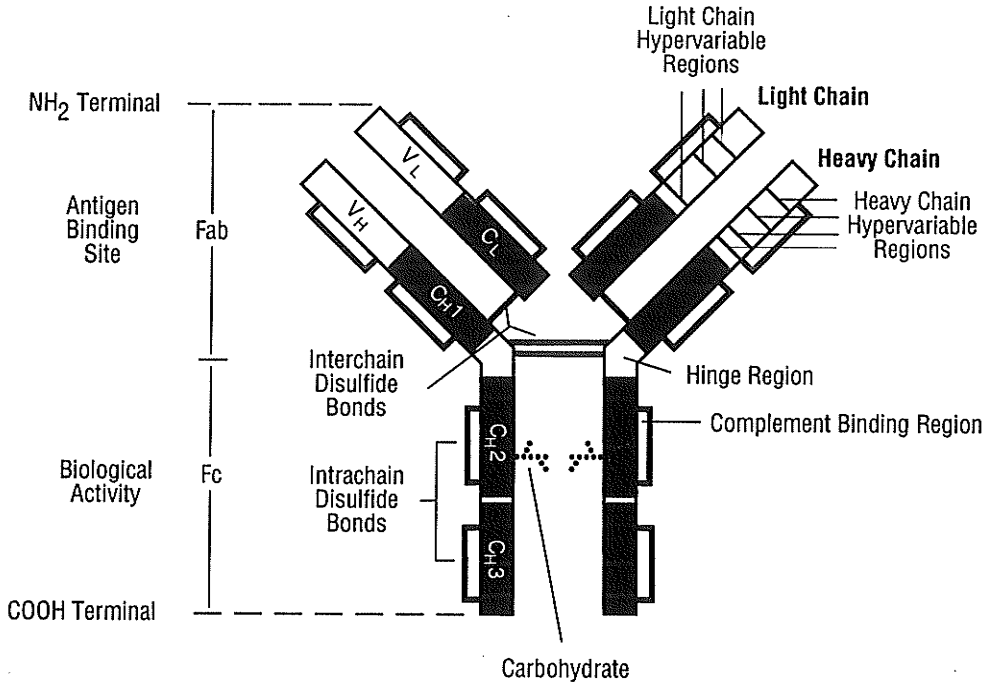


Figure 1.1 Schematic structure of an IgG class immunoglobulin molecule. V_L and V_H represent the variable region domains, C_L and C_H represent the constant region domains.

The amino acid sequences of the other domains of the heavy and light chains are strongly conserved within a class, constituting what is known as the *constant region*. On the light chain, the constant region only comprises a single domain, which is involved in interchain disulfide bonding to the heavy chain. On the heavy chain on the other hand, the constant region consists of the three (or four (see below)) -COOH terminal domains. Each of these is referred to by number, starting with the domain nearest the $-NH_2$ terminal, as indicated in Figure 1.1.

Enzymes such as papain split the immunoglobulin molecule at a point between the C_H1 and C_H2 domains, resulting in the production of three fragments. Two of these contain the variable regions, retain the antigen binding function of the molecule, and are referred to as the Fab fragments (antigen binding). The third fragment is made up of the paired C_H2 and C_H3 domains, and it retains the biological effector functions, *such as binding to the immunoglobulin receptors of various cells*. This fragment can be crystallized, and is therefore referred to as the *Fc fragment* (crystallizable).

It should be mentioned at this point that, in addition to the interaction with Fc receptors alluded to in the last paragraph, the Fc region is also involved in the activation of the *complement system*. The C1 component of this complex group of proteins is activated by an interaction with the Fc portion of antigen-bound immunoglobulin. This activation of C1 induces the cascade of reactions which lead to the production of inflammation, marking of

foreign materials for phagocytosis (opsonization), and the direct killing of various cells and microorganisms.

The stretch of amino acids between the C_H1 and C_H2 domains, within which papain acts to cleave the molecule, is referred to as the *hinge region*. This is a region of approximately 15 amino acids which are important to the overall structure of the immunoglobulin molecule (3). As indicated in Figure 1.1, the cysteine residues of the hinge region are involved in the formation of interchain disulfide bonds, linking the two heavy chains of the molecule. These bonds serve to maintain the spatial relationship between the paired C_H2 domains (quaternary structure). As the name implies, the hinge is also involved in the segmental flexibility of the antibody molecule, allowing the antigen combining regions to assume a variety of orientations relative to one another and the Fc region. Finally, the hinge serves as a spacer, putting some distance between the Fab and Fc. The importance of this function is discussed below.

The spatial arrangement of the Fc domains has been determined by x-ray crystallography (4). Through such studies, it has been determined that the paired -COOH terminal domains (C_H3 in Figure 1.1) associate with one another through high affinity, noncovalent interactions. In contrast, the domains between these -COOH terminal domains and the hinge region (C_H2 in Figure 1.1) *do not* interact and are separated by a solvent filled channel. Furthermore, the immunoglobulins are glycoproteins, and a carbohydrate prosthetic group is attached to each C_H2 domain on the surface of the domain *facing* this

solvent channel (ie. between the domains). This is the case at least for the G class immunoglobulins, which is the only class for which x-ray crystallographic data are available. The spatial relationship of these domains is maintained by the hinge region disulfide bonds on the one hand (as mentioned above), and by the tightly associated -COOH terminal domains on the other.

The three dimensional (quaternary) structure of the Fc region has been found to be crucial to the biological activity of immunoglobulins. Reduction of the hinge region interchain disulfide bonds for example destroys the spatial relationship of the two C_H2 domains by allowing them to move relative to one another. This alteration in the molecule's quaternary structure strongly inhibits both Fc receptor binding and complement activation (3). Actually, since the C_H3 domains remain together under these conditions, these findings are quite informative in that they tend to indicate the importance of the C_H2 domains, and their orientation relative to one another and the C_H3 domain, in receptor binding and complement activation.

Further evidence for the importance of the C_H2 domains in these functions comes from studies with hinge deleted proteins. These studies have indicated that, in the absence of a normal hinge region, Fc receptor binding and complement fixation are inhibited. This might be due to an unstable spatial relationship between the two C_H2 domains, resulting from a lack of the hinge region interchain disulfide bonds (as discussed above). However, X-ray crystallographic studies on these proteins have failed to show any significant

conformational alteration in the Fc region itself (5). It has been suggested therefore, that the inhibition is a result of steric interference, due to the close proximity of the Fab regions and the C_H2 domains (3). Therefore, in addition to the implications that these findings have concerning the location of the functional sites on the immunoglobulin, they also indicate the importance of the hinge region as a spacer between the Fab and Fc, mentioned above.

Immunoglobulin classes

All of the structural information presented in the previous section was in reference to the G class immunoglobulins, as was mentioned. There are in fact *five* known classes or *isotypes* of these proteins in most higher mammals. The present nomenclature recommended by the WHO is presented in Table 1.1 (1).

The various classes are distinguished from one another on the basis of variations in the structure of their heavy chains. These structural differences give rise to differences in physicochemical, functional and antigenic characteristics. Thus it has been possible to separate the classes on the basis of physicochemical differences, and to produce antibodies capable of recognizing the specific antigenic determinants of each immunoglobulin class (antibodies to antibodies). These class specific antisera have subsequently assisted in further purification of specific immunoglobulin classes, and have provided a valuable means of comparing the immunoglobulin classes of different species.

Table 1.1 Immunoglobulin nomenclature

Class	Abbreviation	Synonyms
Immunoglobulin G	IgG	gamma-G globulin 7 S gamma-globulin
Immunoglobulin A	IgA	gamma-A globulin beta 2 A-globulin
Immunoglobulin M	IgM	gamma-M globulin 19 S gamma-globulin
Immunoglobulin D	IgD	----------
Immunoglobulin E	IgE	reagin IgND

The heavy chains of a particular class are referred to by the Greek letter corresponding to the class name: mu for IgM, alpha for IgA, gamma for IgG, delta for IgD and epsilon for IgE. These structural variations in the heavy chains which set each class apart are represented schematically in Figures 1.2 and 1.3. The human immunoglobulin classes are presented here since the data is most complete in this species, but one rat class (IgE) is also illustrated (Figure 1.2). Comparison with the analogous human IgE shows that the structure of a particular class is quite similar from one species to another.

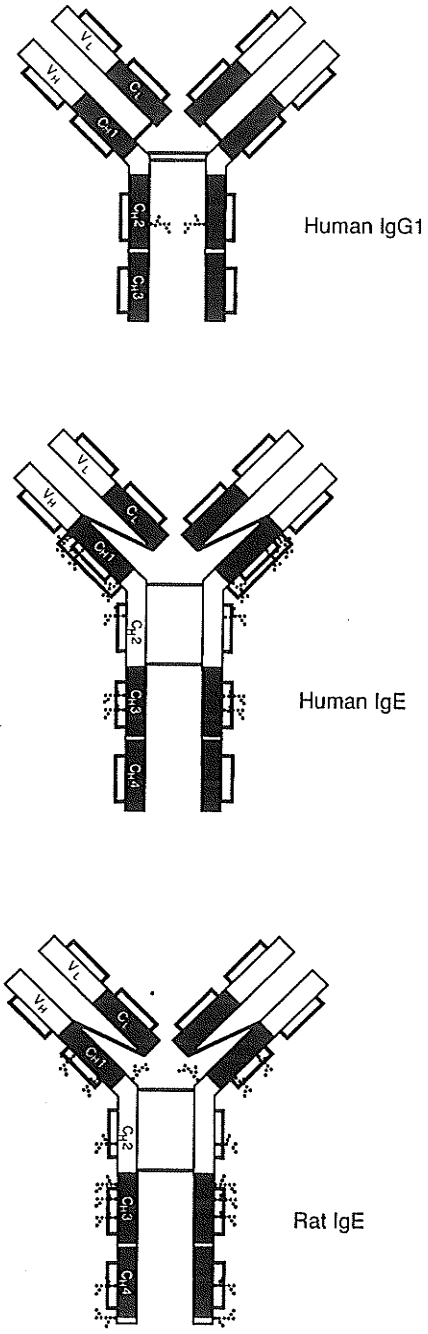


Figure 1.2 Schematic structures of human IgG_1 and IgE, and of rat IgE. Comparison of the human and rat IgE's shows that, even across such diverse species differences, the structure of a particular immunoglobulin class is quite similar. The symbols used are the same as those used in Figure 1.1.

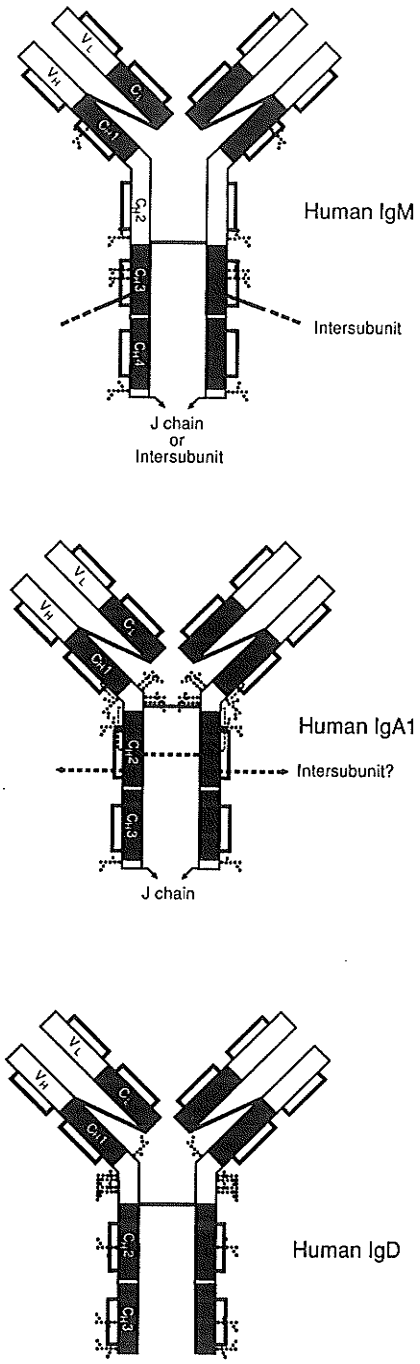


Figure 1.3 Schematic structures of human IgM, IgA_1 and IgD. The symbols used are the same as those used in Figure 1.1 In the case of IgA, the disulfide bonding of several cysteine residues remains uncertain and these bonds are indicated by dashed lines.

IgG

IgG is the predominant immunoglobulin class in the serum of higher vertebrates, accounting for 70-75% of the total immunoglobulin pool, and is of particular interest in the work to be described. IgG isolated from normal serum exists as a monomeric protein with a molecular mass of approximately 150,000 daltons. The purified protein however has a tendency to form aggregates, and it precipitates on isoelectric focusing. On electrophoresis, IgG shows a broad range of mobilities, from so called slow gamma to alpha 2. This range of net charge is also seen on isoelectric focusing and ion-exchange chromatography, and is partially attributable to variations in such things as the protein's carbohydrate content and the amino acid sequence of the variable region.

Another source of charge heterogeneity is the existence of subclasses. In fact, differences in electrophoretic mobility and ion exchange characteristics of immunoglobulin preparations supposedly containing only one *class*, suggested that it might be possible to further divide the IgG class into these *subclasses* (6). The existence of the subclasses has been further confirmed, and finer divisions have been made, based on subsequent studies of the immunoglobulins produced constitutively by a variety of immunocytomas (7). Each of these tumors produces a single specific immunoglobulin in vast quantities, providing an ample source of a single homogeneous protein for biochemical studies. In fact, it would be quite fair to say that much of our present knowledge of immunoglobulin structure and function is directly attributable to the discovery of these tumors.

Thus, human, mouse and rat each have been found to possess four different subclasses of IgG. In the human, these are referred to as IgG_1, IgG_2, IgG_3 and IgG_4. In the mouse, they are called IgG_1, IgG_{2a}, IgG_{2b} and IgG_3. In the rat, the subclasses are named IgG_1, IgG_{2a}, IgG_{2b} and IgG_{2c}. In guinea pigs, so far only two subclass have been discovered, IgG_1 and IgG_2. The rabbit is unique in that only one subclass of IgG has thus far been described. Unfortunately, while the immunoglobulin *classes* of different species are directly comparable, the *subclasses* are not. This is because the classes of each species are distinguished by physicochemical, functional and antigenic criteria, while the subclasses are distinguished on the basis of physicochemical characteristics alone. Thus, for example, the IgG_1's of the various species are not necessarily functionally analogous.

The structures of the human, mouse and guinea pig IgG subclasses are represented schematically in Figure 1.4.

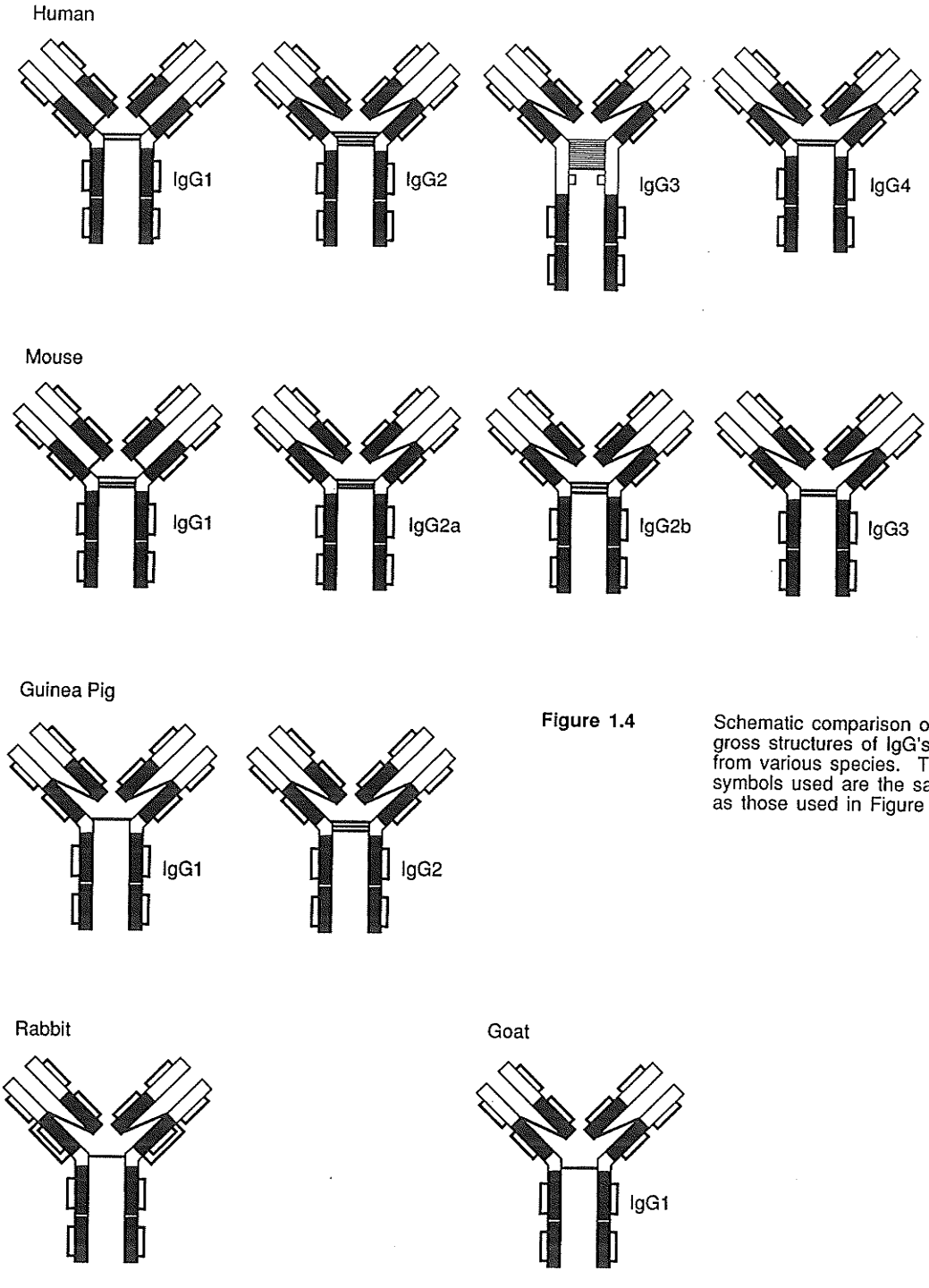


Figure 1.4 Schematic comparison of the gross structures of IgG's from various species. The symbols used are the same as those used in Figure 1.1.

Unfortunately, the structures of the rat subclasses have not yet been determined. Several features of these structures should be pointed out. First, there is the variation in the location of the disulfide bond linking the heavy and light chains. In both the human and mouse IgG_1 subclasses, the cysteine residue involved in this linkage resides between the C_H1 and C_H2 domains, close to position number 220 in the amino acid chain. In the other subclasses however (and in most other immunoglobulin classes), the residue involved is between the V_H and C_H1 domains, near position 130 in the heavy chain. In fact this is not as major a structural difference as it might appear from the representations of Figure 1.4, since x-ray crystallographic analysis has indicated that, due to the folding of the C_H1 domain, positions 130 and 220 are actually very close to one another (1). The other point of interest is the unusually long hinge region of the human IgG_3 subclass. The number of disulfide bonds contained in this region range from estimates of five to *fifteen* (1), although more recently this range has been narrowed to 10-11 (8).

IgE

The other immunoglobulin class of interest in subsequent chapters is IgE (Figure 1.2). IgE exists at the extreme opposite end of the scale from IgG in terms of abundance in serum, being present only in extremely low concentration and accounting for only 0.003% of the total immunoglobulin in normal serum (9). None the less, it plays a major role in immediate hypersensitivity reactions (discussed below). However, much of the chemical information on this class is only available as a result of the discovery of IgE secreting tumors, similar to those mentioned in the discussion of IgG subclasses above (10, 11).

As with IgG, IgE exists as a monomer, but unlike IgG it is heat labile and looses its biological activity on heating at 56°C. The IgE molecule is substantially heavier than IgG, having a molecular mass of approximately 190,000 daltons. This increased weight is accounted for by two significant structural differences between IgE and IgG. First, as indicated in Figure 1.2, the heavy chain is *extensively* glycosylated, and covalently attached carbohydrate accounts for about 12% of the molecule by weight. The second obvious structural feature is the presence of an extra domain in the heavy chain, with five heavy chain domains instead of the four seen in IgG. The 'extra' domain appears to be the C_H2 domain, which is in a position analogous to the hinge region of IgG. The molecule would appear to lack an IgG-like hinge region therefore, but Bennich has proposed that the hinge region of IgG might actually represent a 'collapsed' domain, and thus be equivalent to the C_H2 domain of the IgE heavy chain (1). In fact, the location of the interheavy chain disulfide bonds on either side of the IgE C_H2 domain suggests the 'insertion' of the domain in a hinge type region, and fits well with Bennich's proposal.

There is no evidence thus far for the existence of any subclasses of IgE. However, in view of the extremely low serum concentration of the class and the lack of large numbers of IgE myelomas, subclasses would have been difficult to detect. It has been possible however to determine the amino acid sequences of both human and rat IgE (12), and the structures based on these sequences are presented in Figure 1.2. As mentioned above, comparison

of the two structures emphasizes the similarity of proteins of the same class from different species.

IgA

IgA (Figure 1.3) only accounts for about 10 to 15% of the total serum immunoglobulin, but it is the predominant class of immunoglobulin in extravascular secretions. Most of the IgA in mammary gland, digestive tract and nasal secretions, and in saliva and tears, is a dimeric form, referred to as secretory IgA (SIgA). The SIgA consists of two IgA monomers, a small joining molecule called J chain, and a glycoprotein called secretory component (SC) (9). This structure is illustrated in Figure 1.5. In man, two subclasses of IgA have been identified, IgA_1 and IgA_2.

IgM

In the human, IgM accounts for 5 to 10% of total serum immunoglobulin, and normally exists as a pentamer in association with a J chain subunit , as shown in Figure 1.6. The molecular mass of this entire structure is approximately 950,000 daltons. As with IgA, a more detailed illustration of one of the pentamer's subunits has already been presented in Figure 1.3. Similarly to IgE, the heavy chain consists of five domains, with the C_H2 domain in the hinge position. Each of the five subunits in the intact molecule is held together by disulfide bonding through cysteine residues very close to the -COOH terminal, and, in the human, on the C_H3 domain. There are apparently no subclasses of the IgM class.

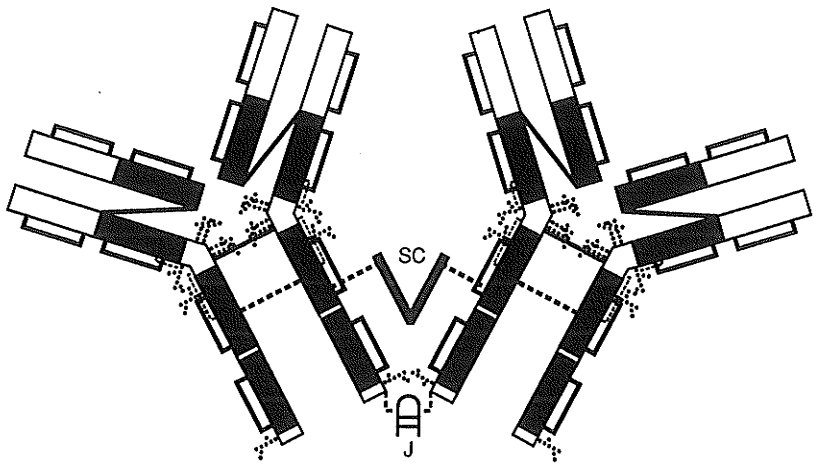


Figure 1.5 Schematic structure of dimeric (secretory) IgA. SC, secretory component. J, J chain. Dashed lines indicate uncertainty as to the precise pattern of disulfide bonding. All other symbols are the same as those used in Figure 1.1.

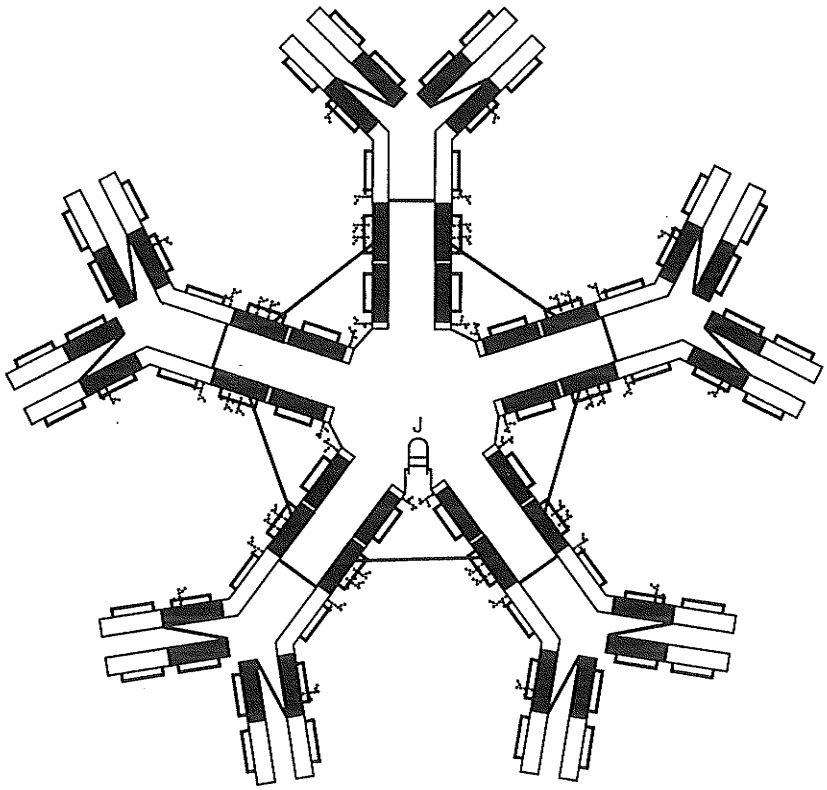


Figure 1.6 Schematic structure of pentameric IgM. J, J chain. All other symbols are the same as those used in Figure 1.1.

A special form of monomeric IgM exists on the surface of B cells, where it is thought to serve as one of the antigen receptors involved in the initial activation of the B cell. This form of IgM is actually an integral membrane protein of the B cell; it is not bound through Fc receptors (see below), and it is not secreted from the cell in this form (13).

IgD

IgD (Figure 1.3) is only present in very small amounts in the serum, accounting for just 0.3% of total serum immunoglobulin (9). However, in addition to the membrane IgM mentioned above, a membrane form of IgD exists which also plays an important role as an antigen receptor in B cell activation. In this membrane form IgD is unusual in that about 50% of the molecules appear to lack the inter heavy chain disulfide bonds and exist as 'half' molecules, consisting of a single heavy chain and a single light chain, with a single antigen combining region (13).

The structure of IgD was presented in Figure 1.3. There are a couple of remarkable features in the structure. First, the hinge region of human IgD is unusually long, contains covalently attached carbohydrate and includes a segment containing many charged amino acid residues, such as those of lysine and glutamic acid. The other feature of note is the glycosylation of the C_H3 domains. Once again, there is no evidence for IgD subclasses thus far.

Rat immunoglobulins

As previously mentioned, the majority of available data relates to human immunoglobulins, with the mouse ranking a very close second. The species of interest in the following work however is the *rat*. Unfortunately, there is very little structural data available for the rat immunoglobulins, but Table 1.2 presents a summary of the physicochemical characteristics and biological functions of each of the rat classes and subclasses, based primarily on the data of Bazin (reviewed in 14, 15).

Table 1.2 Physicochemical properties and biological functions of rat immunoglobulins

	IgG_1	IgG_{2a}	IgG_{2b}	IgG_{2c}	IgM	IgA	IgE
Synonyms	7S-γ_1	IgG_a	IgG_b				
Sedimentation Constant	6.7 S	6.4 S	6.5 S	6.7 S	17-19 S	7 S	7.6 S
Molecular Weight (Daltons)	156,000	156,000	-	156,000	-	163,000	183-198,000
Allotypic variants[1]	-	-	yes	-	-	yes	-
Serum concentration (mg/ml)	5.85	8.00	-	2.60	0.56	0.13	0.02
Half life (days)	2.20	5.0	-	-	2.6	1.12	0.5
Protein A binding	+	-	±	+	+	+	
Antibody activity	yes	yes	yes	yes	yes	yes	yes
Complement fixation	yes	yes	yes	yes	yes	no	no
Placental transfer	yes	-	-	-	-	-	yes
Mucosal secretion	no	no	no	-	no	yes	-
Thermolability (56°C)	no	no	no	no	no	no	yes
Persistence in skin	hours	-	-	-	-	-	days
Passive cutaneous anaphylaxis	no	yes	no	-	-	-	yes

1. Allotypic variants have also been described for the *kappa* light chain

The Receptor

As indicated at the outset, the other half of the system of interest is the receptor, which is a part of the cell membrane, and which selectively binds the immunoglobulin proteins. This introductory discussion therefore continues with a general description of the cell membrane and its components, providing an introduction to the cell membrane's receptor proteins.

Structure of the cell membrane

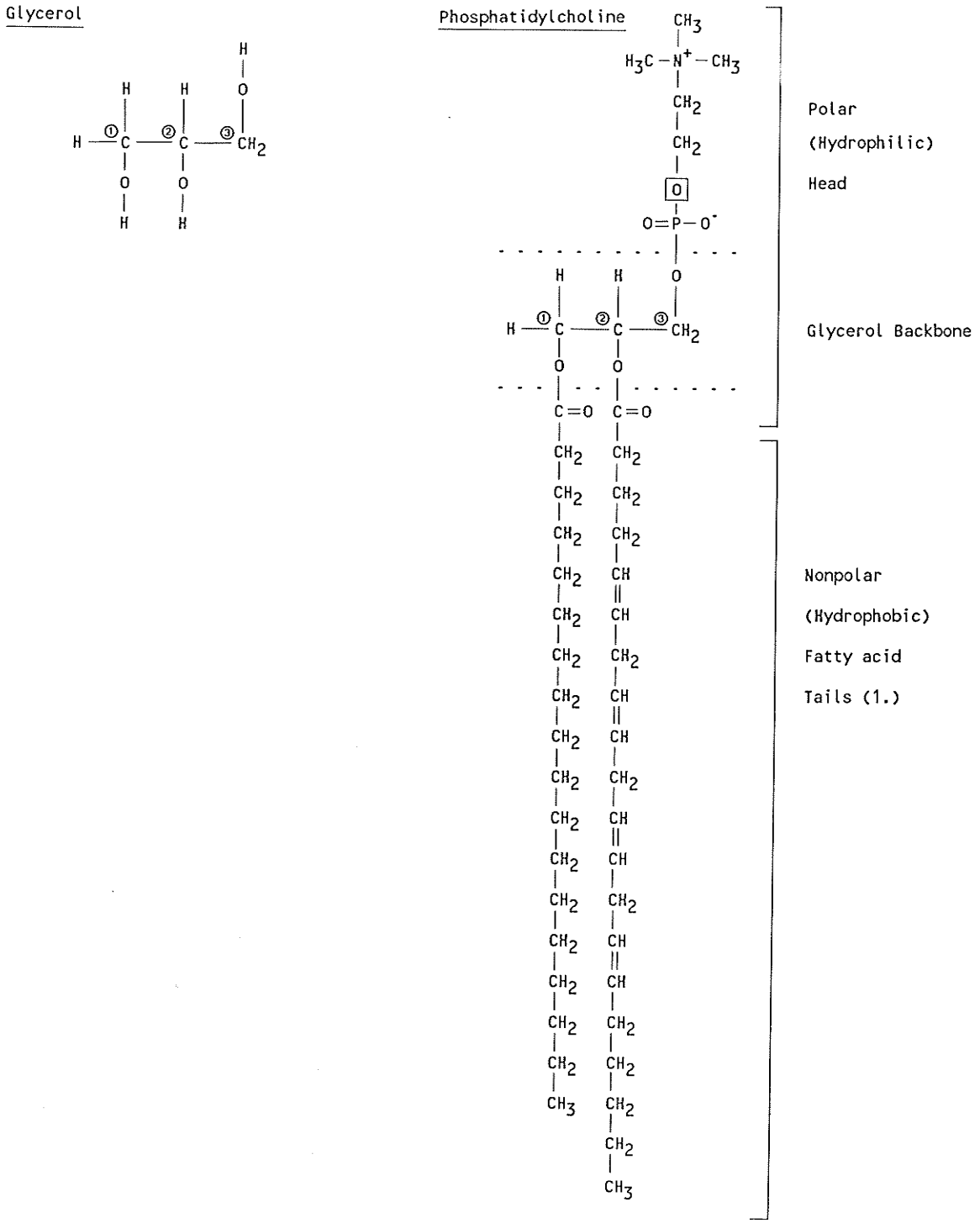
The membranes of most cells consist of about 40% lipid and 60% protein by weight (16). The lipid portion of the membrane is predominantly made up of cholesterol and the three phospholipids phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, the relative amounts of each of these varying somewhat from one cell type to another. There are other lipids present as well, although in smaller quantities. A group that perhaps deserves mention here is phosphatidyl inositol and its phosphorylated derivatives phosphatidyl inositol-4-phosphate and phosphatidyl inositol-4,5-bisphosphate. Although the latter two species only account for about 1% and 0.4% respectively of the total membrane phospholipids, they appear to play an important role in the transmission of signals across the cell membrane (17, 18).

Phospholipids

Figure 1.7 illustrates how the structures of the phospholipids are based on the three carbon backbone of glycerol, with long chain fatty acids esterified to the hydroxyl groups at carbon atoms 1 and 2. The third carbon is substituted with a phosphate group, which in turn is joined through an ester linkage to a group such as choline (Figure 1.7), ethanolamine, serine or inositol (Figure 1.8). At the normal physiological pH of 7.4, these phosphodiester linked groups either carry a net charge or exhibit a charge assymetry (polarity) as shown. The result is that phospholipid molecules have a dual (amphipathic) nature. The long chain fatty acid tails are non-polar and tend to associate with one another, excluding charged molecular species such as water and ions. The phosphodiester linked groups on the other hand form a polar head group that interacts quite well with water molecules, which are also highly polar. The fatty acid tails are therefore referred to as *hydrophobic* (water fearing), while the polar head groups are referred to as *hydrophilic* (water loving).

As a result of this dual nature, in an aqueous environment the phospholipids tend to arrange themselves in a bilayer, with the hydrocarbon chains of the fatty acids extending into the bilayer perpendicular to the surface (Figure 1.10). Water and ions are thus excluded from the hydrophobic membrane interior, while the polar head groups line the bilayer surfaces, where they interact with the aqueous environment. The phospholipids of the cell membrane appear to be similarly arranged in a single bimolecular layer, held together entirely by hydrophobic and polar interactions.

Figure 1.7 Structures of glycerol and phosphatidylcholine, illustrating the polar head group, non-polar fatty acid tails and the glycerol backbone of the phospholipid. The head group esterified to the phosphate group is choline in this case, however the structures of all of the phospholipids are similar, with various head groups bound to the phosphate group through the oxygen atom enclosed in the box. (See also Figure 1.8).



1. As examples, stearic and arachidonic acids are shown attached to carbons 1 and 2 respectively.

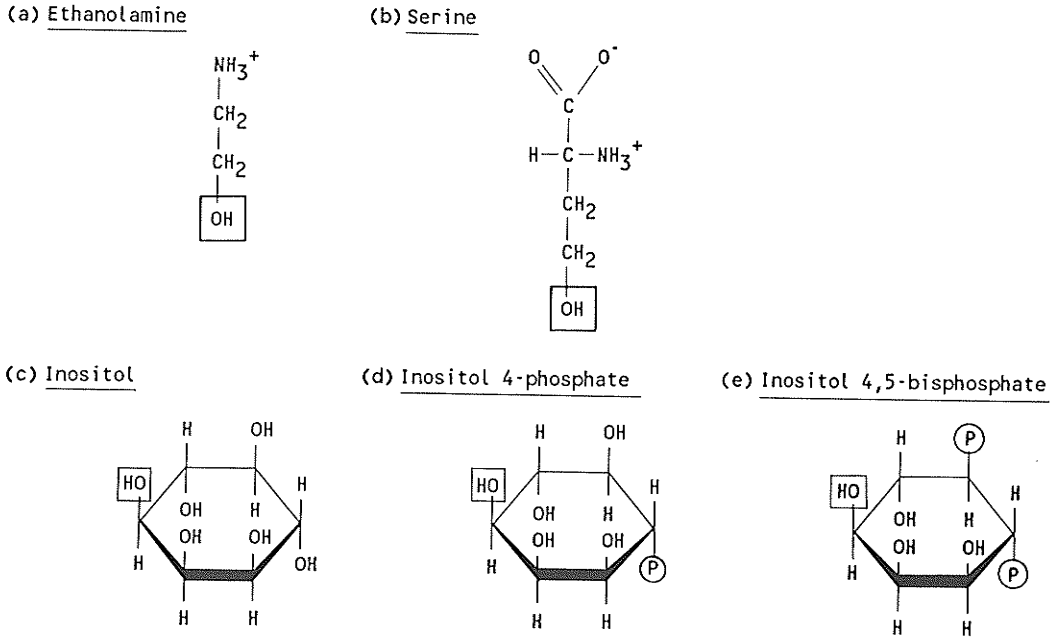


Figure 1.8 Structures of several of the alcohols which constitute the polar head groups of (a) phosphatidylethanolamine, (b) phosphatidylserine, and (c-e) the phosphatidylinositols. The hydroxyl groups esterified to the phosphate group of the phospholipid are enclosed in boxes. (See also figure 1.7)

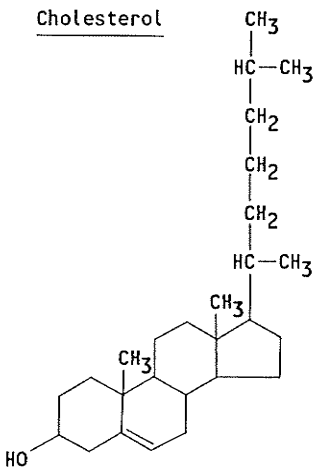


Figure 1.9 Structure of cholesterol. Although obviously different from the structure of the phospholipids, cholesterol is an important component of the cell membrane. It is considerably hydrophobic and mixes with the phospholipids.

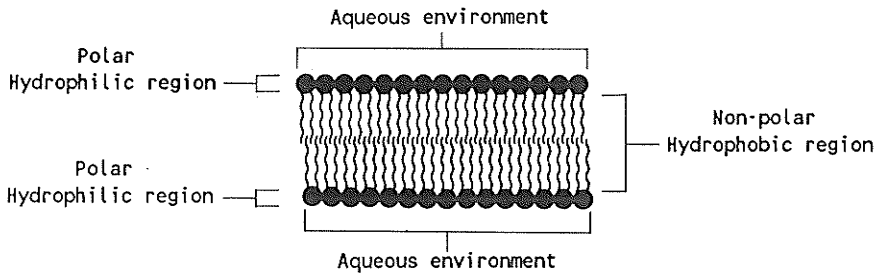


Figure 1.10 Structure of the phospholipid bilayer in an aqueous environment.

Proteins

The twenty common amino acids, from which the proteins are made, vary in their hydrophobicity/hydrophilicity. In an aqueous environment, the amino acid chain of a protein will tend to fold so as to allow the hydrophobic groups to interact with one another in the interior of the protein molecule, while the hydrophilic groups will be exposed to the environment. This is similar to the association of phospholipids in an aqueous environment, as discussed above. Within the cell membrane however, the hydrophobic amino acids interact well with the fatty acid tails of the membrane phospholipids. In this environment, the proteins can exist in conformations that present the hydrophobic amino acids to the similarly hydrophobic environment of the membrane interior.

The integral proteins of the membrane therefore have a dual nature similar to that of the phospholipids, with the regions of hydrophobic and hydrophilic amino acids largely confined to separate parts of the protein molecule. These proteins are thus thought to be oriented in the cell membrane in such a way that their hydrophobic regions interact with the fatty acid tails of the membrane phospholipids in the membrane interior, while the hydrophilic regions extend beyond the membrane surface and are exposed to the aqueous environment (Figure 1.11).

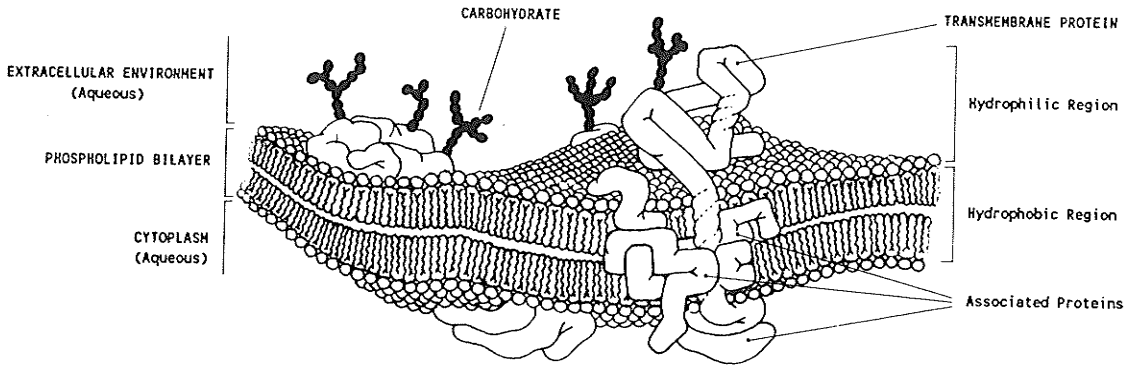


Figure 1.11 Section of the cell membrane illustrating the arrangement of various membrane proteins in the phospholipid bilayer. The carbohydrate moieties on the glycosylated membrane proteins are found exclusively on the extracellular regions of the proteins. Some proteins are found only on one surface of the membrane, while others span the bilayer. Still others may be completely buried in the hydrophobic interior of the membrane.

The proteins are prevented from turning over by the fact that this would require the immersion of the hydrophilic regions of the protein in the hydrophobic environment of the membrane interior. They are quite free however to move *laterally* through the surrounding lipid, which at normal physiological temperatures exists in a fluid phase. This is the "Fluid Mosaic Model" of membrane structure, proposed by Singer, Nicolson and Poste (19, 20), in which the membrane proteins "float" in a continuous lipid bilayer; a "sea of lipid".

It should be apparent that transferring these integral membrane proteins to a completely aqueous environment would require a radical change in the protein's conformation to hide the exposed hydrophobic regions. Such a conformational change would require a substantial amount of energy. In chemical terms, this is the same as saying that this hydrophobic interaction is quite strong. As a result, these proteins are held quite tightly within the membrane, a feature that has made membrane proteins exceedingly difficult to study. The characterization of a particular protein invariably involves its purification. The purification of an integral membrane protein requires its removal from the membrane. This removal involves the use of detergents or organic solvents to dissolve the cell membrane, disrupting the protein's hydrophobic interaction with the membrane lipids. Unfortunately, many of these proteins seem to require the surrounding membrane lipid in order to function normally. Consequently, their removal from the membrane often results in the loss of, or some alteration in, their activity. Furthermore, since membrane proteins also interact with *one another* through hydrophobic and/or hydrophilic interactions, the use of detergents and solvents also disrupts these protein/protein interactions. Since the function of a particular

protein may require interaction with another protein, once again this can result in the purification of a non-functional protein, or in the loss of a subunit of interest.

On the other hand, not all of the membrane proteins are held so tenaciously. The so called *extrinsic* membrane proteins are typical globular proteins with hydrophilic exteriors. These interact strongly with charged or hydrogen-bonding groups on the membrane surface, but they can be removed from the membrane by increasing the ionic strength or changing the pH of the aqueous buffer.

In concluding this introductory discussion of membranes, it should be pointed out that the membrane is not symmetrical with respect to the composition of the inner and outer leaflets. There is an asymmetric distribution of both phospholipid and protein on the two surfaces of the membrane (21). Thus, although many of the membrane proteins are glycosylated, their covalently attached carbohydrate is only present on that portion of the protein located on the *outer* surface of the membrane (Figure 1.11). The distribution of the *proteins* themselves is quite asymmetric, with different types of proteins being located exclusively on the inner or outer surfaces of the membrane, although some span the membrane and are exposed to both the cytoplasm and the extracellular environment. This is rather reasonable, in view of the different functional requirements on either side of the membrane. In addition, there is generally considerably *more* protein on the inner surface than on the outer surface . With respect to the phospholipids, although each phospholipid is generally present on both sides of any particular membrane, the distribution is

asymmetric. In the case of the erythrocyte membrane for example, the majority of the phosphatidylcholine and sphingomyelin is found in the outer layer of the membrane, while the inner (cytoplasmic) layer contains the majority of the phosphatidylserine and phosphatidylethanolamine. The functional importance of this lipid asymmetry is presently under investigation.

Receptor proteins

The ability of proteins having a particular amino acid sequence to fold in such a way as to interact only with a very limited number of other components is one of the central tenets of biochemistry. Thus, over time proteins have evolved that interact with specific molecular species (*ligands*) to do a specific job. These specific ligands include everything from other proteins, peptides and sugars, to inorganic ions. The key point here is the *specificity* of the interaction. Enzymes for example require their *specific* substrates in order to perform their particular function, and the specificity of the antibody/antigen interaction is well known.

Similarly, each of the membrane proteins have their specific ligands. Some of these are cellular components. For instance, the *phospholipid methyltransferase* membrane proteins specifically bind both phosphatidylethanolamine (from the membrane) and the methyl group donor *S*-adenosyl-L-methionine (from the cytoplasm), converting phosphatidylethanolamine to phosphatidylcholine (22). On the other hand, some of these membrane proteins

specifically bind *extracellular* components. The idea that these proteins serve to provide some means of interaction between the cell and the extracellular environment has led to them being referred to as *receptors.*

Fc Receptors

A relatively large number of different cell types possess membrane proteins which specifically bind extracellular immunoglobulin molecules (reviewed in 23, 24, 25, 26, 27). This binding can generally be inhibited by Fc fragments of the immunoglobulin, implying that this is the part of the immunoglobulin molecule responsible for the interaction. The proteins are therefore referred to as *Fc receptors* (FcR). This is an operational term (3), and does not imply that the receptors on different cell types are structurally related or that the same site on the immunoglobulin is recognized by all cell types. In fact, clear differences are found in the receptors on different cell types with respect to their molecular masses (range 12,000 to 130,000 daltons, average approximately 45,000 to 65,000 daltons (24, 28)), binding constants (range 10^4 to 10^{10} M^{-1} (29, 30)), relative affinities for monomeric and aggregated immunoglobulin, antigenicity of the receptor as probed with anti(FcR) antibodies, species and subclass specificity of immunoglobulin binding, and the sensitivity of the receptors to proteases (31). Moreover, several biochemically distinct types of FcR can even exist *on the same cell.*

None the less, this does not preclude the possibility that the various FcR are structurally *related.* A structural similarity of different Fc receptors has been suggested by some on the

basis of their binding the same type of immunoglobulin (32). However, since much of the work to be described in the following chapters is concerned with such ligand cross reactivity, further discussion of the conclusions that can be reached on the basis of these studies will be delayed until a later chapter. Suffice it to say at this point that despite the *suggestion* of relatedness based on cross reactivity, the ultimate assessment of FcR similarity will depend on the amino acid sequence of the FcR proteins. Fortunately, these sequences are quickly becoming available as various groups succeed in cloning the genes for various Fc receptors (33, 34, 35, 36, 37, 38, 39, 40, 41).

The apparent heterogeneity of the FcR proteins is enhanced somewhat by disagreement in the results of different groups supposedly studying the *same* receptor protein. Actually, given the variety of different methods used in Fc receptor studies, disagreement between different groups could almost be anticipated. Moreover, collaborative studies have shown that the FcR on cells of the same line, maintained in different laboratories (sub-lines), have somewhat different characteristics, even when studied using exactly the same methods (42).

The *biological function* of the Fc receptors appears to depend on the type of cell on which they are expressed (23, 24). There is much evidence that FcR are involved in the recognition and specific lysis of antibody coated target cells by K cells, the effector cells of antibody-dependent cell-mediated cytotoxicity (ADCC). Fc receptors have also been implicated in the regulation of antibody production by B cells, and they play an important role in the placental transport of antibodies to the fetus by fetal yolk sac membranes and

placental cells. On mast cells and basophils, FcR binding of immunoglobulin enables the otherwise 'blind' cells to recognize the antigens that the antibodies were produced in response to. As will be discussed below, it is this antibody/FcR mediated recognition that subsequently initiates the release of the mediators of immediate hypersensitivity contained in these cells. Precisely *how* the antibody/FcR interaction induces these various biological effects remains to be determined.

It is quite reasonable to expect that Fc receptors having different functions might also have different structures and induce different biochemical reactions. Conversely, it is possible that Fc receptors involved in similar functions on different cell types may have similar structures and be involved in similar biochemical pathways. Thus it might be possible to group FcR into several 'classes', each having a particular biological function. On the other hand, it is not inconceivable that all FcR induce the same biochemical signals, which might have different effects on the various types of differentiated cells. If this were the case however, it would be difficult to understand why a single cell would carry more than one type of Fc receptor.

Fc receptors for IgE on mast cells and basophils

Basophils and mast cells both contain prominent cytoplasmic secretory granules that stain with certain basic dyes. This characteristic staining of the granules is due to the storage of large amounts of acidic proteoglycans, such as heparin in the mast cells and chondroitin

sulfate in basophils. However, the cells also produce and store a variety of biogenic amines, neutral proteases, acid hydrolases, oxidative enzymes and chemotactic peptides (43). The biogenic amines, such as histamine in human mast cells and histamine and serotonin in rodent mast cells, are the dominant components on a molar basis in the granules of these cells. Basophils and mast cells therefore represent a major source of chemical mediators involved in a variety of inflammatory and immunologic processes and disorders, and they play a particularly important role in diseases of immediate hypersensitivity (allergy).

In all mammalian species yet studied, both mast cells and basophils have been found to express Fc receptors that are specific for IgE (termed $Fc_{\epsilon}R$), and bind antibodies of this class with high affinity. This IgE binding results in the 'sensitization' of the cells, and subsequent exposure of these sensitized cells to a multivalent antigen specifically recognized by the cell bound IgE, triggers the cells to release the contents of their granules into the surroundings. Such triggering also induces the secretion of newly generated mediators such as prostaglandin D_2, platelet activating factor and leukotrienes (discussed below). Both the preformed and newly generated components released by the activated cells are potent vasoactive and chemotactic compounds, the release of which induces the typical symptoms of the allergic reaction: wheal and erythema reactions in the skin for example, rhinitis and/or asthma in the respiratory system.

A similar reaction can be initiated by antibodies directed either against the Fc receptor bound IgE or against the receptor itself, suggesting that it is the crosslinking of the cell

surface Fc receptors that produces the response (44). This exocytotic degranulation can also be induced in response to a variety of immunologically *nonspecific* agents, including certain components of the activated complement system, lymphokines, several naturally occurring and synthetic basic peptides, lectins, and some neurotransmitters, although sensitivity to many of these stimuli varies considerably according to species and cell type (45).

Recently, evidence has been presented implicating mast cells and basophils in certain cell mediated immune responses, and it is thought that mast cell mediators might also affect the function of T lymphocytes and other cells involved in the immune response (45).

Despite their many similarities, mast cells and basophils also display many differences. Basophils differentiate and mature in the bone marrow, circulate in the blood, and are not normally found in the connective tissues. Mast cells on the other hand, are ordinarily distributed throughout normal connective tissues (45). They are often situated adjacent to blood and lymphatic vessels, near or within nerves, and beneath epithelial surfaces which are exposed to environmental antigens, such as those of the respiratory and gastointestinal systems and the skin. In some species, such as the rat, mast cells are also abundant in the fibrous capsules of internal organs and in physiological exudates such as the peritoneal fluid. They are also a normal, although minor, component of the bone marrow and lymphoid tissues.

Studies in mice have suggested that mast cells originate predominantly in the bone marrow and circulate to various connective tissue sites (45). Unlike mature basophils however, mature mast cells function as sessile cells, and do not normally circulate in the blood. They mature locally in the connective tissues from the bone marrow derived, mitotically active precursor cells, which contain few cytoplasmic granules. As they mature, their proliferative capacity declines markedly, and they acquire larger numbers of cytoplasmic granules.

In addition to these differences between mast cells and basophils, studies of mast cell populations in rats and mice have established the existence of mast cell *subclasses* (46). Thus, the mast cells of the intestinal mucosa of the rat (mucosal mast cells) appear to be distinct from those of connective tissue sites, such as the skin or peritoneum. Both of these subclasses can be activated with IgE and specific antigen as mentioned above, but the finding that mast cells in different anatomical locations vary in morphology and histochemistry suggests that they might also differ in biochemistry and function. Furthermore, *rodent* mast cell granules appear to be of uniform density, whereas *human* mast cell granules display a unique crystalline appearance (43). This suggests species differences in the composition of the granules, and possibly in the species' response to mast cell activation.

Mast cells and basophils are normally sparsely distributed in the tissues or blood respectively, and it has been difficult to obtain them in sufficient numbers and purity for definitive studies. Although modest numbers of mast cells (1-2 x 10^6/animal) could be

obtained in rather high purity (90%$^+$) from the peritoneal exudates of rats (47), the situation was vastly improved in 1973 with the development of a basophilic leukemia in rats treated with beta-chloroethylamine (48). These rat basophilic leukemia (RBL) cells were subsequently adapted to *in vitro* culture, providing a homogeneous population of cells in sufficient numbers for biochemical studies. Unfortunately, although these cells contained the cytoplasmic granules characteristic of mast cells and basophils, they had a defect in the IgE/Fc receptor mediated release mechanism, and they failed to secrete histamine (49). However, Siraganian's group has since isolated a variety of functionally diverse clones of the RBL cell line, and has managed to obtain a histamine releasing line, called RBL-IV HR+ (50). Their work has further suggested that, in the non-releasing cells, the defect is not in the $Fc_\epsilon R$ (50), but rather may be the result of a phospholipid methyltransferase deficiency (51).

Although the cells of the original chemically induced leukemia were described as basophil-like, recent studies have suggested that the RBL cell line may also be closely related to the rat mucosal mast cell subset (46). This would therefore tend to support the use of the RBL cell as a model system in the study of certain types of allergic disease involving the mucosa (rhinitis and asthma for instance), provided that mucosal mast cells are similar in different anatomical locations. As mentioned above however, connective tissue mast cells, such as those of the peritoneum, are a distinct subset. Since much work has also been done using these peritoneal mast cells as a model system, the *potential* functional differences between them and the RBL cell should be borne in mind when comparing the two systems.

Care should also be exercised in comparisons across species barriers, due to the potential functional differences in analogous cell types in different species also mentioned above. None the less, because the RBL cell lines are capable of providing cells in sufficient numbers and purity for biochemical studies, and because of the availability of large quantities of homogeneous IgE from a rat immunocytoma (11), most of the information available on the molecular nature of the IgE/mast cell interaction has been obtained from the rat system.

The work of Mendoza and Metzger (52) has indicated that the Fc receptor for IgE on rat peritoneal mast cells (RPMC) and RBL cells is univalent. This conclusion was based on their finding that two distinguishably labelled rat IgE's, bound to the same RBL cells, failed to comigrate when one of the labelled IgE's was induced to redistribute itself on the cell surface by interaction with an antibody specific for only one of the labelled IgE's. Concurrent laser fluorescence photobleaching recovery (FPR) studies of Schlessinger and coworkers (53) tended to support the concept of univalence. These studies were somewhat similar to those of Mendoza and Metzger in their use of two distinguishably labelled, fluorescent IgE's. Reacting one of these IgE's with an antibody was found to severely inhibit the lateral mobility of that particular IgE/receptor population without affecting the mobility of the other receptor bound IgE.

Studies of the maximum IgE binding capacity of the cells have indicated that the RPMC carry about 2-5 x 10^5 receptor molecules per cell, whereas the range for both RBL cells and human basophils is considerably higher, at 0.3-1 x 10^6 per cell (54).

Kulczycki and Metzger have shown that the interaction of IgE and the Fc receptor is reversible (55), and that the binding reaction is a simple bimolecular interaction characterized by the straight forward relationship:

(1) $$\text{IgE + receptor} \underset{\leftarrow k_{-1}}{\overset{k_1 \rightarrow}{\rightleftharpoons}} \text{Receptor-IgE complex}$$

where k_1 and k_{-1} are the rate constants for the forward and reverse reactions respectively. The equilibrium constant, representative of the [complex]/[reactants] ratio at equilibrium, is related to the rate constants by the equation:

(2) $$K_{eq} = k_1/k_{-1}\,.$$

Measurement of the rates of IgE/receptor association and dissociation have indicated values for k_1 of about 10^5 $M^{-1}sec^{-1}$ on intact cells, but about 30 fold greater for cell free receptor preparations (56). While the reason for this difference is presently uncertain, it has been suggested that other glycoproteins and glycolipids on the intact cell surface might limit the access of the ligand to the receptor (56). Alternatively, energy transfer studies of Holowka and Baird (57) have suggested that binding of IgE to the membrane bound Fc receptor

might involve *bending* of the IgE molecule in the C_H2 region. Such bending may not be necessary in the interaction of IgE with cell free receptors, leading to a faster ligand/receptor association and a greater forward rate constant.

While the forward rate constant is not unusually large, the *dissociation* of IgE/receptor complexes is quite slow, with values for k_{-1} only around 10^{-5} (55). The equilibrium constant for the interaction is therefore about $10^{10}\,M^{-1}$, according to equation (2). Equilibrium constants for other immunoglobulin/Fc receptor interactions typically range around 10^4 to $10^8\,M^{-1}$ (30), so, with a K_{eq} two orders of magnitude above the top of this range, the affinity of the IgE/Fc receptor interaction is unusually high by comparison.

The high affinity of the interaction is therefore characteristic of the IgE/$Fc_\epsilon R$ interaction, and this fact has been used to advantage in experimental studies. For instance, the IgE antibodies in serum samples, passively transferred to an animal by subcutaneous injecton, will bind to the $Fc_\epsilon R$ on mast cells at the site of injection. As a result of the high affinity of this interaction, the IgE remains bound to the cells for an extended period of time, whereas the other serum components diffuse out of the area relatively quickly. Subsequent exposure of these sensitized cells to specific antigen, by intravenous injection, results in mast cell degranulation at the site of IgE injection, producing a wheal and erythema. The extent of the reaction, referred to as *passive cutaneous anaphylaxis* (PCA), is proportional to the IgE concentration of the sensitizing injection, and can therefore be used to determine antigen specific IgE concentrations in serum. These concentrations are normally too low

to be measured by any other technique except, more recently, radioimmunoassay (RIA). The affinity of the IgE/receptor interaction is important in that the antigen challenge is usually administered one week after the sensitizing injection. The characteristic persistence of IgE sensitization, even after this delay, allows IgE mediated reactions to be distinguished from those mediated by other immunoglobulin classes and serum components.

Biochemical characterization of the RBL cell IgE Fc receptor

The high affinity of the IgE/$Fc_\epsilon R$ interaction has also been an advantage in the isolation and characterization of the receptor, allowing the specific recovery of relatively large percentages of the total receptor protein from preparations containing a large number of other components. The enhanced expression of $Fc_\epsilon R$ on RBL cells compared to RPMC has also helped the situation, but the isolation and characterization techniques have still relied heavily on radioactive labelling for detection of the receptor.

Labelling

Basically, two approaches have been taken in the labelling of the Fc receptors. The first strategy has involved the enzyme catalyzed labelling of the molecules expressed on the cell surface. For example, in the work to be described in subsequent chapters, the tyrosine residues on the cell surface proteins have been labelled with ^{125}I or ^{131}I using a lactoperoxidase catalyzed coupling reaction. Similarly, the carbohydrate residues on surface components can be labelled using a galactose oxidase catalyzed transfer of ^{3}H from [^{3}H]-$NaBH_4$ (58).

The other tactic that has been employed is to label the receptor by biosynthetic incorporation of [^{3}H]-amino acids, [^{35}S]-methionine, or [^{3}H]- or [^{14}C]-sugars into the nascent protein or its carbohydrate components (59, 60, 61, 62, 63). These incorporation studies have been helpful in providing some indication of the receptor's amino acid and sugar composition. They also have the advantage of labelling parts of the receptor that may not be exposed on the cell surface, and which therefore may not be accessible to surface labelling techniques. Unfortunately, *all* nascent proteins and carbohydrates in the cell are labelled by these methods, and this makes the specific detection of receptor proteins more difficult.

Another method of labelling unexposed parts of the receptor has been through the use of photolysable hydrophobic reagents, such as 5-iodonaphthyl-1-azide (INA), which preferentially label only those components buried in the hydrophobic interior of the membrane (64). This has the advantage of labelling considerably fewer cellular components than biosynthetic incorporation, making receptor detection somewhat easier. On the other hand, not all intramembranous protein segments are labelled with this method. An alternative method has been to label the receptor with ^{125}I *after* isolating it. This has been accomplished with reagents such as chloramine T (63) or N-succinimidyl 3-(4-hydroxy, 5-[^{125}I] iodophenyl) proprionate (Bolton-Hunter reagent) (65). The problem with labelling the solubilized receptor is that the detergent used to extract the protein from the membrane may also be labelled, depending on the type used.

Receptor isolation

As discussed earlier, the isolation of membrane proteins invariably involves the dissolution of the cell membrane with detergents or organic solvents. The extraction of a functional Fc_ϵ receptor from the RPMC and RBL cell membranes was first reported by Conrad, Berczi and Froese (66), who used the non-ionic detergent Nonidet P-40 (NP-40) to solubilize the membrane proteins. This remains the most widely used detergent in $Fc_\epsilon R$ studies, although Metzger's group has made extensive use of Triton X-100, obtaining similar results (67). In attempts to reconstitute the isolated receptor into liposomes (68), and to stabilize the interaction of receptor subunits (69), the same group has also tried a rather wide variety of other detergents, such as Triton X-114 (70), Chaps, octylglucoside, sodium cholate, and several Chaps/phospholipid mixtures.

Fortunately, the solubilized receptor retains its IgE binding capacity. In fact, as mentioned above, the affinity of the receptor/IgE interaction *increases* when the receptor is removed from the membrane. Therefore, while a variety of methods have been employed in the isolation of the receptor, they are all essentially variations on the same theme, relying on the interaction of the receptor with its specific ligand, IgE.

Conrad and Froese (71) originally isolated the receptor by precipitating the IgE/$Fc_\epsilon R$ complex with anti(IgE) antisera. Actually, in this particular experiment the ratio of anti(IgE)/IgE was not such as to cause the direct precipitation of the anti(IgE)-IgE/$Fc_\epsilon R$

complex. Rather, an anti(IgG) antiserum, which recognized the IgG-class anti(IgE), was used at a concentration sufficient to precipitate the whole complex. Alternatively however, the anti(IgE) could be used at such a concentration as to directly precipitate the IgE/$Fc_\epsilon R$ complex, or protein A-Sepharose could be used to isolate the soluble anti(IgE)-IgE/$Fc_\epsilon R$ complex (72). Protein A is a cell wall protein obtained from certain strains of Staphylococci. It has a high affinity for IgG class immunoglobulins, such as the anti(IgE) antibody. Coupled to an insoluble matrix such as Sepharose, it has been of great use in the isolation of various IgG's, although its normal biological function in the bacterium remains unknown.

Several forms of affinity chromatography, such as that employing protein A-Sepharose, have been used extensively in the isolation of the receptor. The most straight forward of these methods has simply involved the direct isolation of the solubilized Fc_ϵ receptor through its interaction with IgE-Sepharose. In some cases however, more complicated methods have been developed in an attempt to reduce non-specific binding of other membrane proteins. For instance, our laboratory has used dinitrophenylated (DNP) IgE and anti(DNP)-Sepharose as an alternate method of receptor isolation. In experiments involving the biosynthetic labelling of the receptor, this technique has helped to reduce the high background resulting from the large number of labelled components (see above) (61). Similarly, Metzger's group has used azobenzenearsonate (ARS) derivatized, DNP specific, monoclonal IgE antibodies and either anti(ARS)-Sepharose or trinitrophenylated (TNP)

Sepharose to isolate the Fc_ϵ receptor. TNP is structurally similar to DNP, and the anti(DNP) IgE binds quite well to the TNP-Sepharose.

These indirect isolation procedures, which rely on the relatively low affinity interaction of the DNP or ARS hapten with an anti(hapten) antibody, have an additional advantage in that the bound IgE/Fc_ϵR complex can be eluted from the affinity matrix by simply increasing the concentration of free hapten. Thus, the DNP-anti(DNP) interaction can be disrupted by free 2,4-dinitrophenol (73), while *p*-nitrophenylarsonate or [(*p*-arsonatophenyl)-azo]-tyrosine can be used in the case of ARS-anti(ARS) (63). The advantage of the method lies in the gentleness of these elution procedures. In contrast, elution of the Fc_ϵR directly bound to IgE-Sepharose requires the use of 3 *M* potassium thiocyanate, 0.5 *M* acetic acid, or 6 *M* guanidine hydrochloride. While these conditions are rather severe, approximately 50% of the eluted receptors retain their IgE binding capacity (42). In many cases however, preservation of the receptor's IgE binding function beyond the isolation procedure has not been required. Such preparations can therefore be eluted from the affinity matrix under conditions which completely disrupt the receptor, such as heating at 100°C in the presence of the detergent sodium dodecyl sulfate (SDS), in preparation for electrophoresis on polyacrylamide gels (SDS-PAGE).

Finally with regard to receptor isolation, there have been a number of anti(receptor) antisera produced which have been used to immunoprecipitate the Fc_ϵR (74, 75). The

production and use of these antisera however is complicated by possible interactions between the receptor and the Fc portion of the anti(receptor).

Biochemical characteristics

SDS-PAGE analysis of the RBL or rat mast cell (RMC) components eluted from IgE-Sepharose resolves two broad bands of material (72), which our group refers to as H and R (76) (Figure 1.12). Comparisons of the electrophoretic mobilities of these bands with those of standard proteins of known molecular mass have indicated molecular masses of approximately 55,000 daltons for H and 45,000 daltons for R (72). Collaborative studies have further established that similar components can be isolated from the RBL cell lines used in other laboratories, although the apparent molecular masses and the ratio of H to R vary somewhat from one line to another (77).

A third IgE binding component has also been reported. Having a molecular mass of approximately 71,000 daltons, the molecule has been referred to simply as 71K (61). However, subsequent studies have indicated that the expression of 71K is related to mycoplasmal infection of the RBL cell line (78). The cell line used in the studies which follow was free of mycoplasma contamination however, and this 71K component was not seen in any of this work.

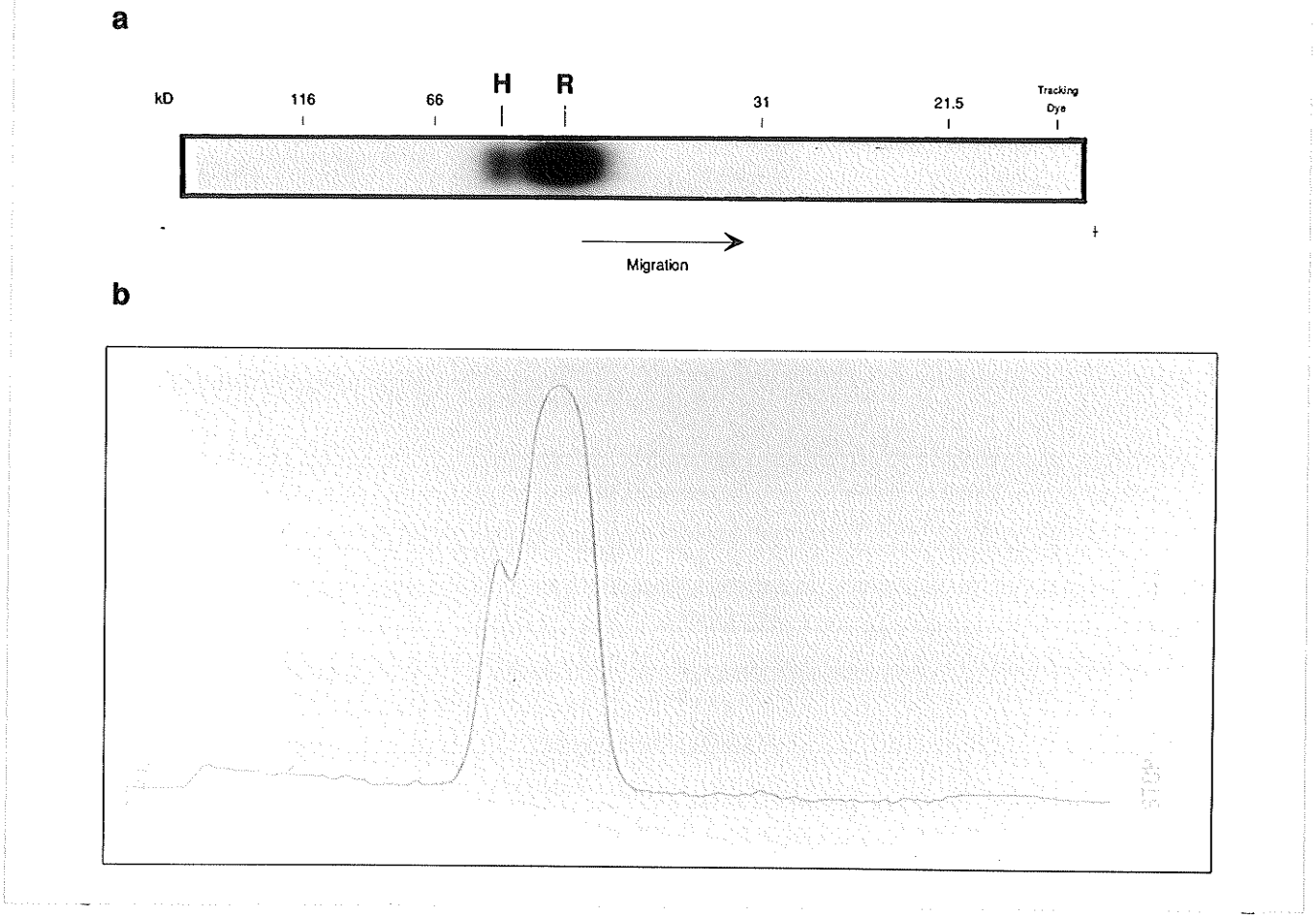


Figure 1.12 SDS-PAGE analysis of RBL cell surface components bound by IgE-Sepharose. RBL cells were surface labelled with ^{125}I and membrane proteins were solubilized with NP-40. IgE-Sepharose was reacted with the solubilized proteins and washed to remove unbound material. Specifically bound components were eluted with sample buffer containing SDS and analyzed by electrophoresis on a polyacrylamide gel, along with proteins of know molecular weight. Following electrophoresis, autoradiography of the dried gel revealed the H and R receptor protein bands (a) and the relative amounts of the two proteins was assessed by scanning densitometry (b). The relative positions and molecular weights of the standard proteins are indicated along the top of panel (a). The top of the gel is on the left in this presentation and the anode is on the right. kD, kilo Daltons.

Interestingly, only the 45,000 dalton R component can be isolated using the indirect methods involving the interaction of the Fc_ϵ receptors with *soluble* IgE, and subsequent isolation of the receptor/IgE complex using anti(IgE) reagents. The reasons for this are not clear at present, although several possible explanations have been put forward. One suggestion is that the 55,000 dalton H component has a much lower affinity for IgE than R has. The IgE concentration in the reaction mixture simply might be too low to provide an adequate number of IgE-H complexes for detection. The results of the present study would tend to support this possibility. Another possibility is that the anti(IgE) antibody might bind to the same site on the IgE molecule as the H component, inhibiting the H/IgE interaction. The other, somewhat similar, suggestion has been that both the H and R components bind to the soluble IgE, but the H/IgE complex dissociates when the IgE reacts with the anti(IgE) antibody (72).

Whatever the explanation, this finding is important in that it strongly suggests that H and R are functionally distinct components, each capable of binding IgE directly. If such were not the case, and one component was co-purifying through some interaction with the IgE binding component, one might argue that the two components should *always* copurify, regardless of IgE concentration. However, there are several other lines of evidence which also suggest that H and R function separately. For instance, H can be preferentially precipitated with an anti(receptor) antiserum (79). The results of the present study also lend further support to the functional autonomy of the two components.

Protease sensitivity (71) and the biosynthetic incorporation of radiolabelled amino acids (59, 61) have confirmed that the Fc_ϵ receptors are in fact *proteins*, and have further established that H and R are quite distinct from one another (80). Furthermore, the biosynthetic incorporation of radiolabelled sugars has indicated that the receptors bear carbohydrate residues, allowing them to be further classified as *glycoproteins* (59). In fact their glycoprotein nature had been anticipated from their behaviour on SDS-PAGE, where they ran as broad bands whose apparent molecular weights varied with the acrylamide concentration of the resolving gel. The breadth of the bands is largely a result of the charge heterogeneity resulting from variations in the glycosylation of each receptor molecule, as has already been mentioned in the discussion of immunoglobulins. If *N*-linked protein glycosylation is inhibited with the antibiotic tunicamycin, the SDS-PAGE bands become much narrower (81). It should also be mentioned that the unglycosylated receptors retain their ability to bind IgE, suggesting that the presence of the *N*-linked oligosaccharide groups is not an absolute requirement for receptor function.

Although amino acid sequence analysis of the R receptor was initially hampered by a blocked NH_2 terminal (82), several laboratories have attempted to circumvent this problem by obtaining sequence data on proteolytic *fragments* of the receptors. It was on the basis of such partial sequence data that Kinet *et al.* were able to prepare a series of oligonucleotide probes that were used to isolate cDNA clones (DNA complementary to receptor messenger RNA transcripts) encoding the receptor protein (83). The nucleotide sequence of the cDNA suggests that the R protein consists of an extracellular portion

containing 180 amino acid residues with two homologous domains of approximately 40 residues each (Figure 1.13 (84)). The protein appears to have a hydrophobic transmembrane segment of approximately 20 residues, and a 27 residue cytoplasmic tail. Sequence comparisons with other proteins have actually indicated numerous homologies with the immunoglobulins themselves. Many other cell surface proteins, such as the T cell antigen receptor, proteins of the major histocompatibility complex, and *other* Fc receptor proteins have similarly been found to be structurally related to the immunoglobulin proteins. These homologies have suggested that these proteins are all encoded by a family of genes referred to as the *immunoglobulin supergene family* (reviewed in 85). The R protein would appear to be yet another member of this family.

The amino acid sequence of the R protein has indicated the presence of five cysteine residues per molecule (83), which may be involved in disulfide bonding to form two immunoglobulin like domains as indicated in Figure 1.13. However, reduction of disulfide bonds prior to electrophoresis has very little effect on the SDS-PAGE mobilities of either H or R. While this would tend to indicate that the receptors are not made up of subunits held together by disulfide bonds, the possibility still exists that intrachain disulfide bonds might be involved in the protein's structure. In fact, studies by Roth *et al.* (86) have shown that somewhat different proteolytic fragments are produced from reduced and unreduced receptors. This would suggest that reduction *does* alter the protein's conformation somewhat, suggesting the involvement of intrachain disulfide bonds in maintaining the native conformation.

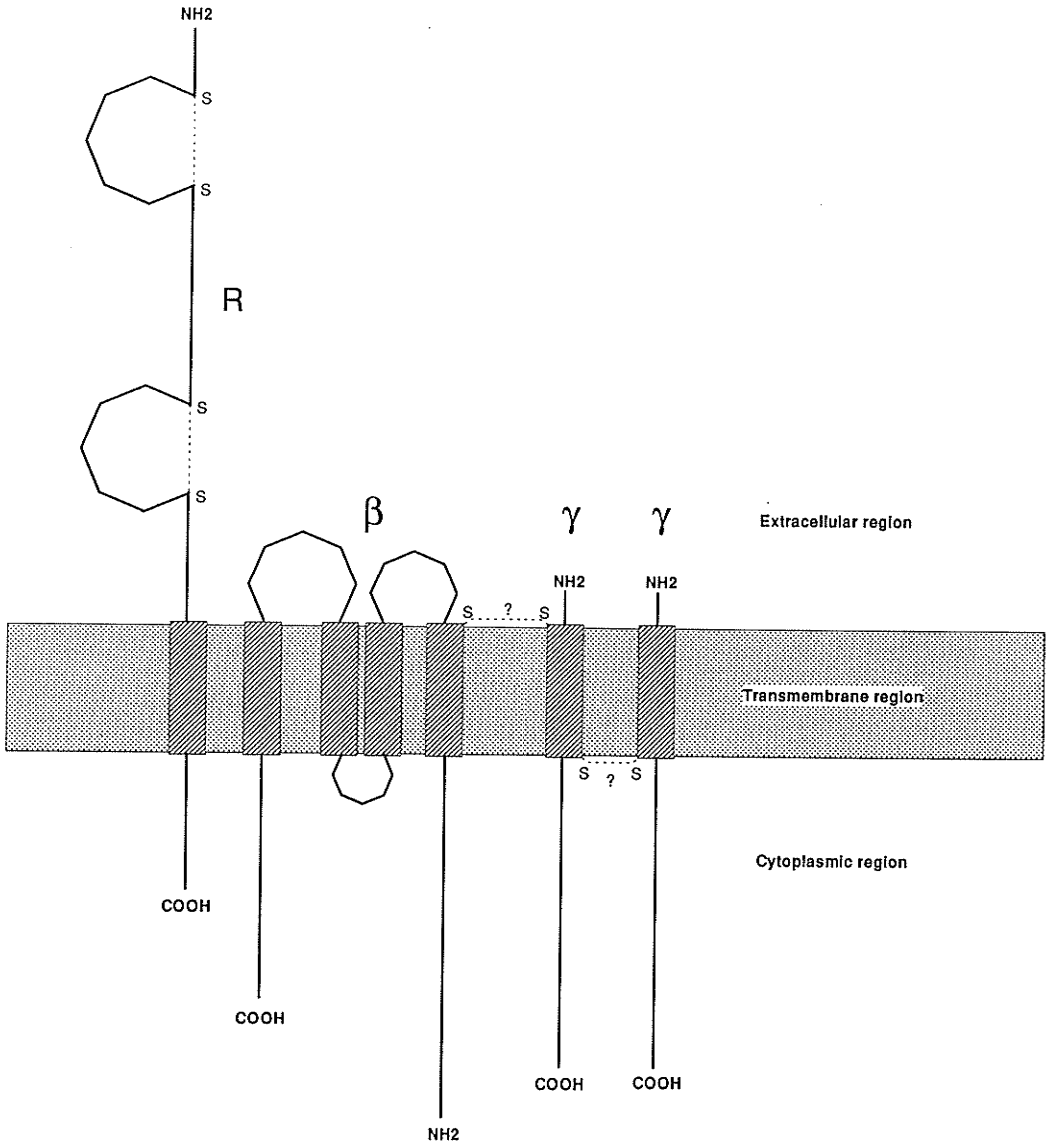


Figure 1.13 Schematic representation of the structure of the rat high affinity IgE Fc receptor complex. The structures of each of the R, beta and gamma components based on cDNA sequence data, although the subunits aren't necessarily drawn to scale. The positions of possible disulfide bonds are indicated by dashed lines. The crosshatched boxes represent transmembrane regions of each protein.

As has already been mentioned, a considerable variety of proteases are stored in the cytoplasmic granules of mast cells and basophils (43). One would expect these to be released into the medium on solubilization of the cells. A variety of protease inhibitors are therefore routinely added to the solubilization buffer in order to afford the solubilized proteins as much protection as possible. Originally however, the solubilized receptors were isolated in the *absence* of protease inhibitors. This would immediately suggest that these proteins are fairly resistant to enzymatic degradation, and in fact this has been the experience of those attempting to prepare proteolytic fragments and peptide maps of the receptors.

Although these receptors are relatively resistant to proteolysis, Rossi, Newman and Metzger (67) have found that the solubilized $Fc_{\epsilon}R$ are quite sensitive to heat. Thus, while the proteins were generally stable at -90°C for periods of several weeks, at 4°C the half life of the receptor decreased to approximately 2 to 6 days. The half life further decreased to 2-5 hours at 25°C, and at 37°C it was only 10-20 minutes. In contrast to the temperature sensitivity of these *free* receptors however, IgE-receptor *complexes* proved to be relatively stable, even at 37°C. For instance, after 1 hour at 37°C or 6 hours at 25°C, there was still no apparent inactivation of IgE bound receptors.

Receptor associated proteins

While the receptors do not appear to be composed of disulfide linked subunits, chemical crosslinking has revealed that two other membrane proteins are closely associated with R

(62, 63, M.-S. Lao, unpublished observations). Metzger's group has suggested that these are in fact receptor subunits, and they refer to them as *beta* and *gamma*. They refer to R as the *alpha* subunit, and in fact the recently established standard nomenclature now refers to R as $Fc_{\epsilon}RI(\alpha)$, but for continuity we will continue to refer to this component as R for the purposes of the thesis.

Only the R component of this complex is modified by lactoperoxidase catalyzed labelling of the cell surface with ^{125}I, suggesting that the beta and gamma components are largely buried in the membrane and are not exposed to any great extent on the cell surface. These associated proteins can however be labelled with hydrophobic reagents such as 5-[^{125}I]iodonaphthyl-1-azide (^{125}I-INA) that preferentially label the intramembranous segments of intrinsic membrane proteins (64). Interestingly, R is *not* labelled by such procedures, suggesting that labelling of its intramembranous region might be prevented by the association of this region with the beta or gamma component. Alternatively, beta and gamma can be labelled by biosynthetic incorporation of radiolabelled amino acids, which originally established that the components are indeed *proteins* (63). However, unlike R, neither of these proteins incorporate radiolabelled sugars, suggesting that they are not glycoproteins. This conclusion is supported somewhat by the narrowness of the SDS-PAGE bands of both of these components in comparison to the broad R band. A lack of carbohydrate would also be consistent with a lack of exposure on the cell surface.

While these receptor associated proteins may not be exposed on the *external* surface of the cell, Holowka and Baird (4) have shown that both beta and gamma can be modified by lactoperoxidase catalyzed iodination of the *cytoplasmic* surface of the membrane. These proteins are therefore exposed on the interior surface of the cell membrane, and may interact with cytoplasmic components in the transduction of signals across the membrane (88).

SDS-PAGE analysis has indicated that the beta protein has a molecular mass of 30,000 daltons, which is unaffected by disulfide bond reduction (89). The gamma component, on the other hand, has a 20,000 dalton molecular mass, but disulfide bond cleavage reduces this to about 10,000 daltons. This suggests that the component is a disulfide linked dimer of two 10,000 dalton polypeptide chains (63).

Based on the work of Perez-Montfort, Kinet and Metzger (63), it appears that R, beta and the gamma dimer exist as a complex in the ratio of 1:1:1. It should be pointed out that, although chemical crosslinking was originally used to stabilize the interaction between R and these associated proteins, the noncovalently bonded complex can be stabilized in the absence of crosslinking by the use of certain detergent/lipid mixtures in the isolation of the receptors (69, 90, 63). Without this stabilization however, the beta and gamma components eventually dissociate from the complex irreversibly, even under the relatively mild conditions normally used. This loss of beta and gamma as a result of their dissociation from R suggests that neither of these components interacts directly with IgE, but rather that the

IgE binding of the complex is a function of the R component. Furthermore, the *simultaneous* loss of both beta and gamma might indicate that they do not interact with R completely independently of one another (91). The recently successful expression of the Fc_ϵ receptor in cells into which cDNA's encoding R, beta and gamma have been transferred (84) suggests that only receptors composed of all four subunits (R, beta, and gamma dimer) reach the plasma membrane. This further underlines the functional interdependencies of the receptor's components. Unfortunately, it is not known whether R interacts with beta or with gamma, or perhaps with both. Beta however was the first of these components to be discovered on the basis of the crosslinking studies. Perhaps this has tended to associate R and beta most closely in people's minds, leading Metzger (91) and Kinet *et al.* (84, 26) to propose the structure represented schematically in Figure 1.13 for the Fc_ϵ receptor complex.

The structures represented in the Figure are based on genetic sequence data. As was alluded to above, cDNA's have recently been isolated encoding both beta (92) and, most recently, gamma (84). The beta protein is perhaps somewhat unusual in that it appears to contain four hydrophobic transmembrane regions and both the NH_2 and COOH terminals appear to be on the cytoplasmic side of the membrane.

The function of these receptor associated proteins remains unclear. There is some evidence for the association of a beta like component with the Fc_ϵ receptor on rat macrophages (93). This raises the possibility that Fc receptors might generally be composed of an

immunoglobulin specific surface component associated with another membrane protein or proteins. These associated components might serve to anchor the immunoglobulin specific subunit in the membrane and/or interact with cytoplasmic components, serving as signal transducing elements. On the other hand, so far there is no evidence that the H protein is associated with similar subunits. It is conceivable however that this might simply be a result of a failure to crosslink H and any associated proteins with the crosslinking reagents employed thus far.

Receptor induced mediator release

Triggering

The interaction of *monomeric* IgE with the Fc_ϵ receptor has no apparent effect on the RBL or mast cell other than to render the cell sensitive to the specific antigen recognized by the IgE molecule. Rather, as mentioned in the discussion of mast cells and basophils, it appears to be the *crosslinking* of the receptors, resulting from the IgE/antigen interaction, that initiates the biochemical events which lead to mediator release. As shown in Figure 1.14(b), such crosslinking can be experimentally induced by preformed, covalently crosslinked IgE aggregates (94). Studies employing well defined preparations, consisting of a known number of IgE molecules per aggregate, have indicated that aggregates as small as a dimer are sufficient to initiate mediator release. Anti(IgE) antibodies have also been used to trigger IgE sensitized cells.(44).

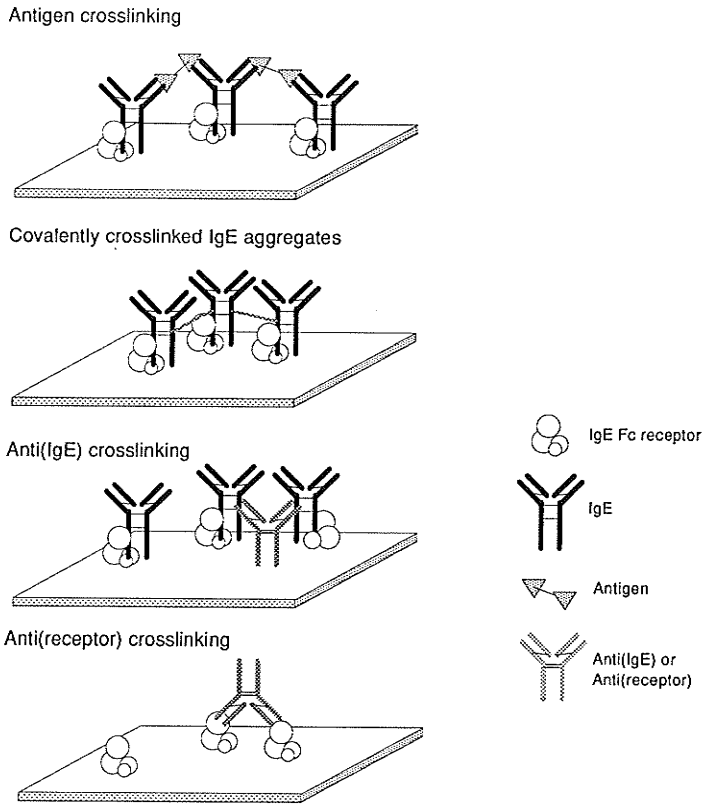


Figure 1.14 Schematic representation of various methods of inducing mast cell mediator release by crosslinking IgE Fc receptors. The top panel illustrates the natural situation in which receptor bound IgE is crosslinked by a multivalent antigen. The lower three panels represent alternative methods for experimentally crosslinking the receptors with: preformed aggregates of chemically crosslinked IgE, anti(IgE) antibodies or anti(receptor) antibodies. Studies have indicated that preformed aggregates of IgE as small as a dimer are capable of triggering mediator release.

Unfortunately, neither of these techniques distinguish between the H and R receptor proteins, since both H and R bind IgE and would be expected to be involved in IgE mediated receptor crosslinking. As an alternative however, the receptors can be *directly* crosslinked with anti(receptor) antibodies, which, in principle, have the ability to bind H or R specifically (95). Such crosslinking also induces mediator release, and it has been through the use of such antibodies that the R protein has been shown to be involved in triggering mediator release (42). The function of the H protein however still remains unclear. The results of the present study have a direct bearing on this question and suggest some possible routes of further investigation.

Biochemical events

The biochemical events induced by Fc_ϵ receptor crosslinking are summarized in Figure 1.15. Briefly, aggregation of the receptors is induced by antigen crosslinking of the bound IgE molecules. This aggregation triggers a transient rise in both phospholipid methylation and cyclic adenosine monophosphate (cAMP) production, typically peaking within 10 to 15 seconds of stimulation (44). These reactions are followed by an influx of Ca^{+2}, perhaps accompanied by its release from intracellular stores, raising the intracellular Ca^{+2} concentration to reach a plateau level in 2 minutes. The release of histamine is accompanied by the release of arachidonic acid, and closely follows the Ca^{+2} influx, reaching maximums within 2-3 minutes (44).

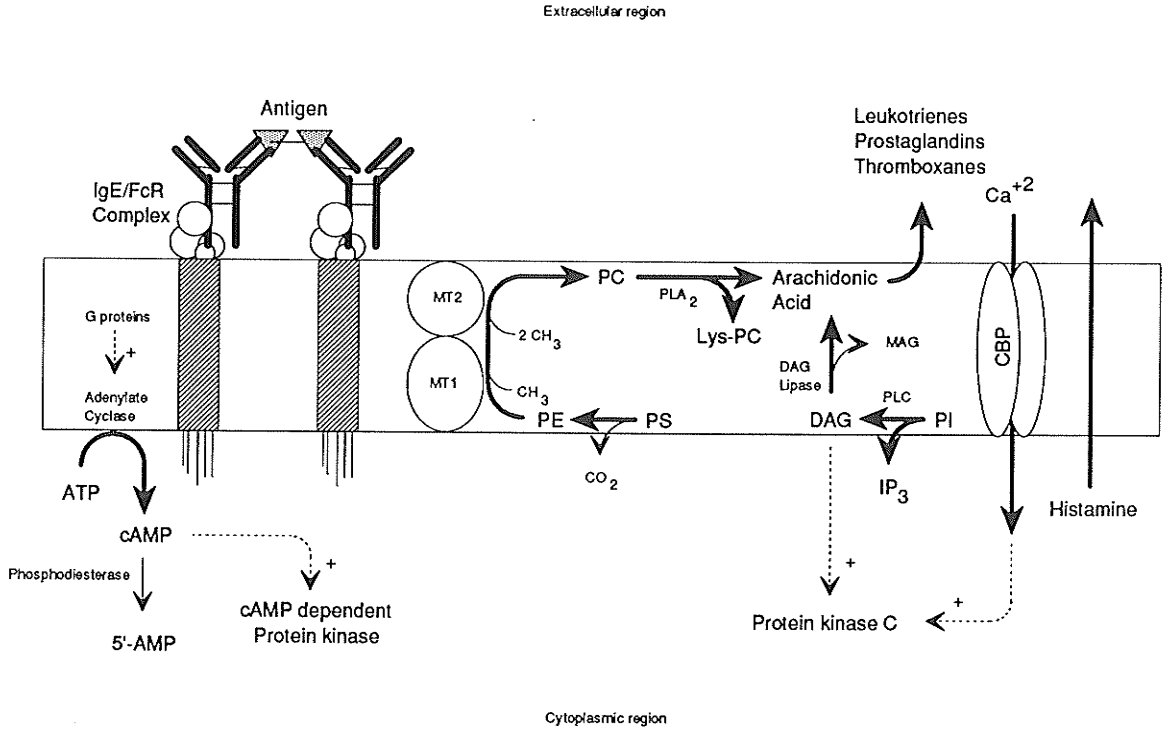


Figure 1.15 Summary of the biochemical events induced by $Fc_{epsilon}$ receptor crosslinking. Aggregation of the IgE/FcR complex is induced by antigen crosslinking of receptor bound IgE molecules. This aggregation triggers transient rises in both phospholipid methylation and cyclic adenosine monophosphate production. These reactions are followed by an influx of Ca^{+2}, apparently via a cromolyn binding protein, which is perhaps accompanied by the release of Ca^{+2} from intracellular stores. Histamine release closely follows the increase in intracellular Ca^{+2} concentration and is accompanied by the release of arachidonic acid through the actions of phospholipase A_2 and phospholipase C/diacylglycerol lipase, leading ultimately to the production of leukotrienes, prostaglandins and thromboxanes. The action of phospholipase C also leads to the production of the intracellular messenger inositol trisphosphate. AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic AMP; CBP, cromolyn binding protein; DAG, diacylglycerol; IP_3, inositol trisphosphate; Lys-PC, lysophosphatidylcholine; MAG, monoacylglycerol; MT1, phospholipid methyltransferase 1; MT2, phospholipid methyltransferase 2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLA_2, phospholipase A2; PLC, phospholipase C; PS, phosphatidylserine.

Considerable gaps remain in our understanding of the biochemistry of mediator release, and the precise mechanisms through which all of these events are connected remain to be determined. For instance, the central question of how receptor aggregation induces the cascade of other reactions remains unanswered. It has been suggested that adjacent receptor molecules may interact to form an active enzymatic complex which activates other membrane associated enzymes. For example, the aggregated receptor complexes may have some proteolytic activity. There is some evidence for the involvement of a protease in the activation reactions, since mediator release can be inhibited by a variety of protease inhibitors. Unfortunately, no enzymatic activity has yet been directly attributed to the Fc_ϵ receptor.

The increase in phospholipid methylation results in the conversion of phosphatidylethanolamine to phosphatidylcholine (see Figures 1.7 and 1.8), in conjunction with its transfer from the cytoplasmic side to the external surface of the cell membrane. This increase in the phosphatidylcholine content of the membrane is associated with an increase in the membrane's fluidity, and may play an important role in the function of various membrane proteins. For instance, membrane fluidity may be an important factor in the coupling of Fc receptors to adenylate cyclase, activating cAMP production.

The role of cAMP remains unclear. As mentioned, the increase in cAMP is only temporary, rising rapidly on stimulation, peaking after about 15 seconds, and returning to normal levels by about 30 seconds (96). On the one hand, this transient rise in cAMP

appears to be required for mediator release. Inhibiting the rise inhibits the release reaction. Augmenting the rise, augments release (97). On the other hand, if the sensitized cell is treated with the anti-asthmatic drug theophylline prior to antigenic challenge, the drug inhibits the phosphodiesterase which normally breaks down cAMP. This results in an accumulation of the cyclic nucleotide produced during normal cell metabolism, and such a cAMP overload *inhibits* mediator release (97). Therefore, while a transient rise in cAMP appears to be required for release, a sustained rise in cAMP prior to challenge seems to be inhibitory. Such is the case at least for *normal mast cells.* Unfortunately, in the RBL cell, no increase in cAMP is detected during the release reaction, casting some doubt on the role of the increase in the release mechanism of normal cells (44).

Cyclic AMP has been implicated in the control of a variety of metabolic processes, such as in the activation of various *cAMP dependent protein kinases* (97). The activities of many proteins depend on their degree of phosphorylation. This phosphorylation is often in turn controlled by a specific protein kinase, which is involved in the transfer of phosphate groups to the kinase's specific substrate protein. A protein's activity can therefore be regulated by the kinases acting in conjunction with specific phosphatases, which catalyze the reverse reaction, dephosphorylating the phosphoproteins. By activating a variety of protein kinases therefore, cAMP can affect a multitude of regulatory systems in the cell. In fact, there is evidence that both the beta and gamma components of the R receptor are phosphoproteins, and that their degree of phosphorylation changes in response to cell activation (98). Similarly, there is evidence that, at least on normal rat peritoneal mast cells,

the R component is also phosphorylated in the release reaction (99). Other studies have indicated that cAMP may act through a cAMP dependent protein kinase to inhibit phospholipid methylation (44). Thus, whereas phospholipid methylation might enhance cAMP production, the cAMP produced might act to reduce phospholipid methylation in a self regulating cycle.

Phospholipid methylation appears to be essential to the subsequent Ca^{+2} influx, since inhibiting methylation inhibits Ca^{+2} uptake (100). However, the Ca^{+2} influx appears to be the critical event in mediator release, since both phospholipid methylation and cAMP production can be experimentally *bypassed* through the use of the Ca^{+2} ionophore A23187 to produce the Ca^{+2} influx (101). Furthermore, in the absence of Ca^{+2}, no release can be achieved despite normal increases in phospholipid methylation (101).

The Ca^{+2} influx appears to be mediated by proteins distinct from the Fc_ϵ receptor (102, 103). These proteins, which aggregate to form the membrane ion channel, bind the anti-asthmatic drug cromolyn, inhibiting the Ca^{+2} influx and mediator release (102). As a result, the protein is simply known as the *cromolyn binding protein* (CBP). Unfortunately, once again, it is not known how Fc receptor crosslinking induces CBP aggregation, opening the Ca^{+2} channel.

Precisely how the Ca^{+2} influx causes mediator release is not known. As with cAMP, Ca^{+2} is involved in the activation of protein kinases, specifically protein kinase C, which have

been implicated in the release reaction (18). Furthermore, in the presence of Ca^{+2}, the membrane phospholipids are broken down by phospholipase A_2 (PLA_2), which cleaves the fatty acid esterified to the number two position of the glycerol backbone, as shown in Figure 1.16 (105). The products of this reaction are a lysophospholipid and a free fatty acid. The lysophospholipid tends to destabilize the membrane, and might facilitate the fusion of the cytoplasmic granules with the cell membrane during the secretory process. With respect to the free fatty acid, a large percentage of the fatty acid in the number two position is arachidonic acid. As indicated in Figure 1.15, this can be further metabolized by the cyclooxygenase and lipoxygenase pathways, leading to the production of prostaglandins, thromboxanes, and the extremely potent leukotrienes (106). This last group, the leukotrienes, were formerly referred to collectively as *slow reacting substance of anaphylaxis* (SRS-A), and they perhaps deserve special attention. Formed via the 5-lipoxygenase pathway, the leukotrienes exhibit a number of biological effects similar to histamine, such as the induction of bronchial smooth muscle contraction, stimulation of vascular permeability, and the ability to attract and activate leukocytes (107). Although they are released in smaller quantities than the preformed mediators contained in the cytoplasmic granules, they are 3 to 4 *orders of magnitude* more potent than histamine and their effects are longer lived (107). They thus may play a very important role in the allergic response.

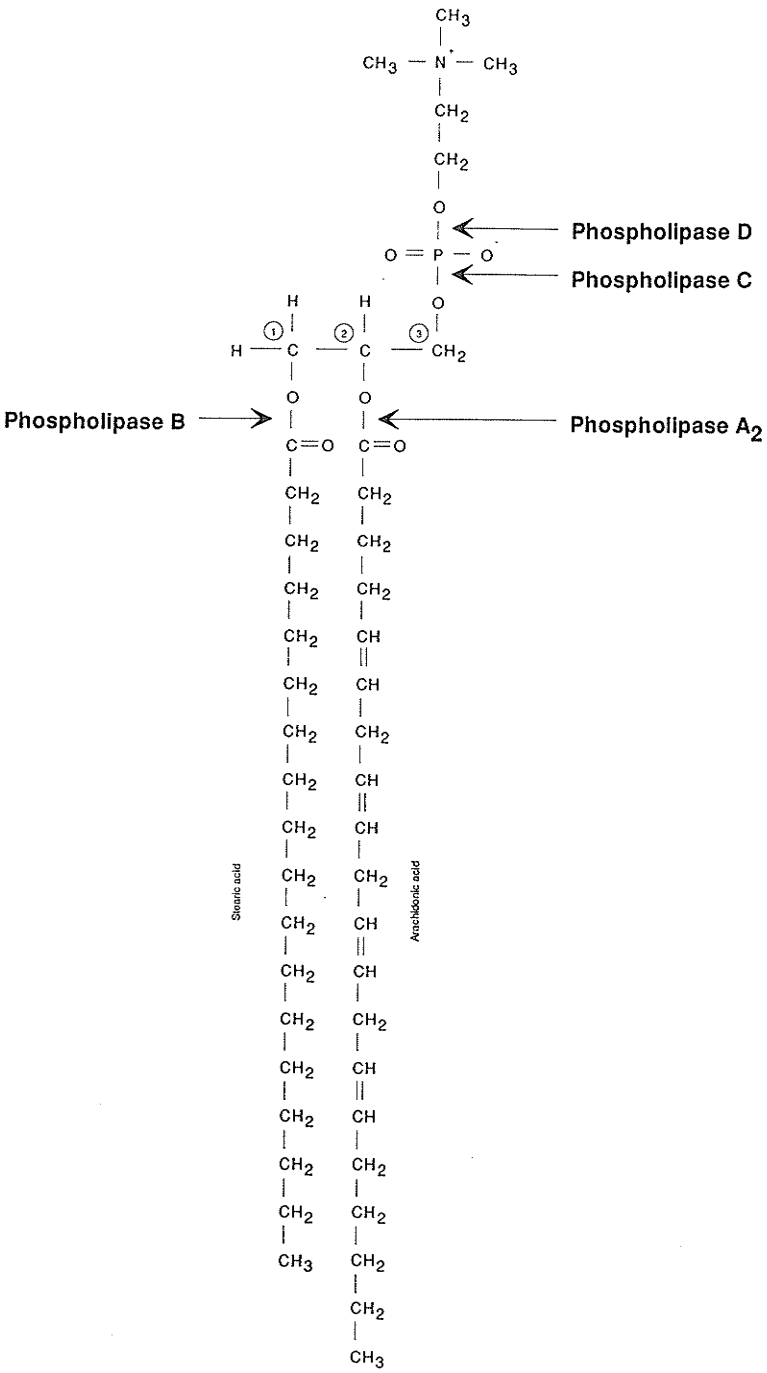


Figure 1.16 Sites of action of various phospholipases. Phosphatidylcholine is used as a model phospholipid, but the phospholipases act at the same sites on other phospholipids.

An additional source of free arachidonate is provided through the activation and sequential action of phospholipase C (PLC) and diacylglycerol lipase (DAG lipase) (108). As shown in Figure 1.16, PLC cleaves the ester linkage between the polar head group of the phospholipid and the glycerol backbone, releasing the phosphorylated head group and diacylglycerol. The release of diacylglycerol (DAG) is important both in that its further degradation by DAG lipase results in the release of arachidonic acid, and in that DAG itself acts as a messenger, stimulating protein kinase C activity. Furthermore, the inositol trisphosphate (IP_3), released through the action of PLC on phosphatidylinositol 4,5-bisphosphate, has been implicated as a second messenger in cellular signal transduction, as mentioned in the initial discussion of lipids (see also Figure 1.8) (17).

The interaction of IgG with rat mast cells and RBL cells

In the early sixties, Rapp reported that the injection of a *rabbit* antibody preparation into the peritoneal cavity of the *rat* appeared to result in the animal's sensitization, such that the subsequent injection of the specific antigen produced an anaphylactic reaction, the rat going into shock (109). This reaction was found to be the result of leukotriene production, with little or no involvement of histamine release from the peritoneal mast cells (109, 110).

This ability to produce a leukotriene mediated anaphylactic response in the absence of histamine release suggested that there might be *two* separate pathways involved in allergic reactions; one involving IgE mediated histamine release, the other involving leukotriene

release induced by an as yet undetermined antibody class (Figure 1.17). Stechschulte, Austen and Bloch were therefore prompted to follow up these initial studies in an attempt to characterize the antibody population responsible for leukotriene release (111).

Their preliminary work indicated that, as with the rabbit antibody preparation, the rat peritoneum could be similarly sensitized with a *rat* antibody preparation. Once again, the subsequent injection of the specific antigen resulted primarily in the production of leukotrienes, with little or no histamine release. Furthermore, an extension of these studies indicated that a passive cutaneous anaphylaxis (PCA) could be obtained by injecting the antibody into the skin, followed by an intravenous challenge with the specific antigen. As with the typical PCA reaction, this resulted in an increased vascular permeability at the site of antibody injection. The reaction was *unusual* however in that the sensitization was short lived relative to that mediated by IgE. Thus, optimal release of leukotrienes was obtained by injecting antigen 1-9 hours after the antibody. As previously mentioned, IgE mediated sensitization is extremely long lived, with an optimal latent period of 24-72 hours. Therefore, the lack of response to antigen challenge 48 hours after antibody injection indicated that IgE was not involved in this reaction. In fact, the physicochemical characteristics of the responsible antibody population indicated that the response was mediated by immunoglobulins of the *IgG class*, or a subfraction thereof.

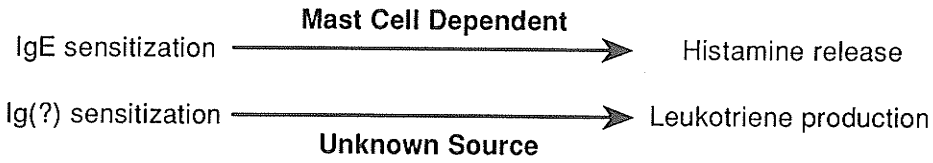


Figure 1.17 Simple hypothesis for the production of mediators of anaphylaxis based on initial studies by Rapp.

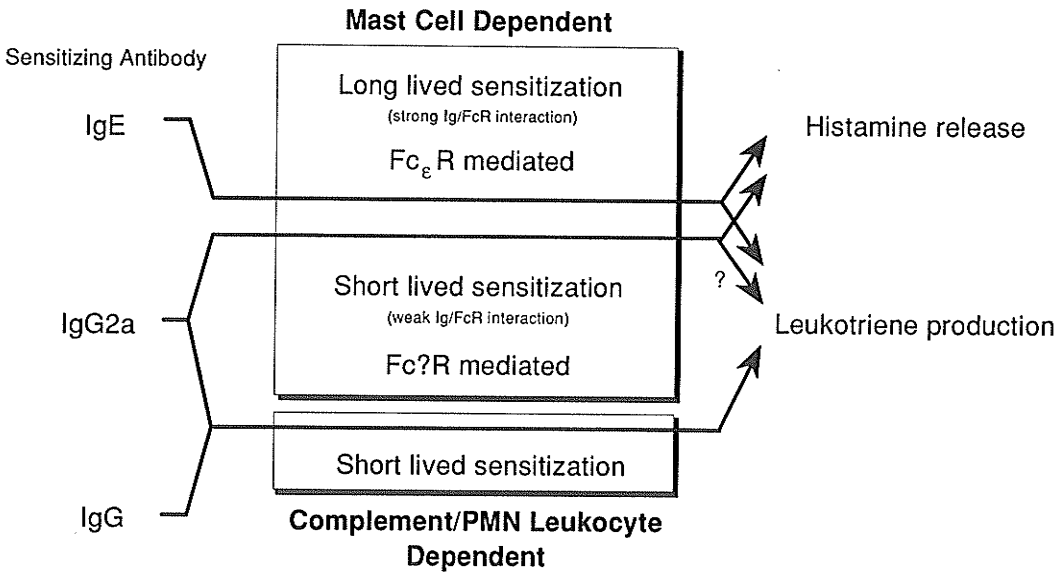


Figure 1.18 Hypothesis for the production of mediators of anaphylaxis based on the work of Stechschulte, Bach, Bloch and Austen.

Subsequent attempts to further characterize the IgG antibodies involved in the reaction led to the isolation of several distinct IgG subfractions by diethylaminoethyl (DEAE) cellulose ion exchange chromatography (112). The first of these fractions to elute from the ion exchange column in 0.005 *M* phosphate at pH 7.5 contained essentially pure IgG_{2a} protein, referred to at that time as IgG_a. Furthermore, this appeared to be the IgG subclass responsible for inducing the leukotriene release (113).

Further studies suggested that the release of leukotrienes was dependent on an intact complement system, appeared to be connected to the presence of polymorphonuclear (PMN) leukocytes, and was unaffected by mast cell depletion (114). Taken together therefore, these results tended to suggest that, rather than simply inducing a different response in the mast cell, IgG_{2a} induced leukotriene release via a *completely different pathway* than IgE induced histamine release. The mechanism appeared to perhaps involve complement activation by antigen-antibody complexes, followed by complement induced leukotriene release involving PMN leukocytes. On the other hand, the inhibition of the reaction by nonspecific antibodies (113) suggested that IgG induced the response through an interaction with something like an Fc receptor.

As mentioned previously, *IgE* is also involved in the induction of leukotriene production. It should be pointed out however that, in contrast to the IgG_{2a} mediated release, the IgE mediated reaction is *independent* of PMN leukocytes, and has *no* requirement for the complement system (115). Rather, this IgE induced release appears to be a direct

consequence of mast cell activation, and is therefore considerably different from IgG_{2a} induced release.

Interestingly, it was found that, whereas *whole* rat antiserum primarily induced the release of leukotrienes in the sensitized rat peritoneum, purified IgG_{2a} induced the release of appreciable quantities of *histamine* as well (114). In contrast to the production of leukotrienes however, this histamine release was quite similar to that induced by IgE, in that it depended on the presence of mast cells, and was independent of the complement system. Furthermore, as in the IgE mediated reaction, the response was inhibited by disodium chromoglycate. This suggested that IgE and IgG_{2a} might share some common biochemical elements in their mechanisms of histamine release.

The question then became one of whether IgE and IgG_{2a} interacted with the *same* cells. This problem was addressed by Bach, Bloch and Austen's *in vitro* studies of isolated peritoneal cells and purified mast cells (116), which indicated that the IgG_{2a} and IgE target cells were indeed the same (117). In fact, a single cell appeared to be capable of binding both immunoglobulin classes simultaneously. Thus, cells sensitized with IgE could be depleted of histamine by IgG_{2a} induced release, leading to a decreased response on challenge with the antigen specifically recognized by the IgE. Moreover, soluble (supposedly monomeric) IgG_{2a} was found to inhibit the sensitization of peritoneal cells with IgE, suggesting that the IgG_{2a} might actually interact with the *Fc_ϵ receptor itself.* The IgG_{2a}/mast cell interaction appeared to be rather weak in comparison with the IgE

interaction however, and sensitizing IgG_{2a} antibody could be removed by simply washing the cells. Figure 1.18 provides a summary of the above information, which shows that the mechanisms involved are considerably more complex than originally anticipated.

The establishment of the rat basophilic leukemia (RBL) cell as a mast cell model, and the availability of homogeneous IgG_{2a} preparations from various immunocytomas provided a considerably more well defined system, and prompted Halper and Metzger to re-examine the IgG_{2a}/mast cell interaction (118). Initial studies failed to demonstrate direct binding of IgG_{2a} monomers by the RBL cells. However, the binding assay involved a washing step which, based on the work discussed above, may have resulted in the removal of any weakly bound immunoglobulin. The IgE/Fc receptor interaction *was* inhibited by IgG_{2a} *immune complexes*, but, contrary to Bach's findings, *monomeric* IgG_{2a} failed to inhibit IgE binding.

There are several possible explanations for this discrepancy. For instance, Bach was unable to determine the relative concentrations of IgG_{2a} and IgE in his antibody preparations. The appropriate assays simply were not available at the time. It is possible therefore that the ratio of IgG_{2a}/IgE which inhibited IgE binding was higher than the range explored by Halper and Metzger. On the other hand, neither contaminating IgE nor IgG aggregates were rigorously excluded in Bach's study, and these, rather than monomeric IgG_{2a}, may have been responsible for the observed inhibition of IgE binding.

Although IgG_{2a} immune complexes inhibited IgE binding, Halper and Metzger could not rule out the possibility that this inhibition was due to steric hinderance of the IgE/Fc_ϵ receptor interaction by IgG_{2a} complexes bound to some *other* receptor protein. They were therefore unable to come to any final conclusion as to whether or not IgG_{2a} and IgE interacted with the RBL cell through the same receptor, as had been suggested by Bach and coworkers.

Subsequent studies by Conrad and Froese found that soluble rat IgG apparently failed to inhibit the binding of detergent solubilized Fc_ϵ receptors to IgE-Sepharose, as shown in Figure 1.19 (72). In comparison, soluble *IgE* inhibited the interaction quite strongly. It should be pointed out that these results were based simply on total ^{125}I labelled RBL proteins bound by the IgE-Sepharose. There was unfortunately no distinction made between the effects of the soluble immunoglobulins on the binding of the H and R Fc receptors. None the less, these results would tend to argue against any interaction between the *solubilized* Fc_ϵ receptors and *soluble* IgG. In other studies however, there were indications that *IgG-Sepharose* was capable of binding at least some of the solubilized RBL cell membrane proteins (Conrad, unpublished observations). These last results therefore prompted a closer look at the interaction of IgG with these solubilized membrane proteins of the RBL cell, in the hope of further characterizing these proteins and determining their relationship (if any) to the Fc_ϵ receptors. The results of these studies are contained in the work which follows.

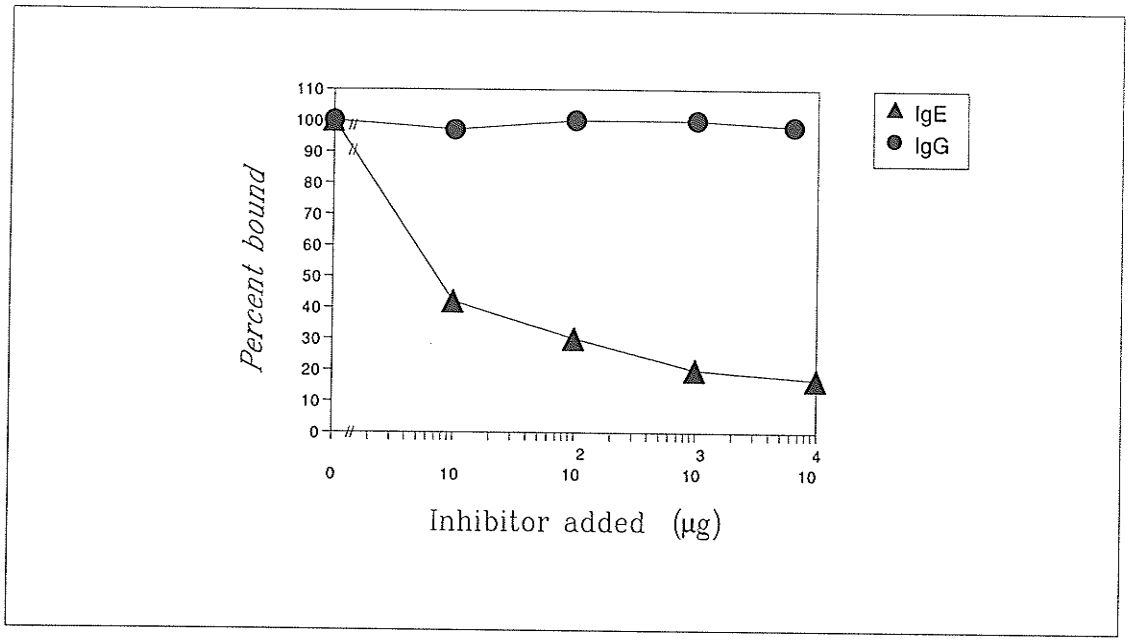


Figure 1.19 Inhibition of the interaction between IgE-Sepharose and RBL membrane components by soluble IgE. RBL cell surface components were labelled with ^{125}I, solubilized with NP-40 and reacted with IgE-Sepharose in the presence of various concentrations of soluble rat IgG or IgE. Whereas the presence of IgE significantly inhibited the binding of the labelled membrane components, IgG failed to produce any detectable inhibition.

Chapter 2

The crossreactivity of the rat basophilic leukemia cell IgE Fc receptors with rat IgG

Introduction

As mentioned in the previous chapter studies by Conrad and Froese indicated that soluble rat IgG failed to inhibit the binding of soluble Fc_{ϵ} receptors to IgE-Sepharose (72). Soluble *IgE* on the other hand inhibited this interaction quite strongly. These results would tend to suggest that the soluble Fc_{ϵ} receptors do not interact with IgG to any appreciable extent. However, in subsequent attempts to use IgG-Sepharose as a non-binding experimental control it was found that the protein-Sepharose conjugate actually retained a substantial amount of radiolabelled material when mixed with membrane extracts of surface labelled RBL cells. This suggested that *some* RBL membrane components interacted with the insolubilized IgG, raising the possibility that the cell line might also express an Fc_{gamma} receptor.

Based on these preliminary findings, the work described in this chapter attempted to first confirm, and then extend these results. The work addresses itself to the specificity and physicochemical characteristics of the membrane components involved in the interaction, and to the relationship of these IgG binding components to the H and R Fc_ϵ receptors.

Materials and methods

Buffers

Acetate Buffer, 0.071 *M* acetate, pH 5.0

Acetate/NaCl, 0.1 *M* acetic acid/sodium acetate, 0.5 *M* NaCl, pH 4.0

Acid Fuchsin, 0.2% acid fuchsin, 50% methanol, 10% acetic acid

Barbital Buffer (2x),0.051 *M* sodium barbital, 0.009 *M* barbituric acid, 0.005 *M* calcium lactate, 0.02% NaN_3, pH 8.6

BBS, 6.25 mM borate, 0.85% NaCl, pH 8.8

BBS/NP-40 contains 0.5% Nonidet P-40 (Calbiochem, San Diego, CA), pH 8.8

BSS/EDTA, Earle's balanced salt solution (-Mg,-Ca) containing 0.005 *M* Na_2EDTA

Coupling Buffer, 0.1 *M* CO_3^{2-}, 0.5 *M* NaCl, pH 9.7

Immunoelectrophoresis Electrode Buffer, 0.0425 *M* sodium barbital, 0.0075 *M* barbituric acid, 0.0012 *M* calcium lactate, pH 8.6

PBS, 0.01 *M* PO_4^{3-} (potassium salt), 0.14 *M* NaCl, pH 7.4

PBS/BSA contains 0.05% BSA (ICN Nutritional Biochemicals, Cleveland, Ohio)

Quenching Buffer, 0.1 *M* CO_3^{2-}, 0.5 *M* NaCl, 0.2 *M* Glycine, pH 9.7

SDS-PAGE Electrode Buffer, 0.05 *M* Tris, 0.384 *M* glycine, 0.1% SDS

SDS-PAGE Sample Buffer, 0.059 *M* Tris-PO_4, 20% glycerol, 2% SDS, 0.2% 2-mercaptoethanol, 0.005% bromphenol blue, pH 7.0

SDS-PAGE Stain, 0.04% Coomassie Brilliant Blue R-250 (Eastman Kodak, Rochester, NY), 27% 2-propanol, 10% acetic acid

SDS-PAGE Destaining Solution, 12% 2-propanol, 7% acetic acid

Tris Buffer, 0.2 *M* Tris-HCl, 0.15 *M* NaCl, 0.05% NaN_3, pH 8.0

Rat Immunoglobulins

Rat IgE was obtained from the ascitic fluid of Lou/M/Wsl rats (Animal Care Facility, University of Manitoba) bearing the IR-162 immunocytoma (11), and was purified as previously described (119), with the exception that Ultrogel AcA34 (LKB Produktor, Bromma, Sweden) was used instead of Bio-Gel P-300. The protein was prepared by Ms. K.D. McNeill, and was routinely available in the laboratory.

Immunoglobulins from the serum of normal Wistar-Lewis rats (Biobreeding Laboratories, Ottawa, Canada) were precipitated with stirring at 4° C by the dropwise addition of an equal volume of saturated $(NH_4)_2SO_4$. The precipitate was sedimented by centrifugation at 12,000 rpm for 30 minutes at 4° C in the SS34 rotor of a Sorval RC2B centrifuge (Ivan

Sorval, Norwalk, CT). The supernatant was discarded and the precipitated protein was dissolved in a minimum amount of distilled water. This preparation was dialyzed 24 hrs. against 30 volumes distilled water to remove $(NH_4)_2SO_4$. Lipoproteins were depleted by dialysis against acetate buffer, distilled water, acetate buffer (24 hrs vs. 30 volumes each). The precipitated lipoprotein was removed by centrifugation as above, and the supernatant was dialyzed against Tris buffer. This preparation was separated in Tris buffer at 4° C on a 3.6 x 175 cm column of Ultrogel AcA34, in lots containing approximately 900 mg. total protein at a concentration of approximately 100 mg protein/ml. The flow rate for the separation was 2.95 cm/hr (30 ml/hr) and the fraction volumes were 8.75 ml (17.5 minutes/fraction). The percent transmission of the column effluent was monitored at 280 nm with a Uvicord II detector (LKB Produktor, Bromma, Sweden). A typical elution profile for a normal rat immunoglobulin preparation is presented in Figure 2.1.

The components of each peak from the gel filtration were determined by immunoelectrophoresis in 1% agar (see below), using a multispecific anti(rat) antiserum for development. Those fractions in the peak containing predominantly IgG were pooled, concentrated by reduced pressure ultrafiltration, and dialysed against Tris buffer. An SDS-PAGE analysis of this crude IgG preparation is presented in Figure 2.2.

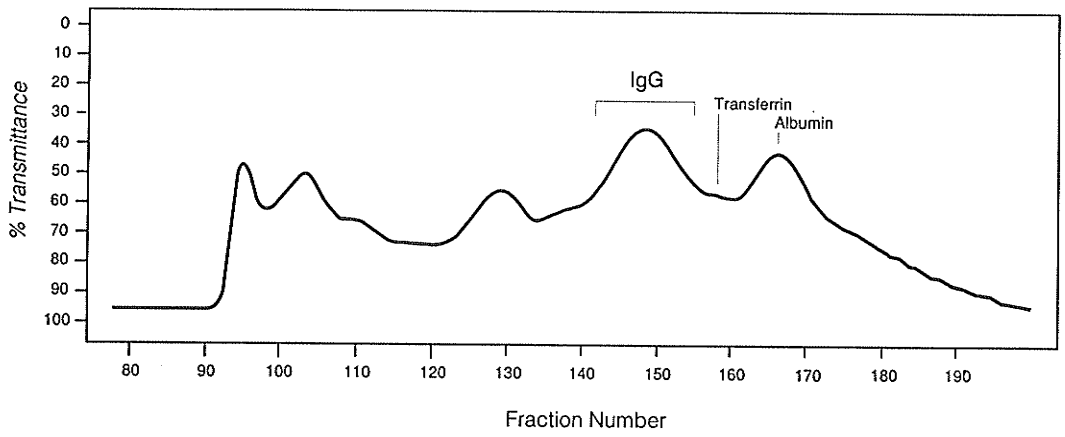


Figure 2.1 Gel filtration elution profile of normal rat serum proteins. The serum proteins were precipitated at 50% of saturation with $(NH_4)_2SO_4$, lipoprotein depleted and separated on a 1.75 m column of Ultrogel AcA34.

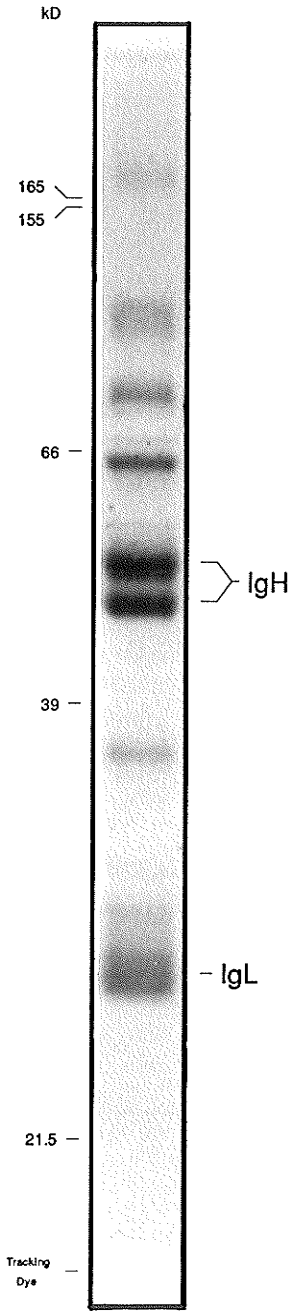


Figure 2.2 Analysis of a normal rat IgG preparation by SDS polyacrylamide gel electrophoresis under reducing conditions. The positions and molecular weights of various standard proteins are indicated. IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; kD, kilo Daltons.

Contaminating IgE was removed from the IgG by absorption with an excess of horse anti(rat IgE) coupled to Sepharose CL-4B. The horse antibody was kindly provided by Dr. K.A. Kelly, this department. Radioimmunoassay (120) indicated that the absorption had reduced the IgE concentration from approximately 700 ng/30 mg protein to below the limit of detectability (30 ng), suggesting a maximum residual IgE contamination of less than 30 ng IgE/30 mg protein, or less than 0.0001%.

$F(ab')_2$ fragments of rat IgG were the kind gift of Dr. K.A. Kelly. These were prepared by pepsin digestion of normal rat IgG, followed by ion exchange chromatography on DEAE-cellulose.

It should be mentioned at this point that methods of IgG purification based on charge (such as ion exchange chromatography) were intentionally avoided because of the possibility of preferentially isolating a particular IgG subclass. The hope was to retain all subclasses in the IgG preparation.

Other proteins

BSA (99% pure) was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio). Carbonic anhydrase B, fibrinogen, and hemoglobin were obtained from Sigma Chemical Co. (St. Louis, Missouri).

Removal of aggregates

Due to the tendency of IgG to undergo spontaneous aggregation, *all* protein solutions were deaggregated immediately prior to use by ultracentrifugation at 90,000 x g for 1.5 hours at 5° C. The top 2/3 of the supernatant was carefully removed for use.

IgE Radioimmunoassay

The radioimmunoassay of total rat IgE was performed according to a method developed by Dr. K.A. Kelly (this department) and has been described previously (120). Very briefly, the assay is a sandwich solid-phase RIA. Purified IR2 IgE is coupled to Sepharose-4B, and this solid phase is treated with an excess of monospecific horse anti(IgE) antiserum raised against IR162 IgE. Thus, on average the antibodies are univalently attached to the solid-phase-coupled IgE, leaving the other binding site available for the competitive binding of $_{125}$I labelled IR2 IgE or unlabelled IgE in the assay sample.

Immunoelectrophoresis

Agar for both immunoelectrophoresis and immunodiffusion was prepared by adding 2 g Noble agar (Special agar - Noble, Difco, Detroit, Michigan) to 100 ml distilled water and bringing the solution to a boil. This 2% agar preparation was then filtered through a Whatman no. 1 filter paper on a warm Buchner funnel, and diluted to 1% by adding an equal volume of barbital buffer (2x). A 13 ml volume of 1% agar was cast in a tray containing 3 1x3 inch microscope slides (precoated with a thin layer of 0.5% agar and dried). Samples of 0.010 ml were applied to wells punched in the agar on each slide. The

samples were electrophoresed for 1 hour at 8 mA per tray of six slides (2 rows of 3 slides). The slide trays, gel punch, and electrophoresis chamber were all manufactured by Gelman (Rexdale, Ontario). The agar in troughs cut adjacent to the sample wells was removed following the electrophoresis, and the troughs were filled with appropriate antisera. The antigens and antisera were allowed to diffuse for 48 hrs. at 4°, developing precipitin bands in the agar. The slides were subsequently washed 4x12 hrs. with 2% saline to remove unprecipitated proteins, and 2x1 hrs. with distilled water to remove NaCl prior to drying. The slides were covered with wet filter paper following this final wash, and allowed to dry at room temperature. The dried slides were stained by immersion in acid fuchsin for 15 minutes (with stirring), and destained in 5% acetic acid until the background was clear. Stained slides were preserved with Vikem spray coating (Bel-Art, Pequannock, New Jersey).

Cell culture

RBL cells or the cloned RBL3114 sub-line were used throughout these studies. The RBL3114 sub-line was cloned by limiting dilution. Briefly, a suspension of the parent cell line was prepared in media (described below) containing 20% supernatant from a growing culture. The cell density of the suspension was adjusted to 10 cells per ml, and 0.1 ml aliquots were plated out on 96 well tissue culture plates (Microtest II, No. 3040, Falcon, Oxnard, CA). The plates were left strictly undisturbed for one week, under the conditions described below, and various sub-lines were then grown up from wells containing single, well defined colonies. The RBL3114 population was one of these. The advantage of the cloned sub-line was an increased stability in the ratio of the H and R receptors, as might

be expected of a more homogeneous population. In all other respects however, the cells appeared identical to the parent line. Tests, kindly performed by Ms. K. McNeil (this laboratory), have shown the RBL3114 cells to be free of mycoplasma contamination.

The cell lines were maintained in 25 or 75 cm^2 tissue culture flasks (Falcon Nos. 3013 or 3024) containing 5 ml Eagle's minimum essential medium (Earle BSS) (Difco cat. no. 5069-23, Difco Laboratories, Detroit, Michigan) per 25 cm^2. The medium was supplemented with non-essential amino acids, TC vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Burlington, Ontario), 0.3% glucose, 0.0262 *M* $NaHCO_3$ (both from Fisher Scientific Co., Canada), and 15% heat inactivated fetal calf serum (Flow Laboratories, McLean, Virginia). The pH of the medium was 7.2-7.4 prior to addition of the fetal calf serum. The cells were grown at 37°C in a humidified, 3% CO_2 atmosphere.

The cells grow attached to the bottom of the tissue culture flasks under these conditions. Rather than forming a uniform monolayer, mature populations tend to show considerable clumping. Originally, the populations in mature flasks were stripped from the plastic by repeated pipetting. In virtually all of the present studies however, the cells were released from the substrate by replacing the normal medium with 2-3 ml BSS/EDTA per 25 cm^2, chelating the Ca^{+2} normally required for attachment. A brief incubation (15 minutes) in this medium at 37°C causes the cells to round up, so that any cells remaining attached to the flask are rather easily released by swirling the medium or by relatively gentle pipetting. On average, a 75 cm^2 flask was capable of yielding $5.5x10^7$ cells.

The cell line was propagated by seeding one drop of the harvested cell suspension per 5 ml of fresh medium in a new flask. The medium was usually completely replaced the third day after seeding and every day thereafter. The flask was generally ready to be harvested 5-6 days after seeding. At this point the population was simply reduced by suspending the cells and removing the suspension. On adding fresh media back to the flask, sufficient cells remained to allow the population to grow up again. Once this second population matured however, the cells were propagated to a new flask. In later work, only this second population of cells was used, harvested as nearly as possible to the same day after the seeding of the culture (day 10), in an attempt to improve the reproducibility of results from one lot of cells to the next.

Labelling

Pasteur pipettes used to handle the cells were fire polished to minimize damage to the cells. Cells were harvested with EDTA/BSS at 37°, and maintained at 0°C prior to labelling. Lots of $5x10^6$ cells were diluted to 4 ml with PBS (0°C) in a 12x75 mm polystyrene tube (No. 2054, Falcon, Oxnard, CA) and centrifuged 20 minutes at 1000 rpm in a PR-J refigerated centrifuge (IEC, Needham Hts., MA) at 4°C. The cells were resuspended in 0.5 ml PBS, and they were surface labelled with 0.5 mCi carrier free $Na^{125}I$ (100 mCi/ml) (Amersham Corporation, Oakville, Ontario) at room temperature. The reaction was catalyzed by the repeated addition of both 0.010 ml lactoperoxidase (166 U/ml) (Calbiochem, San Diego, CA) and 0.015 ml 0.03% hydrogen peroxide (Fisher Scientific Co., Canada), at one minute

intervals for a total of three additions (71). The tube contents were gently mixed after each reagent addition sequence, and at the 30 second interval between additions, on a Vortex mixer (Vortex Genie, Fisher Scientific Co.) at a speed setting of 2. After a total reaction time of 3 minutes, each lot of labelled cells was transferred to 4 ml PBS/BSA (0°C), and centrifuged 20 minutes at 1000 rpm in a PR-J refrigerated centrifuge. To reduce the amount of radiolabelled low molecular weight material (121), the cells were resuspended in 4 ml PBS/BSA per lot and incubated one hour at 37°C in a controlled environment incubator shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm. The cells were subsequently maintained between 0-4°C, and unless otherwise noted, all subsequent centifugations were as described above. The cells were centrifuged, resuspended in 1 ml PBS/BSA per lot, and each lot was washed through 2 ml fetal calf serum (0°C) by careful layering of the cell suspension on the FCS. The cells were washed once more in 4 ml PBS/BSA, and 0.125 ml BBS/NP-40 was added to each of the final pellets ($4x10^7$ cell equivalents/ml). Membrane proteins were extracted by solubilizing 20 minutes at 4°C with rapid shaking (shaker manufactured by Eberbach, Ann Arbor, Michigan), followed by centrifugation at 8000 x g for 2 minutes in an Eppendorf 3200 microfuge (Eppendorf Geratebau, Hamburg, West Germany) to remove insoluble material.

Affinity chromatography

Protein-Sepharose conjugate preparation

Protein preparation

Purified normal rat IgG, IR162 IgE, and BSA were each dialysed against the coupling buffer. Immediately prior to use, the preparations were deaggregated by ultracentrifugation, diluted to 5 mg/ml in coupling buffer, and stored at 4°C until coupling.

Sepharose activation

Approximately 20 g of Sepharose CL-4B (Pharmacia, Upsala, Sweden) were transferred to a 60 ml coarse sintered glass funnel and washed with 500 ml distilled water. The gel was drained to a *wet* cake and 10 g were transferred to a 50 ml beaker. Ten ml of distilled H_2O were added to the beaker, producing a 1:1 suspension.

The beaker containing the Sepharose and a 2.4x0.7 cm magnetic stirring bar was placed in a shallow water bath at room temperature. 1.6 g of CNBr were dissolved in approximately 2 ml acetonitrile and added dropwise with moderate mixing to the Sepharose suspension. (Care was taken at all stages in the preparation and use of the gel to avoid fragmentation and the generation of fines). The pH of the suspension was immediately raised to between 10 and 11 with 1.5 N NaOH and maintained in that range for 15 minutes. The gel was then transferred to a 60 ml coarse sintered glass funnel and washed rapidly with 500 ml coupling buffer, 500 ml distilled H_2O and once again with 500 ml coupling buffer, all at 0°C

(122). The Sepharose was transferred in coupling buffer to a graduated 50 ml conical centrifuge tube and sedimented for 2 minutes at 2000 rpm in a PR-J centrifuge at 4°C. The supernatant was discarded and the gel was resuspended in an equal volume of coupling buffer to give a 1:1 suspension of activated Sepharose (total volume 20 ml).

Coupling

The previously prepared protein solutions were immediately added to aliquots of the activated Sepharose slurry in the ratio of 1 ml protein solution:2 ml slurry (5 mg protein/ml Sepharose). The coupling reaction was allowed to proceed for 48 hours at 4°C with end over end mixing. Coupling efficiency, as determined at the end of this time on the basis of the protein concentration of the supernatant, was typically >98%.

Any unreacted groups on the Sepharose were quenched by reaction with coupling buffer containing 0.2 *M* glycine for 96 hours at 4°C. (A control affinity gel was prepared by simply quenching activated Sepharose at this point without previously reacting it with any protein). After quenching, the gel was transferred to a coarse sintered glass funnel and (based on a Sepharose volume of 10 ml) washed with three rounds of 100 ml coupling buffer, 100 ml BBS, and 100 ml acetate/NaCl. Finally, the gel was washed with 200 ml PBS followed by 200 ml PBS containing 0.5% NP-40 and 0.1% NaN_3, and the coupled Sepharose was stored in this last buffer at 4°C.

Affinity chromatography

Receptors were isolated by reacting 0.05 ml of labelled cell extract (equivalent to $2x10^6$ cells) with 0.1 ml of affinity gel suspended in 0.1 ml BBS/0.1% NP-40 in a 12x75 mm polystyrene tube (Falcon no. 2054). The mixture was incubated 1 hr. at 4°C on an Eberbach shaker. The gel was washed with 4x4 ml of BBS/0.1% NP-40 and finally with 4 ml of 0.059 *M* Tris PO_4^{-3}, pH 7.0, sedimenting the gel in each wash cycle by centrifugation at 2000 rpm for 2 minutes in a PR-J centrifuge at 4°C. Following the last wash and removal of as much of the supernatant buffer as possible, bound material was eluted from the gel by adding 0.1 ml of SDS-PAGE sample buffer to the tube and heating the mixture on a boiling water bath for 1 minute. If not analyzed immediately by SDS-PAGE, the samples were stored at -20°C. Following such storage, the samples were reheated at 100°C for 1 minute immediately prior to use.

Labelled membrane protein bound by the affinity gel was determined on the basis of the radioactivity of the washed sample. Radiolabel cpm bound were determined in a Gamma 8000 spectrometer (Beckman Instruments, Fullerton, CA) immediately prior to electrophoresis of the sample. The percentage of the total sample electrophoresed was determined on the basis of cpm remaining after sample application.

Inhibition experiments were performed by adding various concentrations of soluble, deaggregated protein preparations to the affinity gel slurry prior to the addition of the cell extract to the reaction mixture.

SDS-PAGE

Gel electrophoresis was performed according to a procedure described by Maizel (123) for a discontinuous gel buffer system containing SDS. Briefly, the gels were cast in 6mm I.D. glass tubes (1 mm thick walls), 18 cm in length. The resolving gel was 10% acrylamide, 14 cm long, and contained 0.375 *M* Tris HCl pH 8.9. The stacking gel was 3% acrylamide, 2 cm long, and was buffered with 0.059 *M* Tris PO_4^{-3} pH 6.7. The electrode buffer contained 0.05 *M* Tris, 0.384 *M* glycine, and 0.1% SDS. The resolving gel was cast at least 6 hours prior to use (more often the day before use, stored at 4°C overnight), and the gels were run as soon as possible after the casting of the stacking gel.

As much of the 0.2 ml sample slurry as possible was applied to the top of the gel. The samples were overlaid with electrode buffer and the gels were run at 18°C in a Canalco electrophoresis apparatus (Canalco, Rockville, MD) under a constant voltage of 100 v. Initial current was 3.5 mA/gel. The voltage was increased to 250 v once the samples were through the stacking gel, and the electrophoresis was continued until the tracking dye was within 3 mm of the bottom of the gel. Total run time was typically about 5 hours.

Following electrophoresis, the gels were temporarily stored at 0°C to minimize band diffusion while awaiting fractionation. Each gel was sliced into 2 mm fractions on a Gilson model 200 gel fractionator (Gilson Medical Electronics, Villiers-le-Bel, France). The radioactivity of each fraction was determined in a Gamma 8000 spectrometer, and cpm were

plotted against fraction number. In dual labelling experiments, the gamma counter was programmed to correct automatically for ^{131}I spillover into the ^{125}I channel.

Quantitative analysis of SDS-PAGE profiles

Quantitative comparisons of the cpm vs. fraction plots were made by first correcting the cpm of each fraction for the percentage of the total sample that was actually electrophoresed, since this varied somewhat for different samples. The peaks of the profile, corrected to account for 100% of the sample, were then graphically resolved, and compared on the basis of the total peak cpm minus background.

Results

IgG-Sepharose binding of radiolabelled membrane components

Initial experiments compared the abilities of various protein-Sepharose preparations to bind radiolabelled RBL cell membrane components. The results, presented in Table 2.1, were somewhat surprising. The relatively high binding to BSA-Sepharose was particularly unexpected, since previous results from our laboratory had indicated that this conjugate bound less material than IgE-Sepharose (72). This may have been a result of a different protein/Sepharose ratio on the conjugates. Interestingly, in support of Conrad's unpublished observations, IgG-Sepharose appeared to bind nearly as many counts as IgE-Sepharose.

Table 2.1 Comparison of cpm bound to various protein-Sepharose conjugates relative to IgE-Sepharose

Sepharose conjugate	Ratio of cpm bound relative to IgE-Sepharose
IgE	1.00
IgG	0.79
BSA	1.86
Activated/Quenched	0.40

SDS-PAGE analysis

An entirely different picture emerged however, when the material bound by the various Sepharose preparations was eluted and analyzed by SDS-PAGE (Figure 2.3). The eluate from IgE-Sepharose yielded the expected peaks, corresponding to the H and R receptors (Figure 2.3a). Two peaks with very similar mobility were also eluted from IgG-Sepharose (Figure 2.3b). However, over several experiments, the total counts in these two peaks only amounted to 26 ± 9% of those found in the corresponding two peaks from IgE-Sepharose. This would suggest that, if these are the same proteins bound by IgE-Sepharose, they have a somewhat lower affinity for IgG-Sepharose.

In contrast, as shown in Figure 2.3c, the profile of material eluted from BSA-Sepharose was surprisingly flat, despite the large number of counts bound by this preparation. In particular, no peaks were found in this profile that might correspond to either of the H or R receptor proteins. In fact, the fate of the BSA-Sepharose bound counts is uncertain. Although the majority of counts were applied to the SDS-PAGE gel, the peak of low molecular weight material migrating with the tracking dye was too small to account for all of the applied counts, and they did not appear to be trapped in the stacking gel.

The SDS-PAGE profile obtained with activated/quenched Sepharose (not shown) was very similar to that for BSA-Sepharose, and further supported the conclusion that the material bound by IgG-Sepharose was *not* bound by some non-specific interaction.

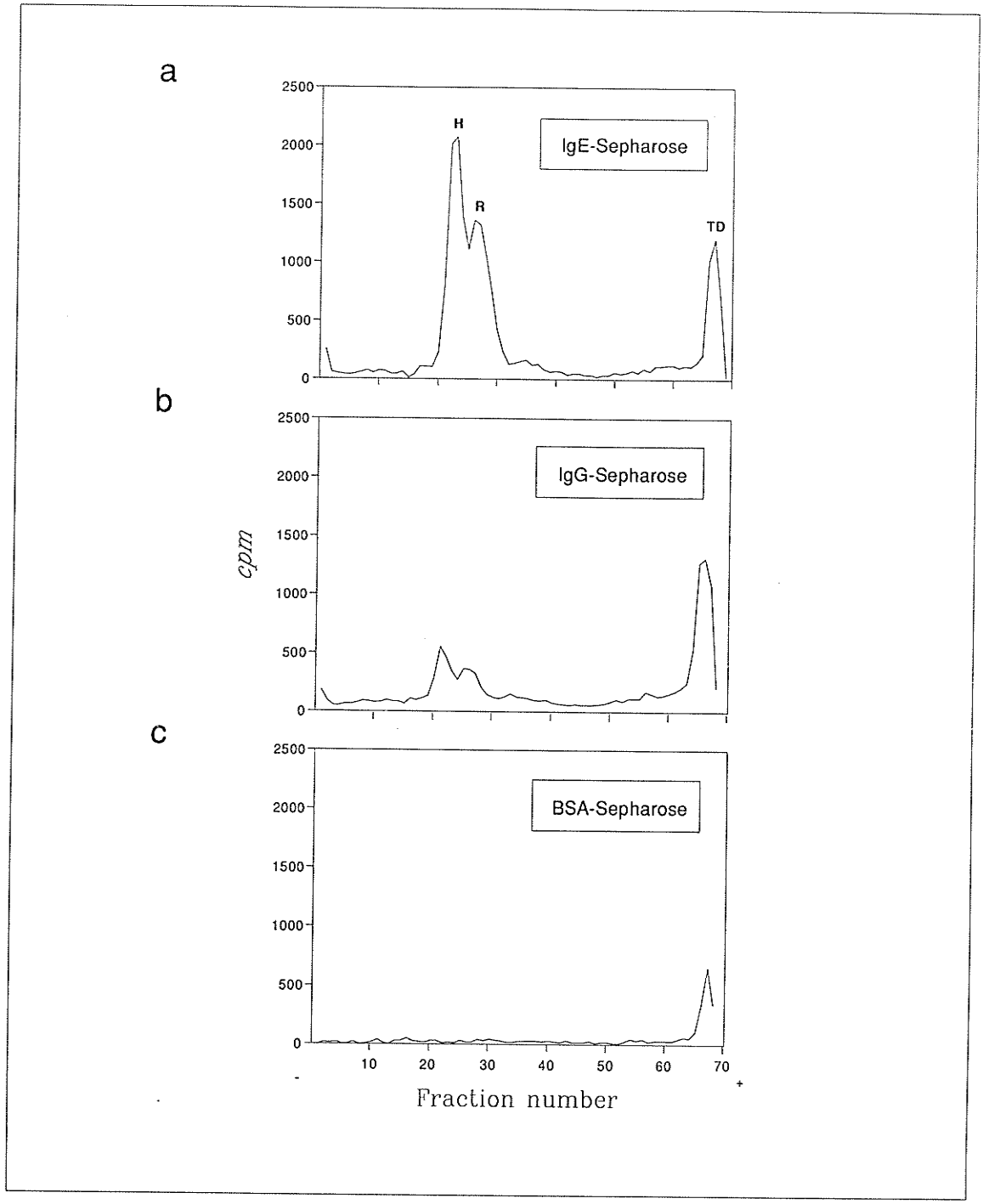


Figure 2.3 SDS-PAGE analysis of radiolabelled RBL surface components eluted from various protein-Sepharose conjugates. Gels were sliced into 2 mm fractions and results are plotted as cpm/fraction. TD indicates the position of the bromphenol blue tracking dye.

Dual labelling SDS-PAGE analysis

While these results suggested that the components bound by IgG- and IgE-Sepharose had *similar* electrophoretic characteristics, in order to establish that the mobilities of the IgG bound components were actually *identical* to the H and R proteins, a dual labelling experiment was performed. One lot of cells was labelled with ^{125}I, and the detergent extract of this preparation was reacted with IgG-Sepharose. Another lot from the same cell population was labelled with the ^{131}I radioisotope, and this extract was reacted with IgE-Sepharose. Eluates from the two Ig-Sepharose conjugates were combined, and the mixture was analyzed by SDS-PAGE. As shown in Figure 2.4, the ^{125}I labelled molecules binding to IgG-Sepharose migrated with mobilities *identical* to the ^{131}I labelled H and R receptor proteins bound by IgE-Sepharose. The apparent similarity in the peak heights of the IgG-Sepharose bound material compared to that bound by IgE-Sepharose was due to the low specific activity that seems to be characteristic of ^{131}I labelling.

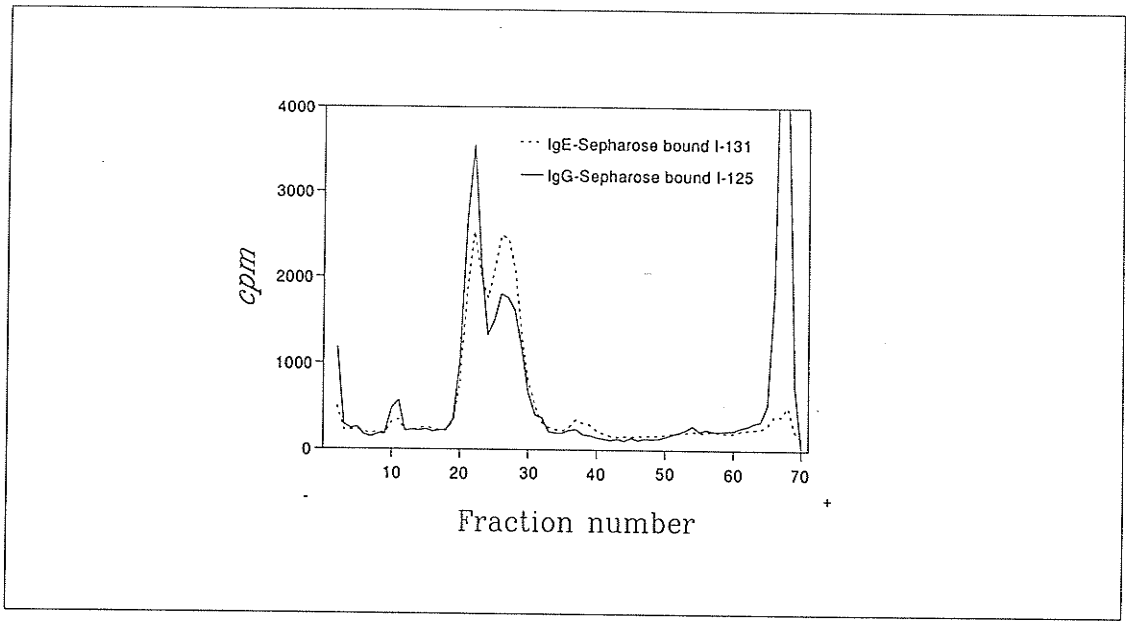


Figure 2.4 Dual labelling experiment. SDS-PAGE analysis of a single sample containing ^{131}I labelled material eluted from IgE-Sepharose and ^{125}I labelled material eluted from IgG-Sepharose.

Absorption studies

Conclusive evidence indicating that IgG-Sepharose was binding the *same* H and R proteins as are bound by IgE-Sepharose was provided by a series of absorption experiments, presented in Figures 2.5 and 2.6. These figures illustrate the effects of absorbing the solubilized cell preparation with various types of Sepharose prior to reaction with IgE-Sepharose (Figure 2.5) or IgG-Sepharose (Figure 2.6). Whereas absorption with BSA-Sepharose had no effect on the profile of material subsequently bound by IgE-Sepharose (Figure 2.5a), absorption with IgG-Sepharose significantly reduced the subsequent binding of both H and R, with a preferential decrease in H binding (Figure 2.5b). Conversely, as shown in Figure 2.6, while absorption with activated/quenched Sepharose (or BSA-Sepharose, separate experiment) similarly failed to affect the profile of material subsequently bound by *IgG-Sepharose* (Figure 2.6a), absorption with IgE-Sepharose eliminated essentially all of the material normally bound by the IgG conjugate.

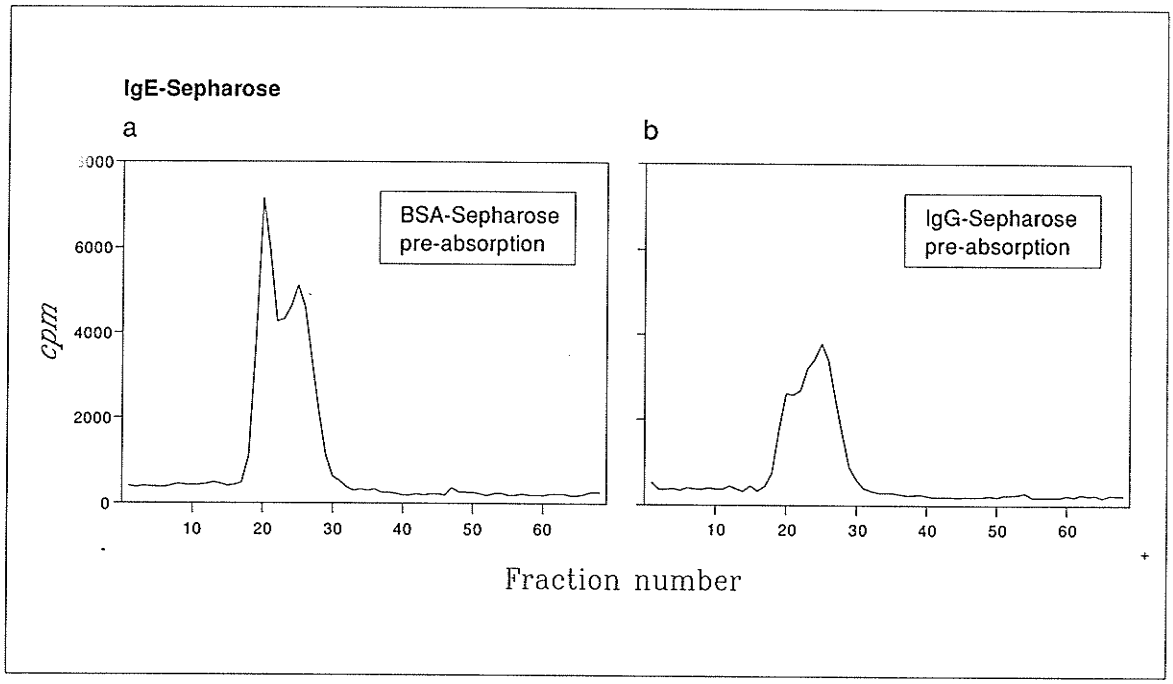


Figure 2.5 The effects of pre-absorbing the cell extract with BSA-Sepharose or IgG-Sepharose on the profile of RBL membrane components bound by IgE-Sepharose.

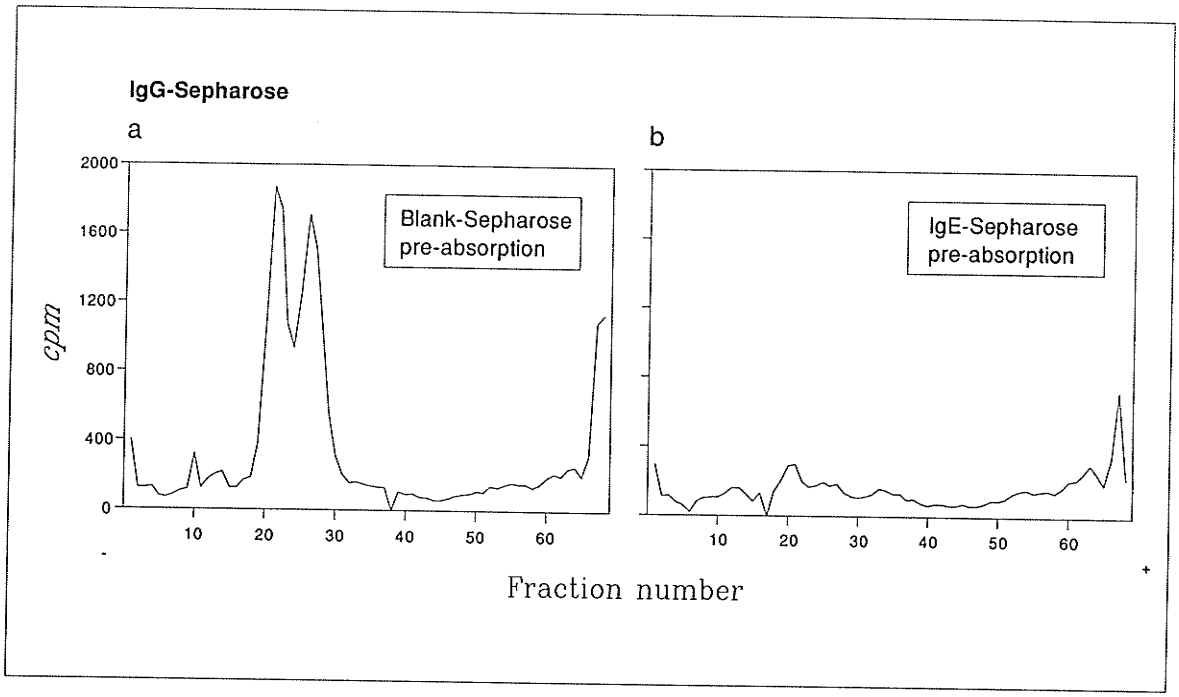


Figure 2.6 The effects of pre-absorbing the cell extract with Blank-Sepharose or IgE-Sepharose on the SDS-PAGE profile of RBL membrane components bound by IgG-Sepharose.

Inhibition studies

Subsequent experiments explored the interaction in solution between the H and R receptor proteins and monomeric IgE and IgG. Various concentrations of the immunoglobulins were used in an attempt to inhibit the binding of the two receptors to IgE- or IgG-Sepharose.

Figure 2.7 shows that, solely on the basis of total counts bound, IgG (•) apparently failed to inhibit binding to either IgE- or IgG-Sepharose to any great extent. In neither case did the inhibition exceed that produced by an equal concentration of BSA (♦). IgE (▲), on the other hand, significantly reduced the total counts bound by both IgE- and IgG-Sepharose.

However, subsequent analysis of the electrophoretic profiles of the material eluted from each of these inhibited samples provided considerably more information than could be obtained from the straight binding data of Figure 2.7. Quantitative analysis of these profiles both eliminated the effects of non-specifically bound radiolabelled material, and allowed the effects of the inhibitors to be assessed separately for each of the H and R peaks. Figures 2.8 and 2.9 show the effects of the soluble inhibitors on the receptor region of the SDS-PAGE profiles, and Figures 2.10-2.12 provide quantitative analyses of these profiles.

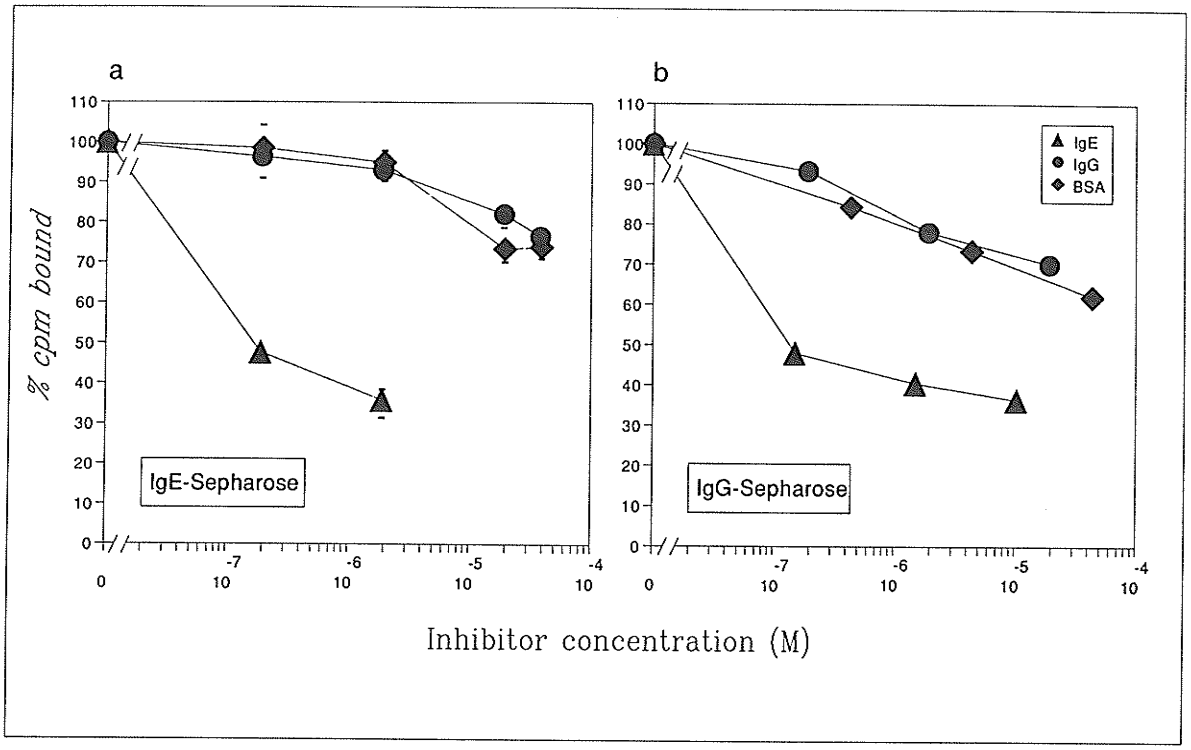


Figure 2.7 The effects of various concentrations of soluble IgE, IgG or BSA on total RBL extract cpm bound by IgE-Sepharose and IgG-Sepharose.

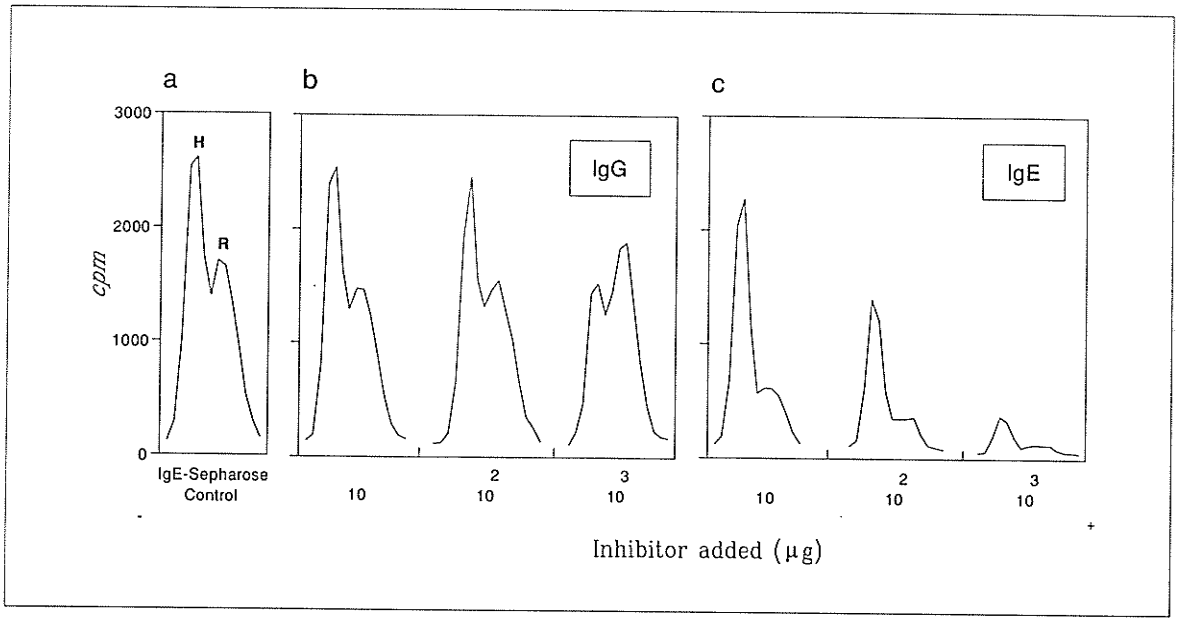


Figure 2.8 The effects of various concentrations of soluble IgG or IgE on the SDS-PAGE profiles of RBL membrane components bound by IgE-Sepharose. Only the receptor region of each profile is shown.

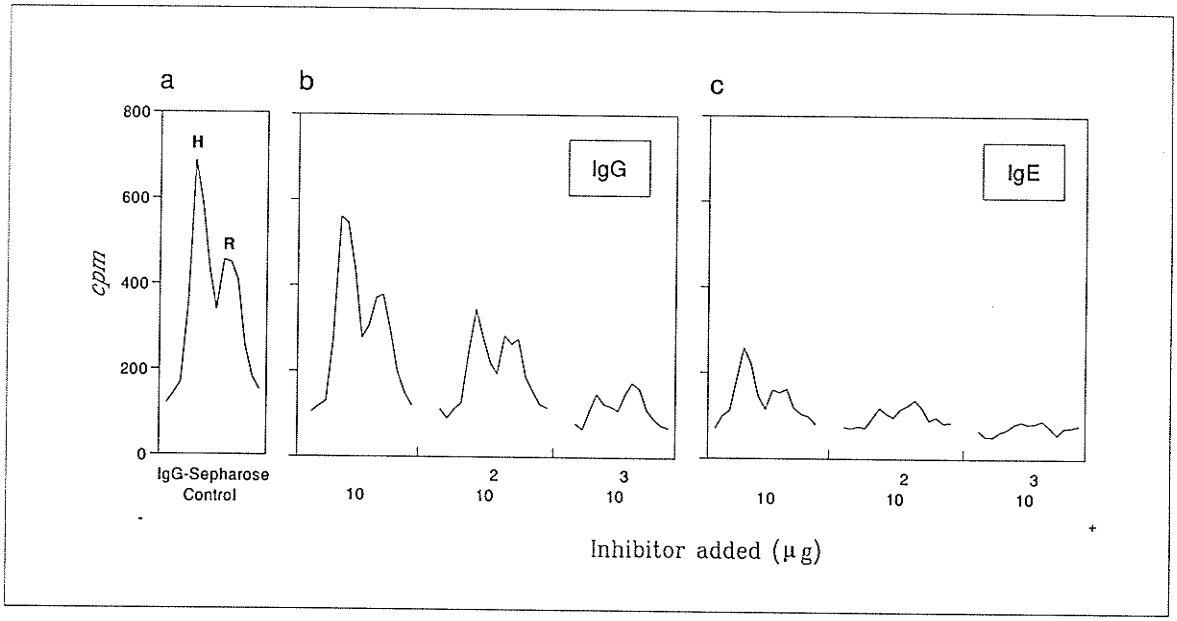


Figure 2.9 The effects of various concentrations of soluble IgG or IgE on the SDS-PAGE profiles of RBL membrane components bound by IgG-Sepharose. Only the receptor region of each profile is shown.

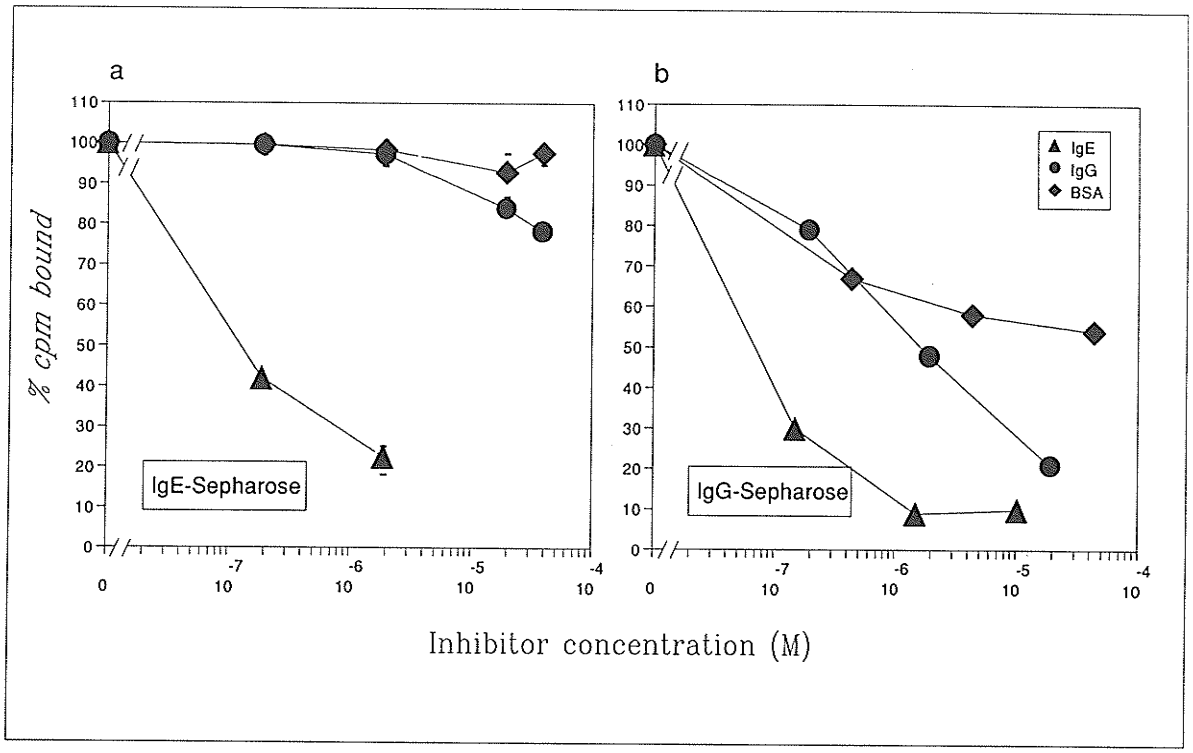


Figure 2.10 The effects of various concentrations of soluble IgE, IgG or BSA on total receptor-related cpm (H+R) bound by IgE-Sepharose and IgG-Sepharose.

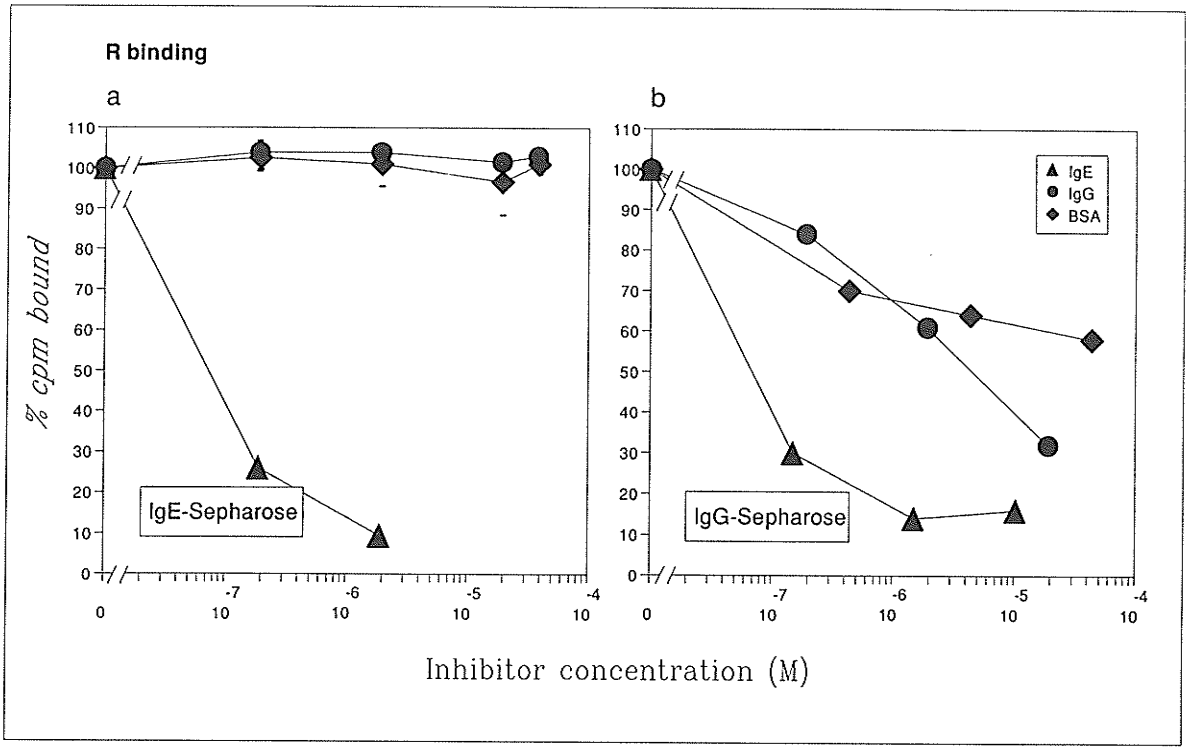


Figure 2.11 The effects of various concentrations of soluble IgE, IgG or BSA on R receptor-related cpm bound by IgE-Sepharose and IgG-Sepharose.

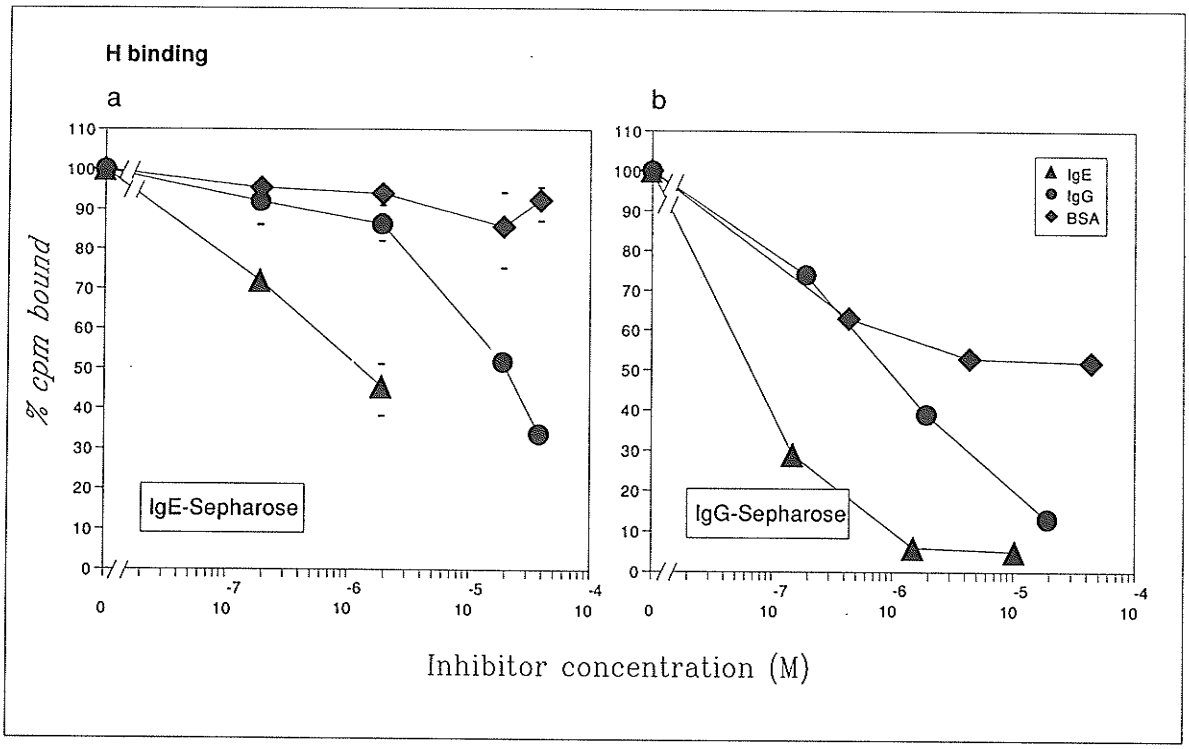


Figure 2.12 The effects of various concentrations of soluble IgE, IgG or BSA on H receptor-related cpm bound by IgE-Sepharose and IgG-Sepharose.

It is immediately apparent from the profiles presented in Figures 2.8 and 2.9, that *both* IgG (panels (b)) and IgE (panels (c)) have profound effects on the binding of H and R to IgE- and IgG-Sepharose. This is contrary to the simple analysis of total cpm bound, presented in Figure 2.7, and suggests that this previous analysis failed to accurately reflect the effects of the soluble proteins on actual *receptor* binding. The large number of non-receptor cpm bound non-specifically by the control Sepharose preparations (Table 2.1) suggests that high background binding probably obscured the effects of the soluble proteins on receptor binding in this earlier analysis.

It would be more appropriate therefore to plot binding of *receptor specific* cpm as a function of soluble protein concentration, rather than simply plotting total cpm bound. Based on the SDS-PAGE profiles of Figures 2.8 and 2.9, such an analysis of total *receptor related* cpm is presented in Figure 2.10. Perhaps the most important point made in this figure is that soluble IgG (•) causes significantly greater inhibition of total receptor binding to both (a) IgE- and (b) IgG-Sepharose than BSA (♦) does. This provides considerable support for the specificity of the IgG-monomer/receptor interaction. Furthermore, by restricting the data to receptor related cpm, much of the interference due to non-specific binding of radiolabelled protein was eliminated, and the apparent inhibition by each of the soluble proteins was significantly enhanced.

This type of quantitative analysis was still more informative if the interaction between receptor, soluble protein, and Sepharose conjugate was analyzed *separately* for each receptor

protein, as in Figures 2.11 and 2.12. In Figure 2.11a it can be seen that, in the range of concentrations used, soluble IgG (•) was incapable of inhibiting the interaction of the R protein with IgE-Sepharose. Free IgE (▲) on the other hand inhibited this interaction quite effectively. In contrast, the interaction of R with *IgG*-Sepharose (Figure 2.11b) was inhibited both by IgE (▲) *and* by IgG (•). Although IgE was the more effective inhibitor, these results none the less show quite clearly that *monomeric* IgG *is* capable of interacting with the R protein.

A similar analysis is presented for the H receptor in Figure 2.12. In this case, IgG (•) successfully inhibited the interaction between the H protein and *both* (a) IgE- and (b) IgG-Sepharose. It can be concluded therefore that, like R, H is also capable of interacting with monomeric IgG.

The finding that soluble IgG is capable of causing considerable inhibition of the H/IgE-Sepharose interaction, but no significant inhibition of the R/IgE-Sepharose interaction, suggests that either the H/IgG interaction is *stronger* than the R/IgG interaction, or the H/IgE-Sepharose interaction is *weaker* than the *R*/IgE-Sepharose interaction, or perhaps both. The results of the earlier absorption experiments tended to suggest a preferential interaction between H and IgG-Sepharose (Figure 2.5). The present series of experiments would tend to support this conclusion, since IgG seems to inhibit the H/IgG-Sepharose interaction *somewhat* more effectively than it inhibits the R/IgG-Sepharose interaction

(compare the slopes of $-\circ-$, Figures 2.11b and 2.12b). However, there does not appear to be any *marked* difference in the affinities of H and R for IgG. This might suggest therefore, that H *also* has a lower affinity for IgE-Sepharose than R has (the "both" option above).

The data presented in Figures 2.11a and 2.12a for the IgE (▲) mediated inhibition of R and H binding to IgE-Sepharose tend to support this suggestion. A lower affinity for IgE-Sepharose would imply a lower affinity for *IgE*. A lower affinity for IgE would mean higher concentrations of the *soluble immunoglobulin* would be required to inhibit the receptor/solid-phase interaction to any particular extent, say 50%. A comparison of Figures 2.11a and 2.12a shows quite clearly that the R/solid-phase interaction is inhibited by soluble IgE much more strongly than the H/solid-phase interaction is. It would appear therefore that the H protein has a *considerably* lower affinity for IgE than the R protein has.

To briefly summarize the results of this analysis therefore, both H and R proteins are capable of binding *monomeric* IgG and IgE. The affinity of the H/IgE interaction appears to be lower than that of R and IgE, whereas *IgG* seems to interact preferentially with the H protein. However, all of the results thus far are also consistent with both receptors having a higher affinity for *IgE* than for IgG.

Specificity of the interaction of H and R with IgG

Although soluble BSA appeared to cause significant inhibition of total receptor binding to IgG-Sepharose (Figure 2.10b), BSA-*Sepharose* failed to specifically bind either of the H or R receptors (Figure 2.3c). This suggested, therefore, that the inhibition by soluble BSA was a result of non-specific protein/protein interaction, whereas soluble IgG caused a *specific* inhibition of receptor binding. In order to confirm this point however, the inhibitory capacities of IgG and BSA were compared with those of a variety of readily available proteins having isoelectric points (pI) similar to IgG. $F(ab')_2$ fragments of rat IgG were included in the series of proteins to determine the Fc specificity of the receptor/Ig interaction.

The results of this experiment, presented in Table 2.2, indicated that IgG and IgE consistently inhibited receptor binding to IgG- and IgE-Sepharose to a significantly greater extent than any of the various control proteins. While the $F(ab')_2$ fragments appeared to inhibit the receptor/IgG-Sepharose interaction somewhat, this preparation was subsequently found to be contaminated with intact IgG. None the less, the inhibitory capacity of the preparation was still significantly less than that of undigested IgG, suggesting that even the *partial* removal of the Fc portion of the protein interfered significantly with the immunoglobulin's ability to bind the H and R receptor proteins.

Table 2.2 Inhibition of receptor binding by various proteins

Affinity Gel	Inhibitor	Concentration (molar)	% Receptor Bound ± S.E. H	R
IgG-Sepharose	---	---	100 ± 3	100 ± 5
	IgE	1.9×10^{-6}	8 ± 2	27.5 ± 0.7
	IgG	"	57 ± 0.7	66 ± 1.4
	$F(ab')_2$	"	70 ± 5	83 ± 4
	BSA	"	76 ± 3	87 ± 10
	Hemoglobulin	"	80 ± 9	86 + 5
	Carbonic Anhydrase	"	84 ± 10	83 ± 15
	Fibrinogen	8.3×10^{-7}	88 ± 9	107 ± 9
IgE-Sepharose	---	---	100 ± 0.5	100 ± 7
	IgE	1.9×10^{-6}	45 ± 9	10 ± 2
	IgG	3.7×10^{-5}	34 ± 1	103 ± 2
	BSA	"	92 ± 5	101 ± 2
	Hemoglobulin	"	94 ± 7	96 ± 4

Various proteins were tested at the indicated concentrations for their abilities to inhibit the interaction between the H and R receptor proteins and IgG- or IgE-Sepharose. Percent receptor binding was determined by quantitative analysis of SDS-PAGE profiles (see Materials and Methods).

As previously, BSA appeared to inhibit the binding of H and R to IgG-Sepharose. Although the inhibition would appear to have been somewhat comparable to that of IgG (at least in the case of the *H*/IgG-Sepharose interaction), the inhibitory capacity of BSA levelled off at this concentration, as shown previously in Figures 2.10 through 2.12. On the other hand, increasing the concentration of *IgG* continued to further inhibit receptor binding. At higher protein concentrations therefore, BSA causes *substantially* less inhibition than IgG. This was shown quite clearly in the inhibition of receptor binding to *IgE*-Sepharose in the lower part of Table 2.2. Unfortunately, keeping all of the inhibitor concentrations the same, the maximum concentration which could be used for the inhibition of binding to *IgG*-Sepharose (1.9×10^{-6} M) was limited by the supply of the $F(ab')_2$ preparation.

These results therefore suggest that the interaction of the H and R proteins with IgG was quite specific, and appeared to depend on the Fc region of the immunoglobulin. The apparent inhibition of the receptor/IgG-Sepharose interaction by BSA appears to have been the result of non-specific protein/protein interaction.

Discussion

The results of these experiments have confirmed *both* of Conrad's previous observations. Thus, (a) soluble IgG does not *appear* to specifically inhibit the binding of radiolabelled RBL cell membrane proteins to IgE-Sepharose, *on the basis of total cpm bound*, and (b) IgG-Sepharose *does* bind a significant amount of solubilized membrane protein from radiolabelled RBL cells. Furthermore, the proteins bound by IgG-Sepharose are the same H and R receptor proteins bound by IgE-Sepharose. These receptors are bound *specifically*, but they bind to the IgG-Sepharose with a lower affinity than they bind IgE-Sepharose. This reduced affinity has been used to advantage to demonstrate an interaction between *both* of the receptors and the low affinity ligand, monomeric IgG.

In fact, with regard to point (a) above, soluble IgG *was* able to compete with IgE-Sepharose for the *H* protein, but this inhibition is obscured by high, non-specific, background binding. The binding of radiolabelled membrane components to BSA-Sepharose (Table 2.1) illustrated the extent of this non-specific binding. As a result, in the data presented in Figures 2.7 through 2.12, only about 30% of the total cpm bound by IgE-Sepharose could be accounted for by the receptor proteins. Of these receptor related cpm, just about one third (approximately 36%) were related to the H receptor, amounting to only 11% of the total cpm bound. At the maximum concentration of soluble IgG, there was a 66% inhibition of H binding (Figure 2.12a), while R binding was unaffected (Figure

2.11a). This resulted in a decrease in *total cpm bound* of only about 7% (ie. (0.30 x 0.36 x 0.66)x100%), which failed to exceed the *non-specific* inhibition by BSA.

If the H/R ratio of the RBL cells was reduced from that of the cell line used in these experiments, then the above problem would be compounded, with IgG producing an even *smaller* apparent decrease in total cpm bound. In fact, this is the most likely explanation for Conrad's failure to notice *any* inhibition by IgG of total cpm bound to IgE-Sepharose (72). A comparison of Figure 8, reference (72) with Figures 2.3 and 2.8, showed that the cells used in Conrad's earlier work *did indeed* express substantially less of the H protein than the cells used in the present study. Therefore, probably as a result of this difference, inhibition of H binding by soluble IgG had no noticeable effect on total cpm bound by the IgE-Sepharose in this earlier work.

The use of an IgG Fc preparation to establish the regional specificity of the IgG/receptor interaction was intentionally avoided at this stage of the project. Rousseaux *et al.* (124) have shown that the different rat IgG subclasses vary in their susceptibility to enzymatic degradation. The possibility existed therefore that an Fc preparation might have had a considerably different subclass composition than the whole IgG preparation used in the binding and inhibition studies. Without a precise knowledge of the IgG subclass specificity of the H and R receptors, the use of such an Fc preparation could easily have yielded misleading results.

Although, as previously mentioned, the $F(ab')_2$ preparation was found to be contaminated with undigested IgG, the results none the less tend to indicate that the IgG/receptor interaction was Fc specific. In fact, subsequent work using pure $F(ab')_2$ and Fc preparations is presented in the following chapters which strongly supports this conclusion.

As previously mentioned, studies by Halper and Metzger (118), and subsequently by Moller and Konig (125), failed to demonstrate any direct binding of monomeric IgG_{2a} to intact RBL cells. Nor were Halper and Metzger able to inhibit IgE binding with monomeric IgG_{2a}, suggesting that the Fc receptors of RBL cells did not bind IgG_{2a}. However, the same authors *could* inhibit the IgE/RBL interaction with IgG_{2a} *immune complexes*, suggesting that perhaps there actually *was* some interaction between these complexes and the Fc_ϵ receptors. To explain these somewhat contradictory results, it was proposed that, whereas the affinity of the receptors for monomeric IgG_{2a} might have been too low to show direct binding or to affect IgE binding, the interaction between the receptors and an IgG *complex* might be stabilized by the simultaneous interaction with a number of receptors (multipoint attachment). Unfortunately, they could not rule out the possibility that the inhibition of IgE binding was a result of steric interference from the IgG_{2a} complexes bound to some receptors *other* than the $Fc_\epsilon R$.

The results of the present study clearly show that both the H and R Fc_ϵ receptor proteins *do* cross-react with IgG, supporting Halper and Metzger's suggestion that the IgG_{2a} complexes inhibited IgE binding by direct interaction with the Fc_ϵ receptors. However, the results also indicate that both receptors are capable of binding *monomeric* IgG. The failure of IgG_{2a} monomers to inhibit IgE binding to intact cells therefore requires some explanation. With respect to the R protein, the results indicate that, although this receptor binds IgG, it has a *much* higher affinity for IgE. Thus, as shown in Figure 2.11a (•), over the concentration range studied, monomeric IgG failed to successfully compete with IgE for the R protein binding site. In fact, the only way that the interaction between R and IgG monomers could be demonstrated was by inhibition of the lower affinity R/IgG-Sepharose interaction (Figure 2.11b, •). To date, this remains the only conclusive demonstration of IgG monomer binding by this high affinity Fc_ϵ receptor.

The problem is that, as shown in Figure 2.12a (•), the interaction between the *H* protein and monomeric IgG appears to be strong enough to compete successfully with the IgE/H interaction. One might therefore expect IgG monomers to inhibit IgE binding to intact cells as a result of an interaction with the H receptor, but such is not the case. It was previously suggested (126) that this discrepancy might be attributable to an increase in the receptor's affinity for IgG on its removal from the membrane (67). On the intact cell therefore, the membrane bound H protein might *not* bind IgG and IgE with comparable affinities, and IgG might not be capable of inhibiting the IgE/H interaction. However,

while this possibility can not be ruled out, it would be difficult to explain an increase in the affinity of the solubilized receptor for IgG without an accompanying increase in its affinity for *IgE*. It is doubtful therefore that the receptor on the intact cell has relative affinities for IgG and IgE that are considerably different from those of the solubilized receptor.

Another explanation for the apparent failure of IgG to inhibit the IgE/H interaction on intact cells lies in the method used and in the similarity of the H receptor's affinities for IgG and IgE. As mentioned in the introduction, Bach *et al.* found that (supposedly monomeric) IgG_{2a} sensitized rat mast cells and induced histamine release on exposure of these sensitized cells to the antigen specifically recognized by the sensitizing antibody (117). The interaction with the IgG was relatively weak compared to that with IgE however, and the sensitizing antibody could be removed by simply *washing* the cells.

Virtually all studies involving intact cells have ultimately depended on an analysis of the direct binding of labelled IgG or IgE. Halper and Metzger, for instance, attempted to show direct binding of ^{125}I labelled IgG_{2a} monomers. Similarly, they attempted to inhibit the direct binding of radiolabelled IgE with IgG_{2a}. Unfortunately, the direct binding assays most commonly employed have required that the cells be *washed*, to remove free, labelled immunoglobulin. But, as shown by Bach and co-workers (117), such washing results in the loss of the weakly bound IgG ligand. Consequently, no one has been able to demonstrate direct binding of monomeric IgG to RBL cells.

As shown in Figures 2.11a and 2.12a, monomeric IgG is only capable of competing with IgE for the *H* receptor. The strength of the IgG/H interaction is therefore *comparable to* (although significantly less than) the IgE/H interaction. As mentioned above, washing disrupts the interaction between monomeric IgG and the H receptor. It is quite possible therefore that the *IgE*/H interaction is also disrupted by washing, since the affinity of the interaction is comparable to that between *IgG* and the H protein. If this *were* the case, then assays based on direct binding to intact cells would fail to *detect* IgE binding to the H receptor. As a result, any *inhibition* of this binding by monomeric IgG would likewise go undetected. Thus, Halper and Metzger failed to *see* any inhibition of IgE binding by IgG monomers, although such inhibition might have taken place none the less.

This explanation was strongly supported by Segal *et al.* in their studies of the interaction between IgG and intact RBL-2H3 cells (127). As in virtually all previous studies of this interaction, they found that the binding of monomeric IgG was disrupted even by the relatively gentle technique of washing the cells through a phthalate oil mixture. However, they *were* able to demonstrate the direct binding of IgG *dimers*, suggesting that multipoint attachment to the cell stabilized the interaction enough for the binding to survive the wash cycle. Fortunately, the dimers were prepared from *rabbit* IgG, and they failed to inhibit IgE monomer binding to the high affinity Fc_ϵ receptor (the R protein). As will be seen in a later chapter, rabbit IgG is unique in its preferential interaction with the H receptor. Had the dimers been of *rat* IgG, they might well have inhibited the IgE/R interaction. In any case, this *lack* of inhibition of IgE binding therefore led the authors to conclude that the

IgG was bound through some other receptor, which they referred to as the *IgG receptor.* In fact, this was probably the first detection of direct binding to the protein that we refer to as H. Once again, for continuity the receptor will be referred to as the H protein throughout the work presented here.

The interaction of the H receptor with *monomeric* IgG could be demonstrated *indirectly* by the monomer's inhibition of IgG dimer binding. Interestingly, the binding of the IgG dimers was also inhibited by monomeric *IgE*, suggesting that the receptor cross-reacted with this immunoglobulin class. In fact, IgE appeared to be a somewhat better inhibitor of the H/IgG dimer interaction than IgG was. Actual estimates of the association constants for each class were $1.6x10^6\ M^{-1}$ for IgE and $4.2x10^5\ M^{-1}$ for IgG (127). The affinities of the receptor for each class *were* therefore within the same order of magnitude. (Recall that the association constant for the IgE/R interaction is on the order of $10^{10}\ M^{-1}$). Furthermore, pretreatment of the cells with IgE followed by washing failed to inhibit the binding of the *rabbit* IgG dimers (which only interact with the H protein). In support of the argument above, this suggested therefore that, despite its significantly higher affinity, *the IgE washed off of the H receptor just like the IgG monomers did.*

If the bond between the IgG/IgE ligands and the H receptor on the intact cell is disrupted by washing, this raises the question of why the H receptor is not similarly washed off of IgE- or IgG-Sepharose. A possible explanation for this is that the Sepharose conjugates

provide quite high local concentrations of immobilized immunoglobulin in the microenvironment of the Sepharose matrix. According to equation (1) in the introduction:

$$\text{(1)} \qquad \text{IgE + receptor} \underset{\leftarrow k_{-1}}{\overset{k_1 \rightarrow}{\rightleftharpoons}} \text{Receptor-IgE complex}$$

this high concentration of ligand pushes the reaction strongly towards complex formation. Furthermore, this high ligand concentration is *not* reduced by washing, since the immunoglobulin is bound to the solid Sepharose matrix. As a result, conditions always favour complex formation and the receptor remains bound to the Sepharose conjugate. Furthermore, by effectively increasing the ratio of the Sepharose-bound immunoglobulin to free receptor protein, washing the Sepharose actually *enhances* complex formation between the Sepharose conjugate and the receptor protein remaining after the wash. With intact cells on the other hand, washing reduces the concentration of the immunoglobulin ligand, and maintainence of the *equilibrium* between product and reactants results in the dissociation of receptor bound immunoglobulin. For the R protein, its affinity for IgE is so high that the rate of dissociation (k_{-1}) is too slow to result in a significant loss of bound IgE in several wash cycles. The R/*IgG* interaction on the other hand, and the interaction of the H receptor with *both* IgG and IgE, has a much lower association constant, probably largely due to a much more rapid rate of dissociation. Virtually *all* of the bound immunoglobulin is therefore able to dissociate from these interactions during the washing of the cells.

The higher affinity of the IgE/R interaction, compared with that between IgE and the H protein, also provides another possible explanation for the preferential isolation of the R receptor by IgE/anti(IgE) (72). It is possible that in these earlier studies the concentration of IgE used was simply too low to result in significant H/IgE complex formation. It should be borne in mind that at lower IgE concentrations, where the supply of IgE might be limiting, H would have to compete with R for IgE (a battle it does not have a chance of winning). It is also possible that the isolation of the FcR/IgE/anti(IgE) complexes on protein A-Sepharose (or of FcR/IgE complexes on horse anti(rat IgE)-Sepharose) fails to produce a concentration of IgE in the microenvironment similar to that of IgE-Sepharose. As a result, the H protein might be lost in the washing. It might be expected that immunoprecipitation of the FcR/IgE complexes would afford the best chance of isolating both H and R, since the IgE/anti(IgE) immunoprecipitate would most closely resemble IgE-Sepharose. However, it is also possible that the anti(IgE) binds the same region of the IgE molecule as the H receptor, inhibiting H binding. This would also tend to suggest that H and R bind somewhat different regions of the IgE protein, since the anti(IgE) does not appear to inhibit the *R*/IgE interaction. It is also possible that the IgE/H complexes dissociate upon interaction with anti(IgE) for some other reason (perhaps a result of some conformational change), as previously suggested (72).

In summary, both the H and R proteins of the RBL cell membrane appear to crossreact with IgE and IgG. However, the receptors differ considerably in their affinities for each of these ligands. The affinity of R for IgE is *much* greater than that for IgG. The H protein on the other hand has an affinity for IgE which is comparable to (but still greater than) that for IgG, and much lower than that of R. The fact that both receptors have a higher affinity for IgE than for IgG has prompted some to argue that both should be considered IgE receptors (126). However, *in vivo*, where the concentration of IgG is much higher than that of IgE, H might serve *primarily* as an *IgG* receptor. It might be best therefore to simply refer to these proteins generically as Fc receptors, perhaps using a number to distinguish between them (eg. FcR_1 and FcR_2), as has been done with the interleukins. This would avoid the somewhat misleading situation of having an IgE receptor that functions primarily as an IgG receptor, or conversely, an IgG receptor that has a higher affinity for IgE than IgG. In fact, as mentioned in the previous chapter, currently accepted nomenclature refers to R as $Fc_\epsilon RI(\alpha)$, and H is referred to as $Fc_\epsilon R_L$, the L standing for low affinity. Actually, according to the guidelines, H should be referred to as $Fc_\epsilon RII$, but this is now the generally accepted name for the the lymphocyte low affinity F_ϵ receptor (CD23). As previously indicated however, for the purposes of the thesis we will continue to refer to the receptors as H and R.

It is tempting to compare these results with those of Ishizaka *et al.* (128) and Daeron *et al.* (129), although these groups studied *human basophils* and *mouse mast* cells respectively,

and these system differences have to be kept in mind. In both instances, the intact cells were found to possess two distinct receptors; one reacting exclusively with IgE, and the other only with aggregated human IgG (128) or, in the case of the mouse, mouse IgG_1 alloantibody (129). However, in their attempts to show inhibition of IgG binding by IgE, these authors washed the cells between their exposure to IgE and the addition of IgG. Therefore, any IgE bound relatively weakly to the IgG receptors could have been washed off, as discussed above for *this* system.

Chapter 3

The subclass specificity of the interaction between IgG and the Fc_ϵ receptors of RBL cells

Introduction

The work presented in the previous chapter established that *both* the H and R receptor proteins of the RBL cell interact specifically with an IgG protein preparation isolated from normal rat serum. However, as was pointed out in Chapter 1, the IgG proteins can be divided into several subclasses. Moreover, it will be recalled that earlier work of Austen's group had implicated the IgG_{2a} subclass as a second mediator of histamine release, in addition to IgE. However, this work also indicated that whereas purified IgG_{2a} induced release, unfractionated antisera did not, suggesting that not only the IgG subclass but also the relative concentrations of the subclasses were important in this IgG induced release. The results presented in Chapter 2 indicated that the affinities of the IgE and IgG interactions with H may be comparable. Since these results were obtained with an unfractionated IgG preparation, at equal concentrations of *total* IgE and IgG protein in the

reaction mixtures used in the binding inhibition studies, the concentration of any particular IgG subclass would still be *less* than the concentration of IgE in the matching samples of the IgE inhibition series. The possibility therefore exists that H may actually bind one of the IgG subclasses with a *higher* affinity than it binds IgE. It therefore became important to determine the subclass specificity (if any) of the IgG/$Fc_\epsilon R$ interaction.

Unfortunately, whereas a variety of purified proteins of each *murine* IgG subclass are commercially available, no commercial preparations of purified *rat* subclass proteins were available at the time this work was undertaken. Consequently, the continuation of the project involved a considerable amount of time and effort being devoted to the purification of the rat IgG subclasses; both from normal rat serum and from the ascitic fluid of rats bearing tumors that produced large amounts of a single IgG subclass (immunocytomas). Unfortunately, the use of these immunocytoma proteins opened the possibility that one might be dealing with an abnormal protein, with unusual characteristics. Therefore, in order to be able to draw any general conclusions about the interaction between the receptor proteins and a particular IgG subclass, it was desirable to test more than one immunocytoma protein representing each subclass. This compounded the problem of subclass purification considerably, since even immunocytoma proteins of the same subclass have somewhat different characteristics, each requiring a somewhat different method of purification, reducing the usefulness of published methods.

Theoretically, the assay described in the previous chapter could have been used without changes to study the abilities of the various IgG subclasses to inhibit the interaction of the Fc receptors with IgG-Sepharose. However, the limited supply of the purified subclass proteins required that the assay reaction volume be reduced to conserve reagent proteins, in order that several replicates could be run over a range of protein concentrations. Furthermore, the large number of subclass proteins to be tested made it highly desirable to modify the assay in such a way as to increase its sample handling capacity. Thus, the micro-assay described in this chapter was developed to meet these needs. Fortunately, as this method was refined, the effort was further rewarded with reductions in background interference, and improvements in the reproducibility of the data obtained.

The main focus of this chapter is the interaction of the various subclasses of rat IgG with the RBL H and R receptor proteins. However, a considerable portion of the text is devoted to a discussion of the technical aspects of the micro-assay, in view of the assay's potential applicability to other studies.

Materials and Methods

Buffers

Acetate Buffer, 0.071 *M* acetate, pH 5.0

Acetate/NaCl, 0.1 *M* acetic acid/sodium acetate, 0.5 *M* NaCl, pH 4.0

Acid Fuchsin, 0.2% acid fuchsin, 50% methanol, 10% acetic acid

BBS, 6.25 mM borate, 0.85% NaCl, pH 8.8

BBS/NP-40/BSA contains 0.5% Nonidet P-40 (Calbiochem, San Diego, CA) and 0.1% BSA, pH 8.8

BBS/NP-40/BSA + Protease Inhibitors contains 0.001 *M* phenylmethylsulfonylfluoride (Sigma, St. Louis, MO), 0.02 *M* 2-iodoacetamide (Eastman Kodak, Rochester, NY), 0.01 *M* benzamidine hydrochloride (Sigma), 0.05 *M* ϵ-aminocaproic acid (Sigma), 10 ug/ml leupeptin (Sigma), 1 ug/ml pepstatin(Sigma), and 100 ug/ml soya bean trypsin inhibitor (Sigma), pH 8.8

Ferric-citrate complex, 6.25×10^{-3} *M* $FeCl_3 \cdot 6H_2O$, 6.25×10^{-2} *M* $Na_3Citrate$

PBS, 0.01 *M* PO_4^{3-} (potassium salt), 0.14 *M* NaCl, pH 7.4

PBS/BSA contains 0.05% BSA (ICN Nutritional Biochemicals, Cleveland, Ohio)

PBSG, 0.01 *M* PO_4^{3-} (sodium salt), 0.15 *M* NaCl, 0.1 *M* glycine, pH 7.3

SDS-PAGE Sample Buffer, 0.0625 *M* Tris-PO_4, 20% glycerol, 2% SDS, 0.005% bromphenol blue, pH 6.7, containing approximately 4.3 kBq Na^{22}/ml (260000

cpm/ml). Sample buffer was 0.2% 2-mercaptoethanol when running under reducing conditions.

SDS-PAGE Fixative, 4% sulfosalicylic acid, 12.5% trichloroacetic acid

SDS-PAGE Stain, 0.04% Coomassie Brilliant Blue R-250 (Eastman Kodak Co., Rochester, NY), 27% 2-propanol, 10% acetic acid

SDS-PAGE Destaining Solution, 12% 2-propanol, 7% acetic acid

SDS-PAGE Preservative, 12% 2-propanol, 7% acetic acid, 4% glycerol

Tris Buffer, 0.2 *M* Tris-HCl, 0.15 *M* NaCl, 0.05% NaN_3, pH 8.0

Rat Immunoglobulins

IR-162 myeloma IgE and normal rat IgG were prepared as described in the previous chapter.

Normal rat IgG_{2a} was prepared by ion exchange chromatography of a crude immunoglobulin preparation, similar to that from which the whole IgG fraction was purified by gel filtration in Chapter 2. This crude fraction was dialysed extensively, 5x24 hrs. against 10 volumes of 0.005 *M* PO_4^{3-} pH 8.0 before storage at -20°C, and 2x24 hrs. *vs.* 50 volumes of the same buffer immediately prior to use. Lots of approximately 590 mg. total protein at a concentration of 25 mg/ml were separated at room temperature on a 2.5 x 30 cm column of Whatman DE52 cellulose equilibrated with 0.005 *M* PO_4^{3-}, running at 60 ml/hr. The first peak to elute under starting conditions was concentrated by reduced pressure ultrafiltration in a Micro-ProDiCon (MPDC) apparatus (Bio-Molecular Dynamics, Beaverton, OR) with

simultaneous dialysis *vs.* PBS. Final concentration of the preparation was 24 mg protein/ml. Yields were typically 2-7 mg protein per run, depending on the immunoglobulin preparation used.

The sera of rats carrying the immunocytomas described below were kindly provided by Dr. H.Bazin (University of Louvain, Brussels, Belgium).

IgG_1, from the serum of LOU rats carrying the IR401 immunocytoma was prepared by flat bed isoelectrofocusing the protein obtained from the IgG peak of an Ultogel AcA34 gel filtration column (LKB Productor, Sweden) (see Chapter 2). As in Chapter 2, the crude preparation applied to the column was obtained by $(NH_4)_2SO_4$ precipitation, and the concentrated IgG fraction was absorbed with anti(rat IgE) Sepharose. This absorbed fraction was dialysed against 0.001 *M* NH_4HCO_3 (18 hrs. vs. 100 volumes), and electrofocused in lots containing approximately 55 mg total protein. The electrofocusing medium consisted of 6 g Ultrodex (LKB Produktor, Sweden) in a total volume of 150 ml, containing 5% ampholytes pH 5-8 (Phamalyte, Pharmacia, Upsala, Sweden). The Ultrodex slurry was cast on an 11x140 cm glass plate, evaporated approximately 28%, and run at 4°C for 18 hrs at 200 V, increasing to 400 V for 24 hrs, and 500 V for 48 hrs (constant voltage). The electrofocusing plate was divided into 7.5 mm fractions using a stainless steel grid, and the protein was eluted from the support medium using 5 ml aliquots of distilled water. IgG_1 was obtained in a single peak having an isoelectric point of 6.4. Ampholytes were removed from the concentrated preparation by gel filtration in tris buffer on a 1.6x52

cm Ultrogel AcA 44 column (LKB Productor, Sweden). Finally, the protein was dialysed against PBS and concentrated in the MPDC apparatus to a final concentration of 25 mg/ml.

IgG_{2c} was prepared from the serum of LOU rats carrying the IR221 immunocytoma. Preparation of the crude immunoglobulin fraction by $(NH_4)_2SO_4$ precipitation, separation of an IgG fraction by AcA34 gel filtration, and absorption of IgE was essentially as described in Chapter 2. IgG_{2c} is euglobulin in nature, and was precipitated from this semi-purified preparation by dialysis against 0.5% boric acid (2x50 volumes, 4°C) (130). The precipitated protein was collected by centrifugation at 5000 rpm for 30 minutes at 4°C in the HB-4 rotor of a Sorval RC2B centrifuge. After a single wash in 0.5% boric acid, the precipitate was dissolved in a minimum volume of BBS pH 8.8. The protein was subsequently dialysed against PBSG (500 volumes) and approximately 17 mg of this preparation, at 10 mg/ml, was applied to a 0.9x6.5 cm column of Protein A Sepharose (131) bearing 2 mg Protein A per ml Sepharose CL-4B (Pharmacia, Upsala, Sweden). The column was washed with PBSG until the absorbance at 280 nm of the effluent returned to baseline. The buffer was changed to PBSG containing 1.0 *M* NaCl, pH 6.0, and the peak eluting under these conditions was desalted on a Sephadex G25 column running PBSG. The desalted protein was concentrated by reduced pressure ultrafiltration with simultaneous dialysis against PBS.

IgA was obtained by $(NH_4)_2SO_4$ precipitation and AcA34 gel filtration of ascitic fluid from rats carrying the IR22 immunocytoma.

Purified IR27 and IR595 IgG_1, IR33 and IR418 IgG_{2a}, IR863 and RAHE-2 IgG_{2b}, and IR1148 IgG_{2c}, were kindly provided by Dr. H.Bazin (Brussels, Belgium). Briefly, the IR27 protein was purified by the use of a monoclonal mouse anti(rat light chain) antibody specific for the rat kappa-1a allotype (132). This antibody was coupled to a Sepharose 4B matrix, which was then used for affinity chromatography of ascitic fluid from kappa-$1b^+$ rats bearing the kappa-$1a^+$ IR27 immunocytoma.

The IR595, IR33, IR418, RAHE-2 and IR863 proteins were prepared by $(NH_4)_2SO_4$ precipitation of ascitic fluid or sera of rats carrying the various immunocytomas, followed by ion exchange chromatography on DEAE Cellulose in 0.05 *M* Tris-HCl, pH 8.0, eluting with a linear gradient of 0-0.2 *M* NaCl. The ion exchange purified protein was equilibrated with 0.05 *M* Tris-HCl, 0.3 *M* NaCl, pH 8.0 and applied to an AcA34 gel filtration column. The IgG peak from this column was subsequently subjected to preparative electrophoresis in 0.075M barbital-HCl, pH 8.6.

The IR1148 protein was purified by affinity chromatography on Protein A-Sepharose.

All of these preparations were dialyzed against PBS and lyophilized for storage. Prior to use, the lyophilized preparations were reconstituted in PBS, passed through a low binding 0.22 μm Millex-GV filter and concentrated in the MPDC apparatus with simultaneous dialysis against PBS.

'ChromPure' normal rat IgG $F(ab')_2$ and Fc preparations were obtained from Jackson Immunoresearch (Avondale, PA). All commercial immunoglobulin preparations were filtered and concentrated as described above prior to use.

The purity of all IgG preparations was assessed by radioimmunoassay of total IgE (as described in the previous chapter), by double diffusion in agar gel against a series of sub-class specific antisera, and by SDS-PAGE.

Other proteins

BSA (99% pure) was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio).

Transferrin (human) was obtained from Sigma (St. Louis, Missouri). Ferritransferrin was prepared by the addition of 0.1 ml ferric-citrate complex to 9.9 ml 0.04 *M* tris, 0.002 *M* $NaHCO_3$ containing 2.5 mg transferrin/ml, producing a final concentration of $6.25x10^{-5}$ *M* $FeCl_3$ and $6.25x10^{-4}$ *M* Na_3Citrate. The reaction mixture was vortexed briefly and dialysed against 100 volumes of 0.04 *M* tris, 0.002 *M* $NaHCO_3$ at 4°C overnight (133).

Immunodiffusion

Agar for both immunoelectrophoresis and immunodiffusion was prepared as described in Chapter 2. A 2 ml volume of the 1% agar was cast on each 1x3 inch microscope slide (precoated with a thin layer of 0.5% agar and dried). Six wells were punched in a

hexagonal pattern around a central well, using a 3 mm gel punch. Samples of 0.009 ml were applied to each well, and the slides were developed, washed and stained according the procedure described in Chapter 2 for the immunoelectrophoresis slides.

Cell culture

The cloned RBL3114 sub-line was used throughout these studies, and was maintained as described previously (Chapter 2).

Labelling

Due to relatively low signals from the receptor proteins, every effort was made to keep the background as low as possible. Cells were therefore handled very carefully to avoid rupturing the membrane and labelling cytoplasmic components, which might have bound to the IgG-Sepharose non-specifically.

In order to obtain as consistent results as possible, cells were consistently harvested for use the same number of days after seeding. BSA was included in all buffers after labelling to stabilize the cells. Omitting the BSA seemed to lead to problems in centrifugation.

The lactoperoxidase catalyzed ^{125}I labelling of the RBL cells was essentially as described in Chapter 2, except that each lot of cells was solubilized in *0.25* ml BBS/NP-40/*BSA+protease inhibitors*, producing an extract containing $2x10^7$ cell equivalents/ml. Furthermore, the cell extract was filtered through a low binding 25mm Millex GV filter

(0.22 um pore size, Millipore, Mississauga, Ontario) after centrifugation, to remove as much particulate and aggregated material as possible. A smaller 4 mm diameter filter was tried in the hope of taking advantage of its smaller dead volume, but it was rapidly blocked with this material. Filtering appeared to produce a significant improvement in the reproducibility of total ^{125}I cpm delivered to each reaction mixture, and reduced the likelihood of transferring labelled material to the reaction tube which could not be subsequently washed out.

Immunosorbent preparation

Preparation of the IgG-Sepharose conjugate was as described previously (Chapter 2).

Affinity chromatography

A substantial number of changes were made in the method of affinity chromatography due to a 10x reduction in the volume of the reaction mixture. Because of the small volume of Sepharose used for each sample in this assay, special consideration was given to certain technical points to ensure the assay's success.

Care was taken to avoid the generation of fines in all stages of Sepharose preparation and handling. Prior to use, 1 ml of IgG-Sepharose (sufficient for 100 samples) was transferred to a 5 ml glass vial and washed by repeated resuspension and settling at unit gravity in a total volume of 30 ml BBS/NP-40/BSA. This settling at unit gravity was important in that it allowed the Sepharose to be de-fined, markedly improving the reproducibility of the

inhibition results. Resuspension in each cycle was by inversion, again in an attempt to minimize the generation of fines. Following the last wash and complete settling, the volume of the supernatant was adjusted to give a 1:1 slurry, and the gel was maintained in suspension by careful stirring with a 1.5x8 mm micro magnetic stirring bar. Although excessively vigorous stirring of the washed slurry was avoided during aliquotting to minimize the generation of fines, if the Sepharose was not maintained in a homogeneous suspension during this procedure, variations could arise in the volume of the solid matrix delivered to each reaction tube.

Aliquots containing 0.020 ml of the suspension (0.010 ml IgG-Sepharose) were carefully transferred to 1.5 ml Eppendorf 'Flextubes' using a Microman 3-25 μl positive displacement pipette (Gilson Medical Electronics, Villiers-le-Bel, France), and the tubes were stored capped at 4°C awaiting the addition of the inhibitor proteins. Although similar, less expensive tubes are available, the Flextubes, were found to be superior in both ease of handling and dimensional uniformity. This dimensional reproducibility was important to keeping the residual volumes as similar as possible during aspiration of the washing buffers (see below). The use of a positive displacement pipette was important to the reproducible delivery of the slurry aliquots. Normal micropipettes were found to be unsuitable due to the adherence of the Sepharose to the walls of the pipette tip. The tip of the positive displacement pipette was marked with a felt pen on the ring just above the tip's opening to make it visible in the Sepharose slurry. The tip was only immersed in the slurry as far as this mark when picking up each aliquot. It was not touched off on the side of the tube

containing the stock suspension of washed Sepharose, but instead it was lifted directly out of the slurry. This procedure seemed to avoid carrying over various volumes of the slurry to the reaction tube on the outside of the tip, at the same time reducing any tendency of the Sepharose in the slurry to fall out of the pipette tip. On the other hand, the pipette tip *was* touched off on the inside of the reaction tube near the bottom, in order to make as complete a transfer of the slurry aliquot as possible.

All of the inhibitor proteins (at an average concentration of about 24 mg/ml) were deaggregated immediately prior to use by ultracentrifugation in an Airfuge (Beckman Instruments, Fullerton, CA) at 105,000 x g for 15 minutes. Sample volumes were typically around 0.090 ml, using 5x20 mm clear centrifuge tubes in an 18° A-100 rotor. Only about the top $4/5^{ths}$ of the supernatant was used. Serial dilutions of the inhibitors were prepared in PBS and 0.010 ml aliquots of these preparations were added to appropriate tubes containing IgG-Sepharose. In general, triplicate samples were run at each inhibitor concentration.

The inhibitor preparations were delivered to the tubes containing the Sepharose slurry using a 2-10 μl Eppendorf pipette. Although a drain time was included in the transfer, the pipette tip was slightly over filled to leave a small residual volume in the tip after delivering the aliquot, and replicate aliquots were delivered with the same tip, touching it off on the inside of the reaction tube just above the Sepharose slurry. A new pipette tip was used when changing to a different concentration of inhibitor protein. This reduced the

reproducibility problems arising from surface tension and tip wetting related to pipetting small replicate aliquots of protein solutions.

The IgG-Sepharose/inhibitor mixtures were vortexed briefly at low speed and centifuged for 2 minutes in an Eppendorf horizontal microfuge (Model 5413) (Eppendorf Geratebau, Hamburg, West Germany) to collect the slurry in the bottom of the tubes. Aliquots containing 0.010 ml of the filtered cell extract were delivered to each reaction tube (in order of increasing inhibitor concentration) with a 2-10 μl Eppendorf pipette, using the same technique as used to deliver the inhibitor protein preparations. Since the extract contained detergent, this helped to reduce the adverse effects of surface tension and wetting on reproducible sample delivery. The entire operation was carried out at 4°C, in view of the free receptor's heat lability. The total reaction volume was 0.040 ml. The tubes were capped and incubated overnight at 4°C inclined at 45° on a Gyrotory shaker Model G2 (Brunswick Scientific, Edison, NJ) set at 325 rpm. For this incubation, the tubes were held tightly in a rack made by boring holes in a styrofoam sheet with a cork borer. This helped to prevent the reaction mixture from splashing inside the tube, contaminating, and subsequently being lost on, the tube cap. Reaction times were varied between 1 and 16 hours with no obvious difference in the results.

To facilitate washing, the caps of the Flextubes were completely removed by cutting the plastic connection at the tube rim, and the tubes were placed in TJ-6 centrifuge racks (1.5 ml tube racks, Beckman Instruments). As in the incubation above, extreme care was taken

to avoid splashing the sample when removing the caps, bracing each tube on the bench top when cutting the cap connection. Each sample was washed 3 times with 1.00 ml BBS/NP-40/BSA followed by 2 washes with 0.0625 *M* Tris PO_4^{3-} pH 6.7, all at 0°. The washing buffer was delivered with an Eppendorf Repeater using a bare 12.5 ml Combitip pipette. Each wash cycle involved centrifugation of the samples at 2700 rpm for 5 minutes in a TJ-6 refrigerated centrifuge (Beckman Instruments) to collect the Sepharose, followed by the careful aspiration of the supernatant. A 25 gauge aspirator nozzle was constructed (Figure 3.1) which was designed to leave a volume of approximately 0.05 ml as reproducibly as possible. In order to minimize the disturbance of the Sepharose, the aspirator nozzle was held in position by a guide which allowed the nozzle to be moved in and out of the tubes with a minimal amount of sideways movement. The tip of the aspirator was only lowered at a rate that could be easily handled by the nozzle's flow rate. Lowering the tip to the bottom of the tube too rapidly resulted in an increase in aspirator vacuum, leading to an increase in buffer flow rate. This increased flow rate, with the nozzle opening close to the Sepharose, tended to entrain Sepharose, leading to losses. Therefore, the tip was lowered at a rate that resulted in considerable air being aspirated along with the buffer. Tests have indicated that the actual mean volume left in the tubes using this apparatus was 0.0541 ml (S.D.=0.00047, n=10).

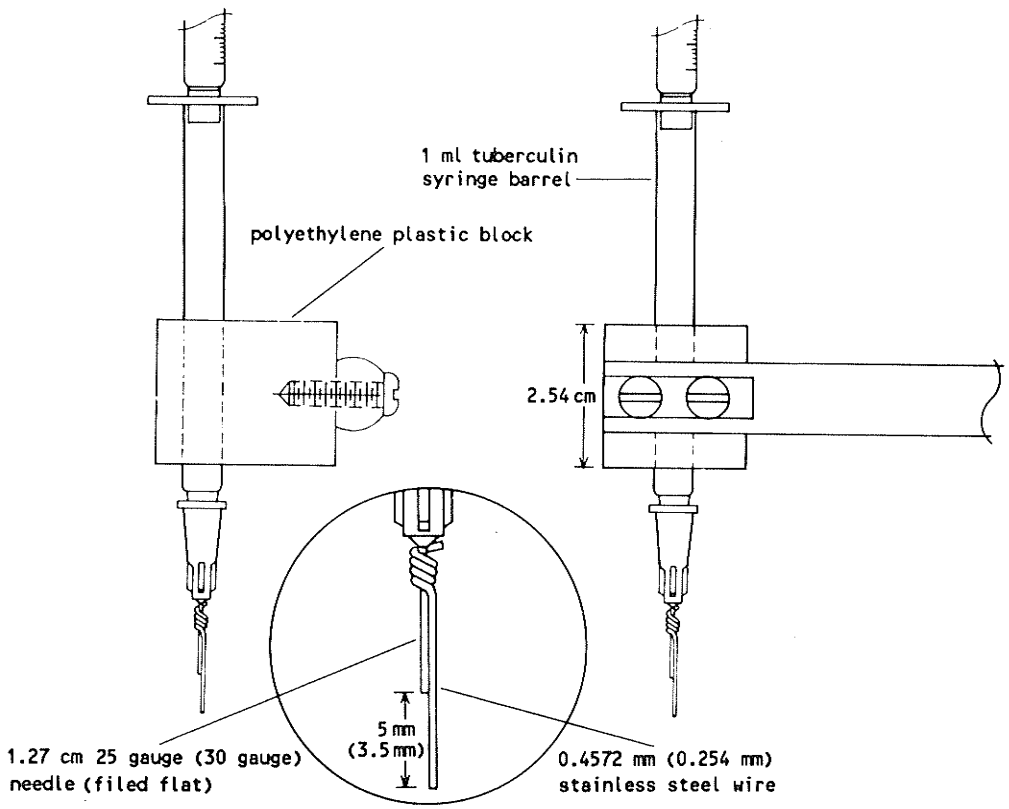


Figure 3.1 Aspiration device. A 25 g needle and a 30 g needle were modified so as to limit their descent into the reaction tubes when aspirating buffers used to wash the protein-Sepharose solid phase. The brace was constructed to hold the tip vertically and make the descent of the nozzle as reproducible as possible. The syringe barrel is held snuggly by the brace, but slides easily up and down. The dimensions in parentheses are those of the modified 30 g needle.

Following the last wash and aspiration of the supernatant, the total volume of the samples was further reduced to approximately 0.02 ml using a second 30 gauge nozzle. The delicate nature of this nozzle made the use of the positioning jig unadvisable. The nozzle was therefore hand held and carefully lowered into each tube while watching through the tube wall. Following this final aspiration, 0.050 ml of sample buffer was added to each tube using an Eppendorf Repeater with a 2.5 ml Combitip pipette carrying a yellow Eppendorf pipette tip. The tubes were recapped, vortexed briefly, centrifuged 2 minutes in an Eppendorf Model 5413 microfuge, heated for 1.5 minutes on a boiling water bath, and stored at 4°C until electrophoresis (overnight).

SDS-PAGE

SDS-PAGE was performed according to standard procedure (134), with few modifications. It should be pointed out, however, that the technique was changed considerably from that used in the previous chapter, in that *slab* gels were employed, rather than *tube* gels. Briefly, the resolving gel was 10% acrylamide, cast in a slab having dimensions of 14.2x12x0.15 cm, and contained 0.375 *M* Tris HCl pH 8.8. The stacking gel was 4% acrylamide, 1.5 cm long from the bottom of the slot to the resolving gel, contained 0.125 *M* Tris PO_4^{3-}, pH 6.7, and was generally cast using twenty well sample combs. The electrode buffer was 0.025 *M* tris, 0.192 *M* glycine, 0.1% SDS, and was pH 8.3 without adjustment. The resolving gel was cast at least 6 hours prior to use (more often the day before use, stored at 4°C overnight), and the gels were run as soon as possible after the casting of the stacking gel.

Storage at 4°C inevitably produced condensation in the sample tubes which was spun down in an Eppendorf horizontal microfuge (Model 5413) for 2 minutes. The samples were vortexed briefly after this centrifugation, collected by a similar centrifugation, and reheated at 100°C for 1.5 minutes. Total ^{125}I and ^{22}Na cpm of each sample were determined in a Gamma 8000 spectrometer (Beckman Instruments) prior to use. Volumes of 0.050 ml of each sample were applied to each gel slot, and the counts remaining in the sample tube were determined. The samples were carefully overlaid with electrode buffer and the gels were run at 20°C in a Protean slab cell (BioRad, Mississauga, Ontario) at a constant current of 20 mA per gel. Initial voltages were typically 67 v. Once the tracking dye was completely into the resolving gel, the current was increased to 30 mA per gel, and the run was continued until the tracking dye was within a few millimeters of the bottom of the gel. Total run time was consistently very close to 5 hours, with final voltages approximately 280 v.

Following the run the gels were fixed overnight, and then stained 2 hours. They were destained until the background was clear, soaked in preservative for at least 2 hours, and dried with heating under vacuum for 2 hours in a Hoeffer slab gel drier (Model SE 1150, Hoeffer Instruments, San Francisco, CA).

Autoradiography

Every effort was made to obtain a linear density increase in the autoradiograph as a function of the radioactivity in the protein bands on the gel. The dried gels were exposed to hypersensitized Kodak X-omat AR film at -70°C (135, 136, 137), using a Cronex Lightning Plus intensifying screen (Du Pont, Wilmington, DE) (138). The film was processed manually at 20°C according to the manufacturer's instructions. The density increase on pre-exposure and maximum band density were monitored on every autoradiograph to ensure that band density was linearly related to band radioactivity. In general, if the uninhibited control samples provided an SDS-PAGE sample containing 6000 cpm (total cpm ^{125}I applied), an exposure of 24 hours was about optimal.

The autoradiographs were scanned on a Helena Quick Scan R&D scanning densitometer (Helena Laboratories, Beaumont, TX), which was standardized using a T-14 Step Tablet (Eastman Kodak Co., Rochester, NY). The densitometer was connected to an HP 3390-A integrating plotter (Hewlett-Packard Canada, Mississauga, Ontario) to allow the determination of peak areas. Scans of each sample track started on the pre-exposed background, 1 cm before the top of the gel's image, and proceeded down the center of the track. The integration baseline was set shortly after the start of the scan and extended horizontally from this point. Instrument settings are listed in Appendix A.

Reproducible scans of the autoradiographs were highly dependent on smooth and consistent operation of the scan drive motor. In order to confirm accurate results, several scans were

performed for each track and the results of these scans were averaged. Values generally agreed between the scans within a maximum range of ±5% of the mean. Rapid scan speeds have the advantage of improved torque on the drive mechanism, and less time for the analysis, but these speeds may lead to decreased resolution of the bands. Slower speeds improve resolution and accuracy of the integrated peak areas, but result in lower torque on the drive motor, making variations in the scan speed more likely. In the final analysis therefore, these two points have to be balanced against one another. In view of the potential for mechanical inconsistencies in the drive mechanism, there would be a considerable advantage to using a photodiode array to scan the entire track at once. Stable line voltage to the scanner was also found to be extremely important. Transient line noise appeared to have a devastating effect on the reproducibility of the integration results.

Data analysis

Each dilution series of a particular inhibitor contained uninhibited and completely inhibited control samples. The uninhibited controls contained 0.0100 ml PBS, in order to maintain the reaction volumes constant for all samples. Completely inhibited controls received 0.0100 ml of a solution containing approximately 6 mg rat IgE/ml. Each SDS-PAGE gel covered one complete inhibitor dilution series, in order to maintain conditions as similar as possible for all samples requiring direct comparison.

The receptor peak areas, determined by scanning densitometry, were corrected to reflect the *total* sample on the basis of ^{22}Na cpm remaining in the sample tube after removing the 0.050 ml aliquot for SDS-PAGE.

The peak areas remaining in the presence of a maximally inhibiting concentration of IgE (0% bound) were subtracted to correct for background binding. The resulting areas were compared to the areas of the uninhibited controls (100% bound). Percent binding of the receptors relative to the uninhibited controls was plotted as a function of the inhibitor concentration.

Results

IgG subclass preparation

SDS-PAGE analysis

An SDS-PAGE analysis of each of the IgG subclass preparations, run under reducing conditions, is presented in Figure 3.2.

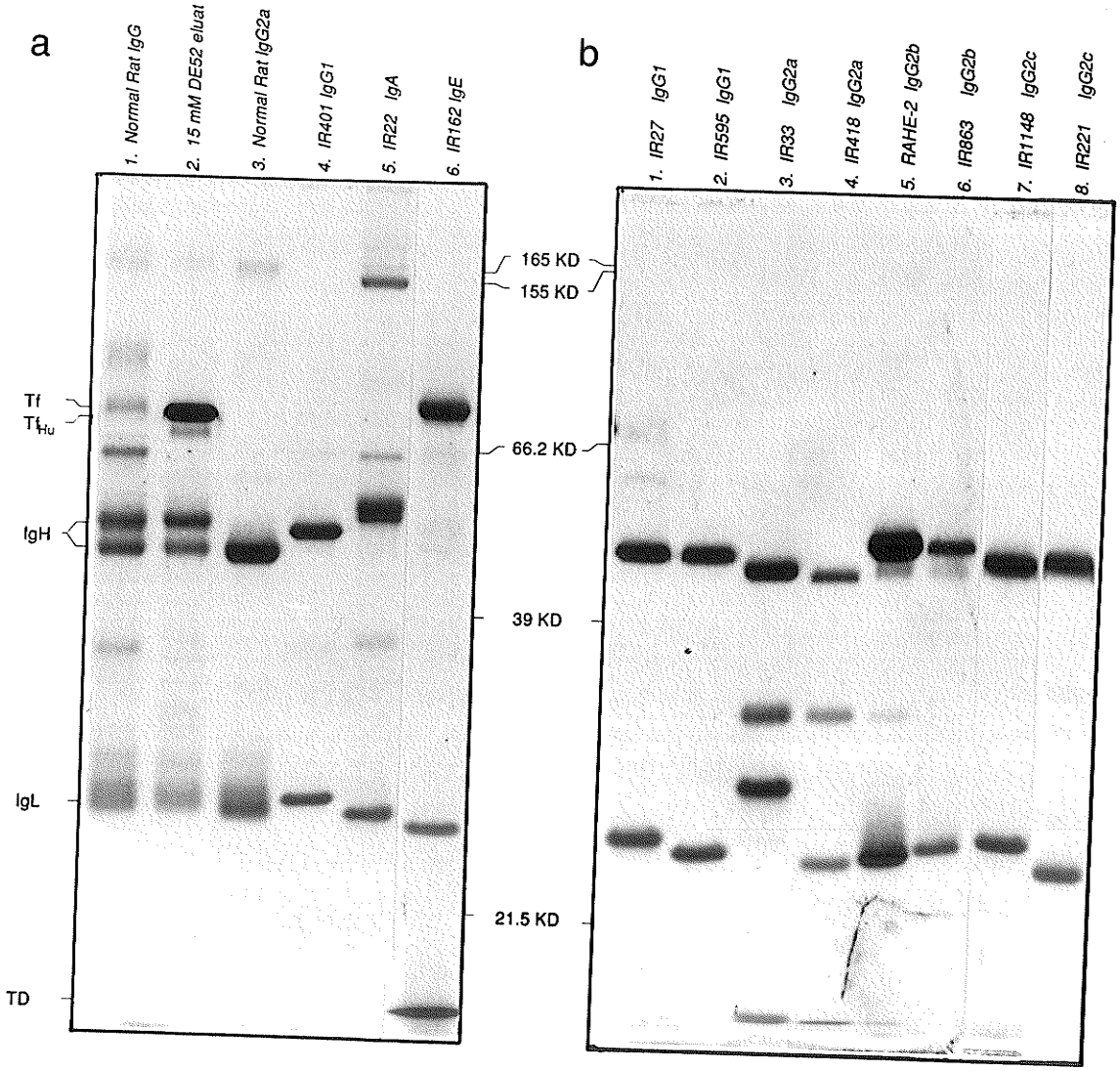


Figure 3.2 SDS-PAGE analysis of inhibitor protein preparations. The positions and molecular weights of various standard proteins are indicated. IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; kD, kilo Daltons; TD, tracking dye; Tf, transferrin; TfHu, human transferrin.

In panel (a), track 1 represents a sample of normal rat serum IgG prepared by $(NH_4)_2SO_4$ precipitation and AcA34 gel filtration. This is the IgG preparation bound covalently to the Sepharose solid phase to produce IgG-Sepharose. The preparation contains all of the rat IgG subclasses, and it is subsequently referred to simply as *rIgG*. While IgG specific bands account for the majority of the components, there was some contamination with albumin, transferrin and several other unidentified minor components.

The band identified as transferrin (Tf) ran with an apparent molecular weight slightly higher than that of a commercial preparation of human transferrin. However, rat serum proteins eluting from DE52 cellulose at 15 mM PO_4^{-3} had the same reddish color as the human transferrin preparation, had a very strong band in this region (shown in panel (a) track 2), and caused the same strong inhibition of transferrin receptor binding as human transferrin (see below). Taken together, these points strongly suggest that the indicated band was indeed rat transferrin.

An analysis of the IgG_{2a} protein, eluted from DE52 cellulose under starting conditions (0.005 *M* Na PO_4^{-3}, pH 8.00), is presented in panel (a) track 3. In contrast to the rIgG preparation (track 1), this IgG_{2a} preparation showed much better purity, with very few non-IgG bands and a single heavy chain band detected. The faint band with a molecular mass of about 150,000 daltons might be attributable to a small amount of unreduced IgG.

The IR401 IgG_1 preparation (panel (a), track 4) appeared to be extremely clean by this analysis, showing virtually *no* non-IgG components. Interestingly, the IR401 heavy chain had an apparent molecular weight somewhat higher than that of the DE52 cellulose purified IgG_{2a} (track 3). This also appeared to be characteristic of both the other two IgG_1 preparations (IR27 and IR595, panel (b) tracks 1 and 2 respectively) and of the two IgG_{2b} preparations (RAHE-2 and IR863, panel (b) tracks 5 and 6). Conversely, the two immunocytoma IgG_{2a}'s (IR33 and IR418, panel (b) tracks 3 and 4) both had heavy chains with lower apparent molecular weights, similar to the IgG_{2a} isolated from normal rat serum (panel (a) track 3). This therefore suggests that the high molecular weight heavy chain might be characteristic of the IgG_1 and IgG_{2b} subclasses, whereas the IgG_{2a} heavy chain runs with a somewhat lower apparent molecular weight. Since the rIgG preparation contained all of the subclasses, these subclass differences in heavy chain molecular weight would tend to explain the double heavy chain bands in the rIgG preparation (panel (a) track 1).

The IgG_{2a} preparations derived from the IR33 and IR418 immunocytomas (panel (b) tracks 3 and 4 respectively) both showed significant contamination with an unidentified (non-immunoglobulin) component which migrated in a position between the immunoglobulin heavy and light chain bands. Furthermore, the light chain of the IR33 protein (track 3) showed an unusually slow mobility, suggesting a higher than normal molecular weight for this part of the immunoglobulin.

One of the IgG_{2b} preparations (RAHE-2, panel (b) track 5) also showed some contamination with an unidentified component similar to that contained in the immunocytoma IgG_{2a} preparations. Furthermore, if a lower molecular weight heavy chain *is* characteristic of IgG_{2a}, then both the RAHE-2 (track 5) and the IR863 (track 6) IgG_{2b} preparations would appear to have contained small amounts of IgG_{2a} subclass protein.

Both of the IgG_{2c} preparations showed good purity (panel (b) tracks 7 and 8). The IgG_{2c} heavy chains seemed to display mobilities similar to those of IgG_1, running at a position somewhat heavier than the IgG_{2a} heavy chains.

Double diffusion analysis in agar gel with subclass specific antisera

The interaction of each IgG preparation with various, commercially available, subclass specific antisera was analyzed by double diffusion in agar gel. The results of this analysis are presented in Figures 3.3 through 3.7, in which the lowest total protein concentration (in mg/ml) still capable of producing visible precipitin bands in the agar gel is plotted for each of the antisera. Actually, the negative of the natural log of this protein concentration has been plotted here, since the protein concentrations analyzed covered several orders of magnitude, and the negative log makes the presentation somewhat more easily understandable. Thus, the higher the bar, the more the IgG preparation could be diluted and still give rise to a visible precipitin band. In other words, the higher the bar, the higher the concentration of the particular subclass in the protein preparation.

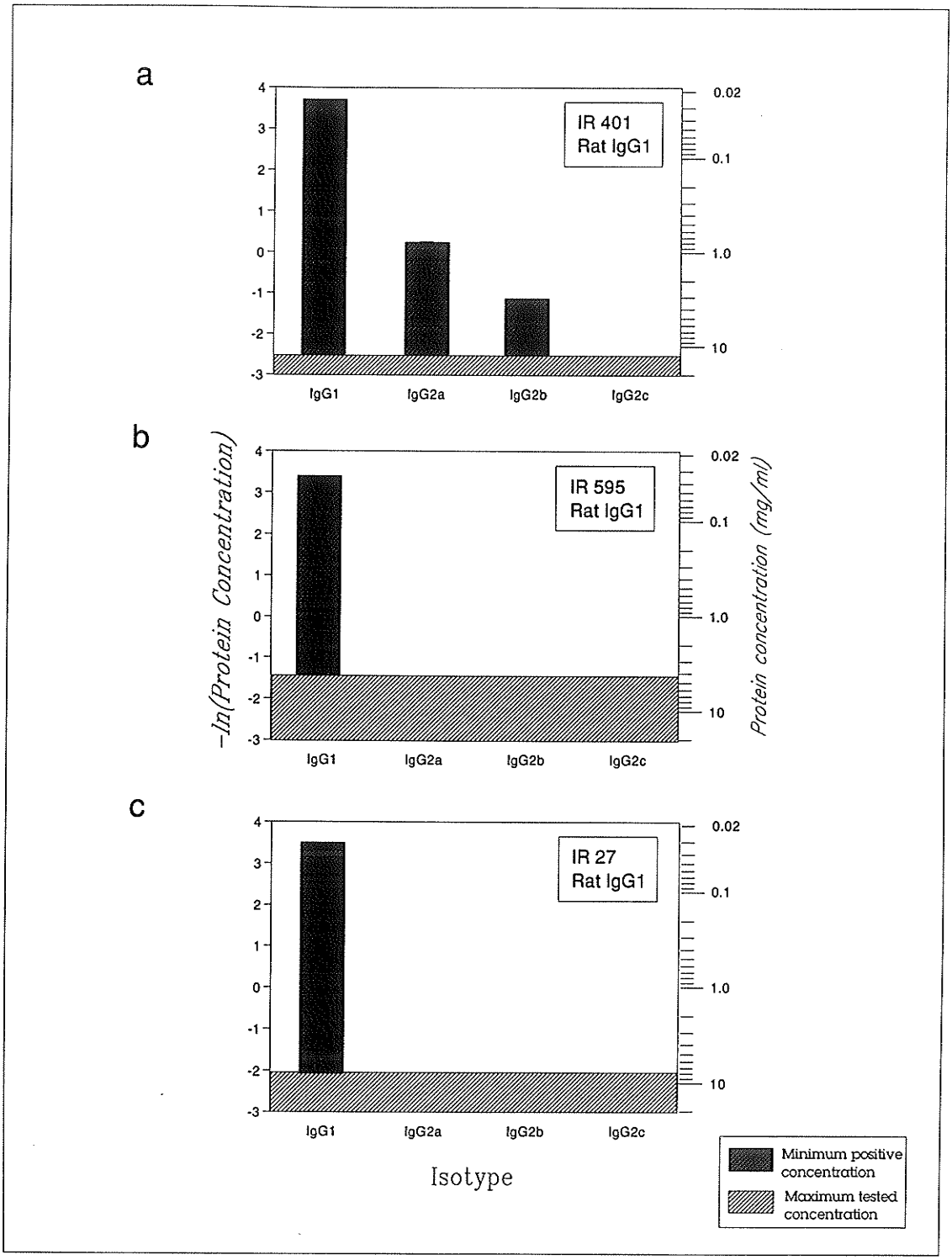


Figure 3.3 Diffusion analysis of IgG_1 subclass preparations. The height of the columns indicate the extent to which the preparation could be diluted and still produce a detectable precipitin band in double diffusion against subclass specific antisera.

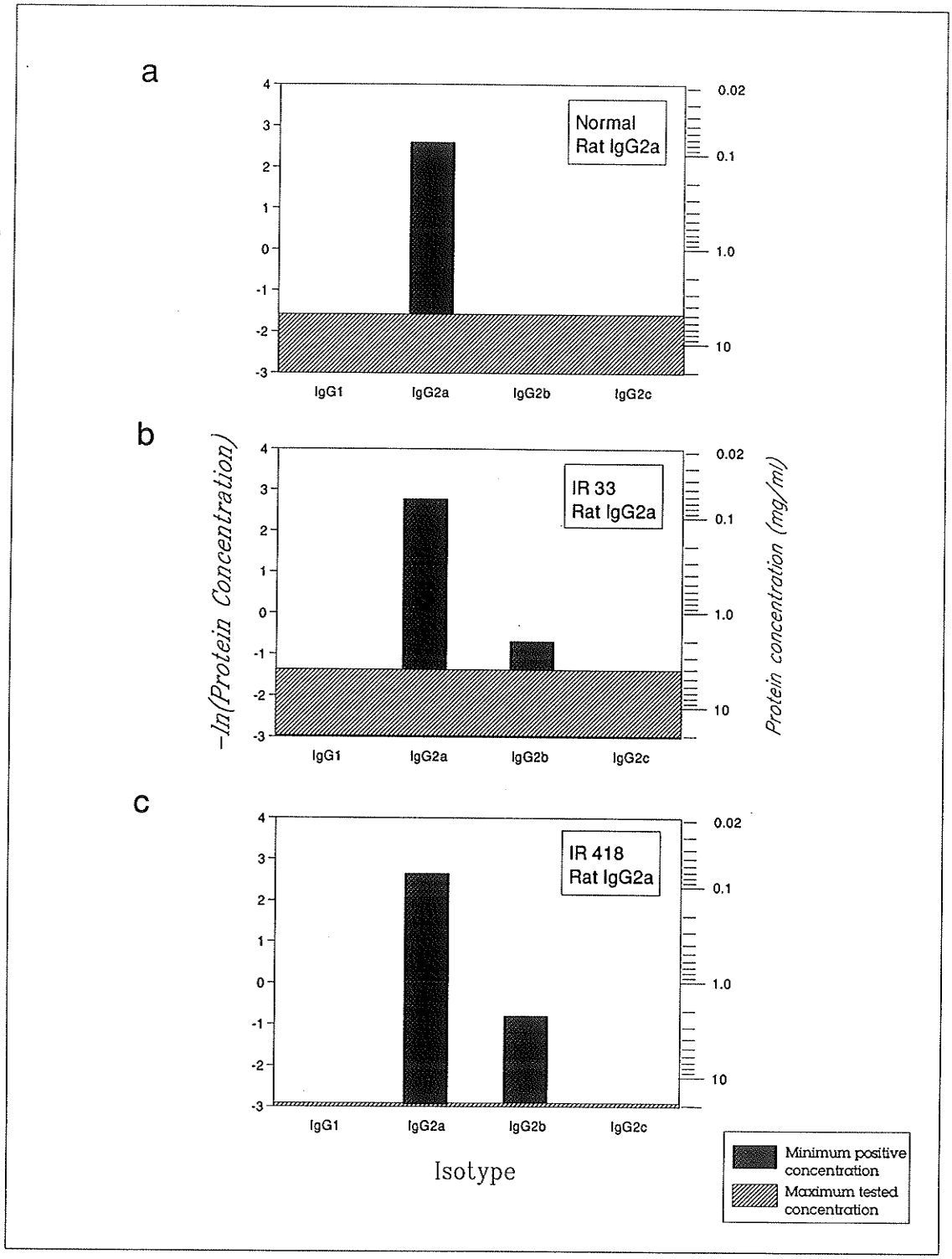


Figure 3.4 Diffusion analysis of IgG_{2a} subclass preparations.

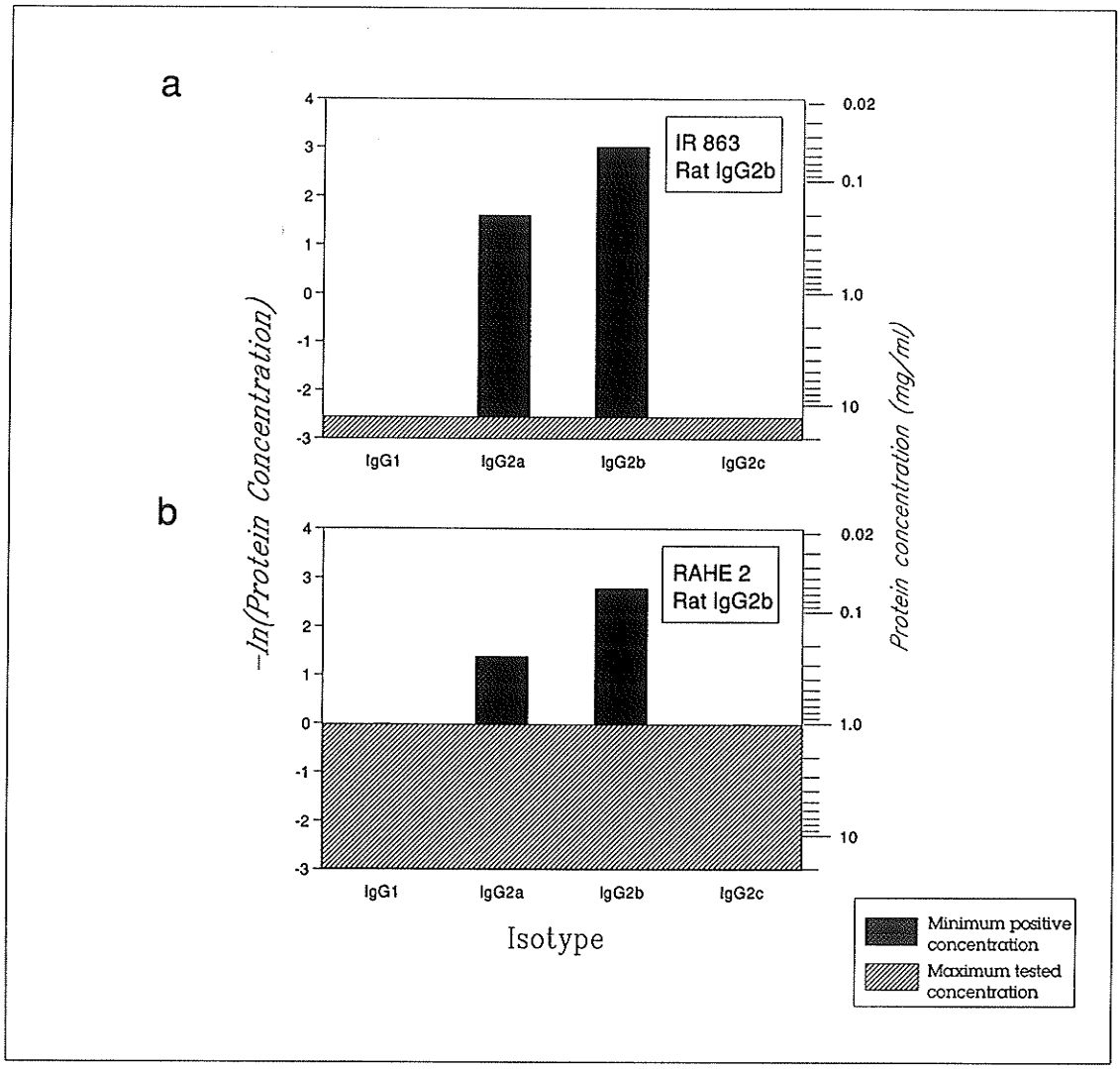


Figure 3.5 Diffusion analysis of IgG_{2b} subclass preparations.

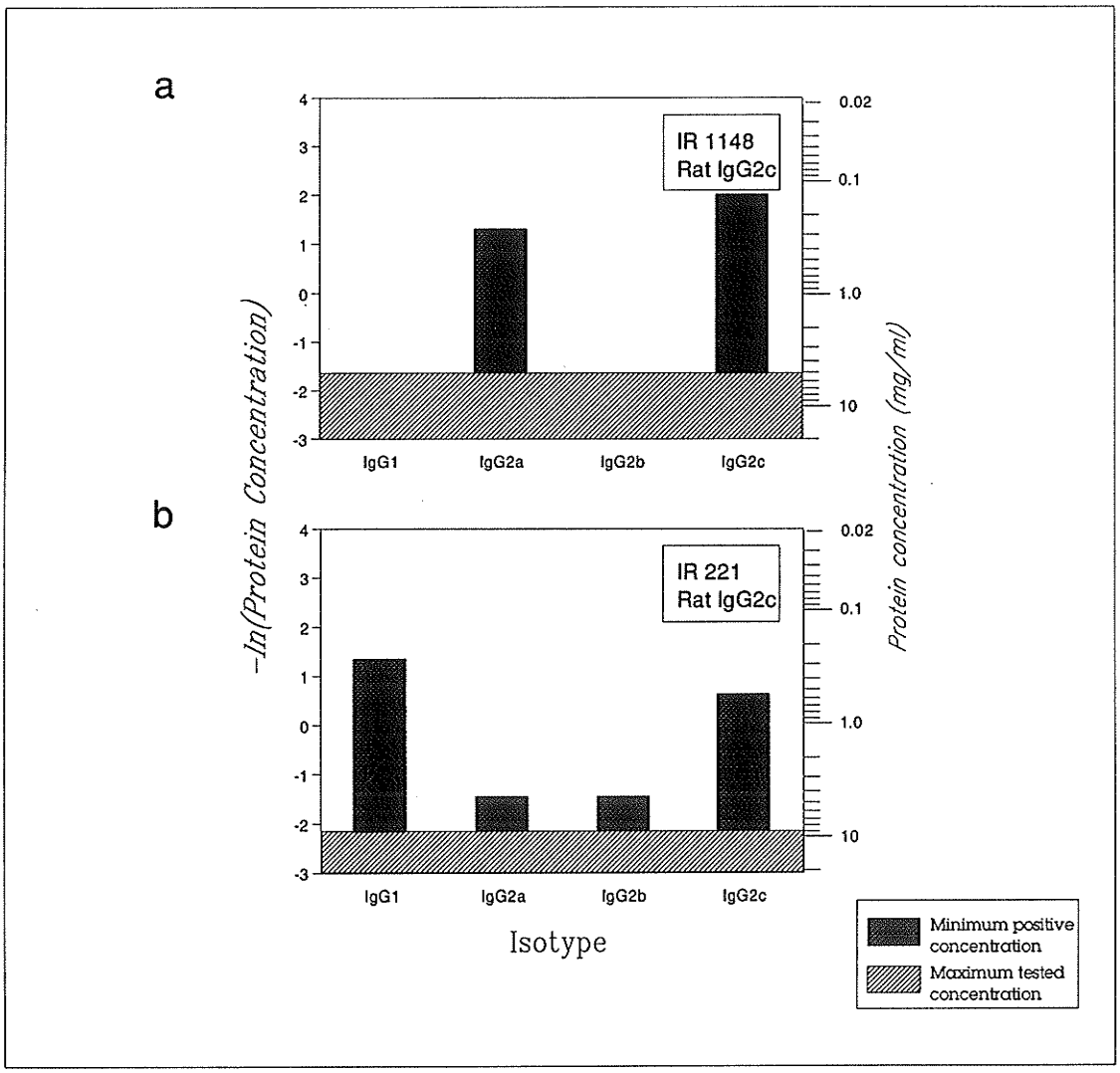


Figure 3.6 Diffusion analysis of IgG_{2c} subclass preparations.

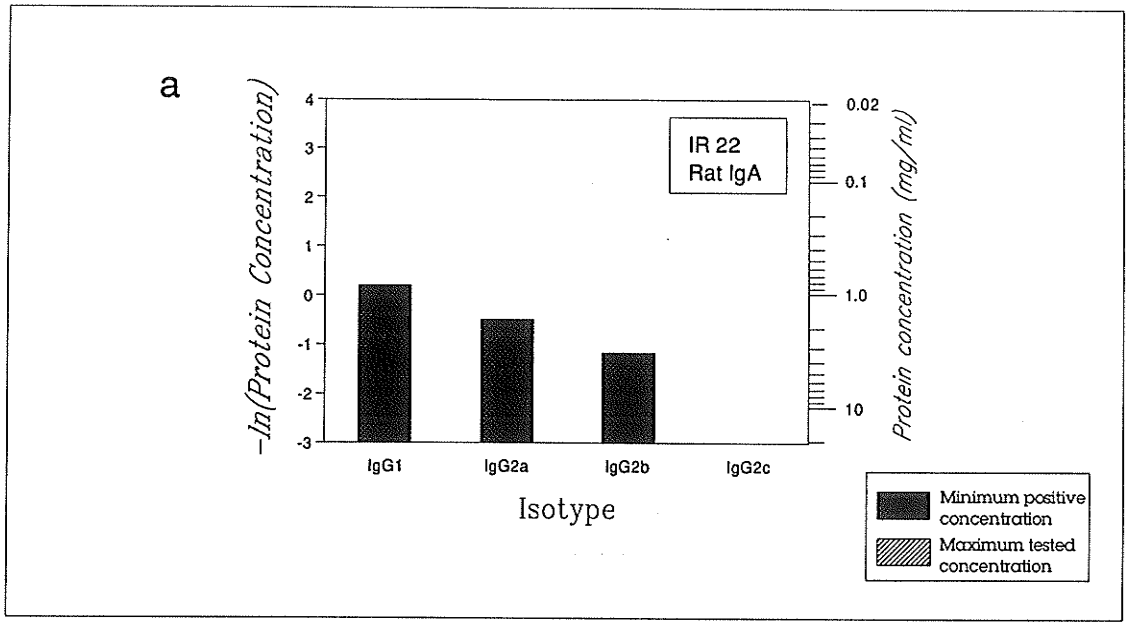


Figure 3.7 Diffusion analysis of the IR22 IgA preparation.

The level of the crosshatched portion of each graph represents the maximum protein concentration analyzed. The lower this level, the higher the maximum protein concentration tested, and the better the chance of detecting contamination of the preparation with other subclasses. Another way of thinking of this portion is as a type of "blind". The higher the crosshatched level (the lower the maximum protein concentration assayed), the greater the possibility that this blind is "hiding" some contaminant whose bar fails to extend above the dotted line.

In the event that the reasoning behind this presentation still remains unclear, the actual data upon which the graphs are based is presented in Appendix B, and actual protein concentrations are indicated down the right side of each graph.

Although SDS-PAGE analysis (above) suggested that the IR401 IgG_1 preparation was extremely clean, the results presented in Figure 3.3 panel (a) indicate that, while the *predominant* protein was IgG_1, the preparation still contained proteins which reacted with IgG_{2a}- and IgG_{2b}-specific antisera. On the other hand, neither the IR27 (panel (b)) nor the IR595 (panel (c)) IgG_1 preparations showed any detectable contamination with the other subclasses.

The diffusion analysis results for the IgG_{2a} preparations are presented in Figure 3.4. There was no detectable contamination of the normal rat IgG_{2a} (panel (a)), however both the

IR33 (panel (b)) and IR418 (panel (c)) preparations showed some contamination with IgG_{2b} proteins.

Both of the IgG_{2b} preparations (presented in Figure 3.5) showed considerable contamination with IgG_{2a}, supporting the suggestion (above) that the lower molecular weight heavy-chain bands seen in the SDS-PAGE analysis of the RAHE-2 and IR863 were attributable to $IgG2_a$ contamination.

Although both IgG_{2c} preparations showed high titres of the appropriate subclass protein, they also showed substantial levels of IgG_{2a} (in the case of IR1148) and IgG_1 (in the case of IR221) (Figure 3.6).

Analysis of IgE contamination by radioimmunoassay

Total IgE was below the limits of detectability (2.5 ng/ml) in all of the Ig preparations except the IR27 IgG_1. However, as shown in Table 3.1, IgE contamination of the IR27 protein only amounted to 0.001% of total protein.

Table 3.1 Evaluation of IgE contamination in rat IgG preparations

Ig preparation	Isotype	Assay Sample Protein Concentration (mg/ml)	Assay Sample IgE Concentration (ng/ml)	%IgE	IgE Concentration at Max. Inhibitor Concentration (molar)
Normal rat IgG	IgG	0.68	< 2.5	< 0.00037%	$< 2.0 \times 10^{-10}$
IR27	IgG_1	0.98	10.00	0.00103%	5.5×10^{-10}
IR595	IgG_1	1.20	< 2.5	< 0.00021%	$< 1.1 \times 10^{-10}$
IR33	IgG_{2a}	1.25	< 2.5	< 0.00020%	$< 1.1 \times 10^{-10}$
IR418	IgG_{2a}	1.12	< 2.5	< 0.00022%	$< 1.2 \times 10^{-10}$
Normal rat IgG2a	IgG_{2a}	0.67	< 2.5	< 0.00037%	$< 2.0 \times 10^{-10}$
RAHE-2	IgG_{2b}	0.58	< 2.5	< 0.00043%	$< 2.3 \times 10^{-10}$
IR863	IgG_{2b}	0.61	< 2.5	< 0.00041%	$< 2.2 \times 10^{-10}$
IR1148	IgG_{2c}	0.20	< 2.5	< 0.00125%	$< 6.7 \times 10^{-10}$
IR22	IgA	0.88	< 2.5	< 0.00028%	$< 1.5 \times 10^{-10}$

It should be pointed out that, although no IgE was detected in the IR1148 IgG_{2c} preparation, it was possible that IgE contamination may actually have been as high as 0.00125% and gone undetected due to the low total protein concentration of the RIA sample. None the less, even this level of contamination would only have amounted to a concentration of $6.7x10^{-10}$ *M* in the inhibition reaction mixture at the highest inhibitor concentration used in the study ($5.37x10^{-5}$ M). As shown below, this is well below the level of IgE found to cause detectable inhibition of the IgG-Sepharose/receptor interaction.

IR22 IgA and IR162 IgE preparations

Based on the elution volume of the IR22 IgA peak on gel filtration, this protein should have been dimeric. SDS PAGE analyses of the preparation is presented in figure 3.2 panel (a) track 5. The Ig heavy chain band showed the higher molecular weight expected for this immunoglobulin class (see Chapter 1), and, based on this analysis, the protein would appear to have been relatively pure for a simple gel filtered preparation. As previously mentioned in the case of the normal IgG_{2a} preparation, the band at high molecular weight on the SDS gel might have been attributable to partially reduced components. Surprisingly, although no IgG heavy chain bands were evident in the SDS-PAGE analysis, diffusion analysis of the IgA preparation indicated substantial amounts of all IgG subclasses except IgG_{2c} (Figure 3.7).

The isoelectric focused IR162 IgE preparation (Figure 3.2, track 6) showed the much more slowly migrating heavy chain (consisting of 5 domains) characteristic of IgE, and extremely

little contamination with any other proteins. The large amount of very low molecular weight material which migrated with the tracking dye is attributable to ampholytes remaining in the sample from the isoelectric focusing run.

Microassay

Typical results obtained with the modified inhibition assay are presented in Figure 3.8, illustrating the effects of increasing concentrations of normal rat IgG_{2a} on the interaction of the H and R Fc receptor proteins with IgG-Sepharose. The autoradiograph clearly shows that both H and R binding (band density) decreased as the concentration of IgG_{2a} was increased, and that the results are highly consistent for each of the replicates at any particular IgG_{2a} concentration.

The transferrin receptor (TfR) band was present on all autoradiographs as a result of transferrin contamination of the IgG preparation coupled to the Sepharose. Such contamination is inevitable in an IgG preparation purified from normal serum by simple ammonium sulfate precipitation and gel filtration, and is due to the poor separation of the IgG and transferrin peaks on gel chromatography (see Figure 2.1 in the previous chapter). The problem is aggravated considerably by the extremely strong interaction between the transferrin receptor and its ligand. None the less, protein purification techniques capable of reducing this contamination were intentionally avoided, in order to prevent the separation of the IgG subclasses present in normal rat serum.

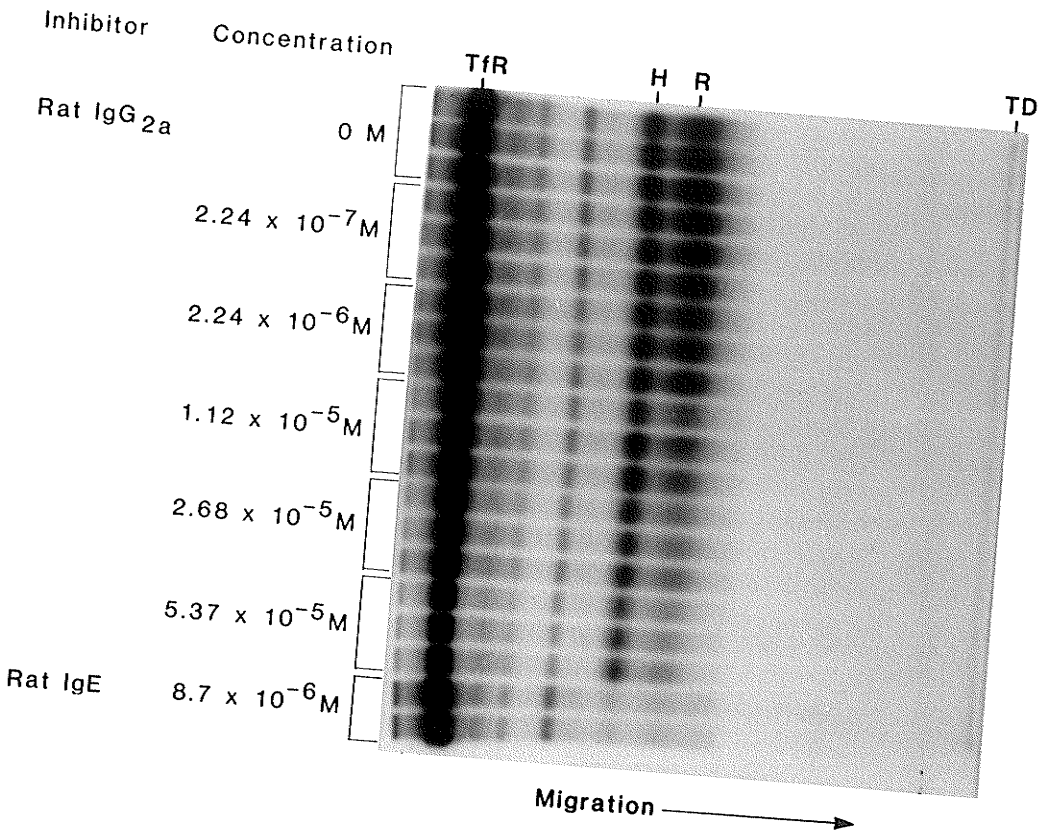


Figure 3.8 SDS-PAGE autoradiograph illustrating effects of increasing concentrations of normal rat IgG_{2a} on the interaction of the H and R Fc receptor proteins with IgG-Sepharose. The autoradiograph clearly shows that both H and R binding (band density) decreased as the concentration of IgG_{2a} was increased, and that the results are highly consistent for each of the replicates at any particular IgG_{2a} concentration. TfR indicates the position of the transferrin receptor band (see Figure 3.9). TD, tracking dye.

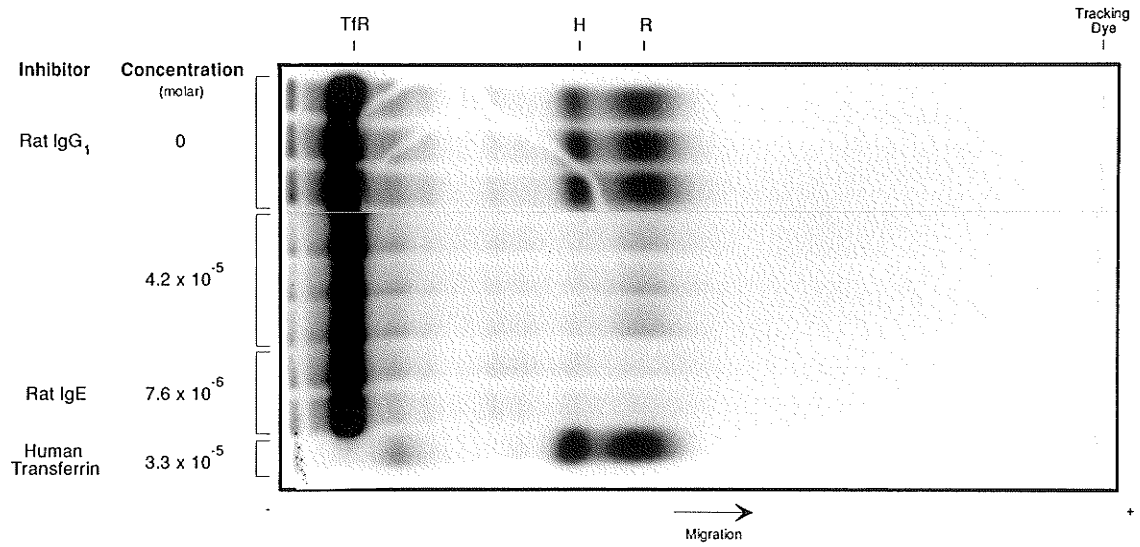


Figure 3.9 Inhibition of transferrin receptor binding to IgG-Sepharose by human transferrin. Transferrin receptor (TfR) binding was unaffected by concentrations of IgG or IgE which strongly inhibited Fc receptor binding, but completely inhibited by comparable concentrations of soluble human transferrin. Conversely, H and R binding are not inhibited (and may be enhanced) by this concentration of transferrin.

The narrow band lying immediately to the left of H in Figure 3.8 is thought to be transferrin itself, bound to the cell surface via the receptor and labelled along with the other membrane proteins. Since the transferrin receptor is divalent (139), receptor bound transferrin is bound to the IgG-Sepharose via the second binding site. As shown in Figure 3.9, the TfR/IgG-Sepharose interaction (and, although not as clear on this gel, the transferrin/TfR/IgG-Sepharose interaction) was completely inhibitible by soluble human ferri-transferrin, whereas H and R binding were unaffected. In fact, FcR binding may actually have been enhanced somewhat in the presence of soluble transferrin, suggesting that the TfR/IgG-Sepharose interaction interferes to some degree with H and R binding to the solid phase (steric interference). The highly purified IgG preparations generally produced only marginal inhibition of TfR binding at their highest concentrations; attributable to some slight contamination of even these preparations with transferrin. Other minor bands also seen on the autoradiographs were not inhibited by any of the immunoglobulin preparations, indicating that they represented membrane components bound non-specifically by the IgG-Sepharose.

Table 3.2 Densitometric analysis data for Figure 3.8

SDS-PAGE Sample Number	IgG2a Molar Concentration	Average Peak Area[1] H	Average Peak Area[1] R	^{22}Na Volume Marker Data Total	Remaining	Fraction Used	Correction Factor	Corrected Peak Area H	Corrected Peak Area R	Percent of Control H	Percent of Control R	Percent Receptor Bound H Avg. ± S.D.	Percent Receptor Bound R Avg. ± S.D.
1.	0.00	1.29	2.30	14548	5114	0.648	1.5421	1.99	3.55	93	91		
2.	0.00	1.43	2.55	14470	4934	0.659	1.5174	2.16	3.87	103	101		
3.	0.00	1.38	2.58	14026	5163	0.632	1.5825	2.18	4.08	104	108	100 ± 5	100 ± 7
4.	$2.24x10^{-7}$	1.38	2.50	14233	4941	0.653	1.5317	2.11	3.82	100	100		
5.	$2.24x10^{-7}$	1.28	2.46	14145	5211	0.632	1.5833	2.03	3.90	95	102		
6.	$2.24x10^{-7}$	1.27	2.25	14357	4924	0.657	1.5220	1.94	3.43	90	86	95 ± 4	96 ± 7
7.	$2.24x10^{-6}$	1.34	2.12	14061	5222	0.629	1.5908	2.14	3.37	102	84		
8.	$2.24x10^{-6}$	1.32	2.08	14200	4968	0.650	1.5381	2.04	3.20	95	79		
9.	$2.24x10^{-5}$	1.30	2.07	13997	5331	0.619	1.6152	2.10	3.35	99	84	99 ± 2	82 ± 2
10.	$1.12x10^{-5}$	1.09	1.36	14293	5305	0.629	1.5902	1.74	2.16	78	44		
11.	$1.12x10^{-5}$	1.28	1.55	14469	5348	0.630	1.5863	2.03	2.47	95	54		
12.	$1.12x10^{-5}$	1.33	1.48	14428	5242	0.637	1.5707	2.08	2.33	98	50	90 ± 9	49 ± 4
13.	$2.68x10^{-5}$	1.13	1.08	14285	4981	0.651	1.5354	1.73	1.66	77	27		
14.	$2.68x10^{-5}$	1.03	1.05	14479	5202	0.641	1.5607	1.61	1.63	70	26		
15.	$2.68x10^{-5}$	1.01	1.04	14164	5216	0.632	1.5829	1.60	1.64	69	26	72 ± 4	27 ± 0
16.	$5.37x10^{-5}$	0.81	0.76	14493	5210	0.641	1.5612	1.26	1.18	49	11		
17.	$5.37x10^{-5}$	0.87	0.71	14253	5076	0.644	1.5531	1.35	1.10	55	8		
18.	$5.37x10^{-5}$	0.80	0.76	14312	4949	0.654	1.5286	1.22	1.16	47	10	50 ± 3	10 ± 1
19.	IgE[2]	0.32	0.62	14412	4933	0.658	1.5204	0.49	0.95				
20.	IgE	0.25	0.48	14319	5175	0.639	1.5659	0.40	0.75				

1. Peak areas are given in arbitrary units.
2. IgE concentration $8.7x10^{-6}$

Quantitative analyses of the autoradiographs were based on densitometer scans of each track, and plots of density *vs.* position were integrated automatically with an HP 3390-A integrating plotter. Table 3.2 lists both the raw peak areas resulting from scans of the autoradiograph presented in Figure 3.8 and the correction factors applied to these data to account for the fraction of the total sample actually analyzed in the SDS-PAGE run. These correction factors are based on the use of a ^{22}Na volume marker, and are discussed in greater detail later in the discussion of this section. Background binding was determined on the basis of the peak areas remaining in the receptor region in the presence of sufficient IgE to produce maximal inhibition of the receptor/IgG-Sepharose interaction (the bottom two tracks in Figure 3.8). This background was subtracted from the corrected peak areas, and the resulting values are reported as a percentage of the uninhibited controls (100%). The relatively low standard deviations of these results confirm the reproducibility of the technique.

Inhibition curves

The results of analyses similar to that of Table 3.2 are presented graphically in Figures 3.10-3.15, where IgG-Sepharose binding of the H and R proteins has been plotted as a function of the concentration of the various soluble test proteins (inhibitors).

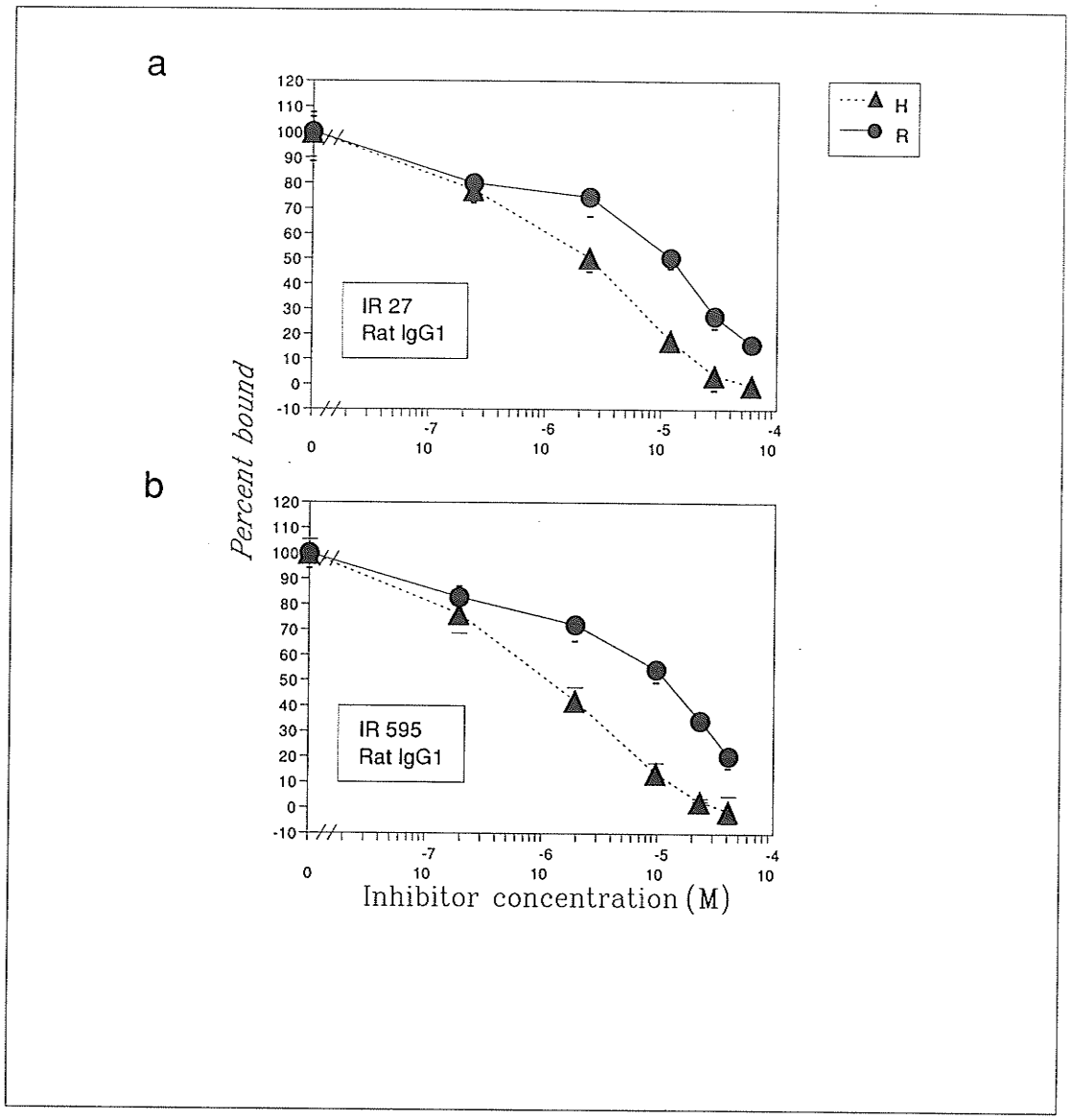


Figure 3.10 Inhibition of H and R binding to IgG-Sepharose by various concentrations of rat IgG_1.

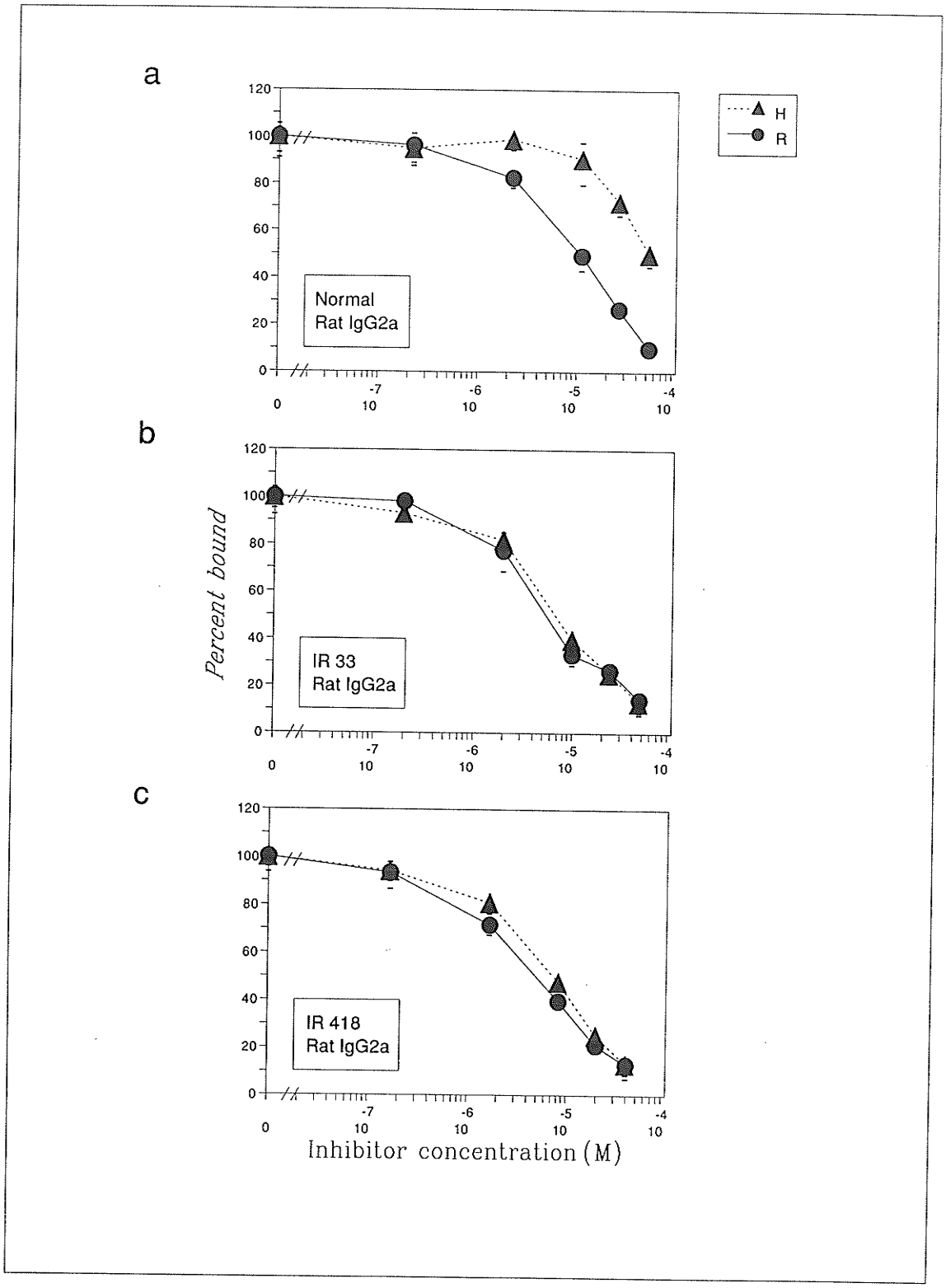


Figure 3.11 Inhibition of H and R binding to IgG-Sepharose by various concentrations of rat IgG_{2a}.

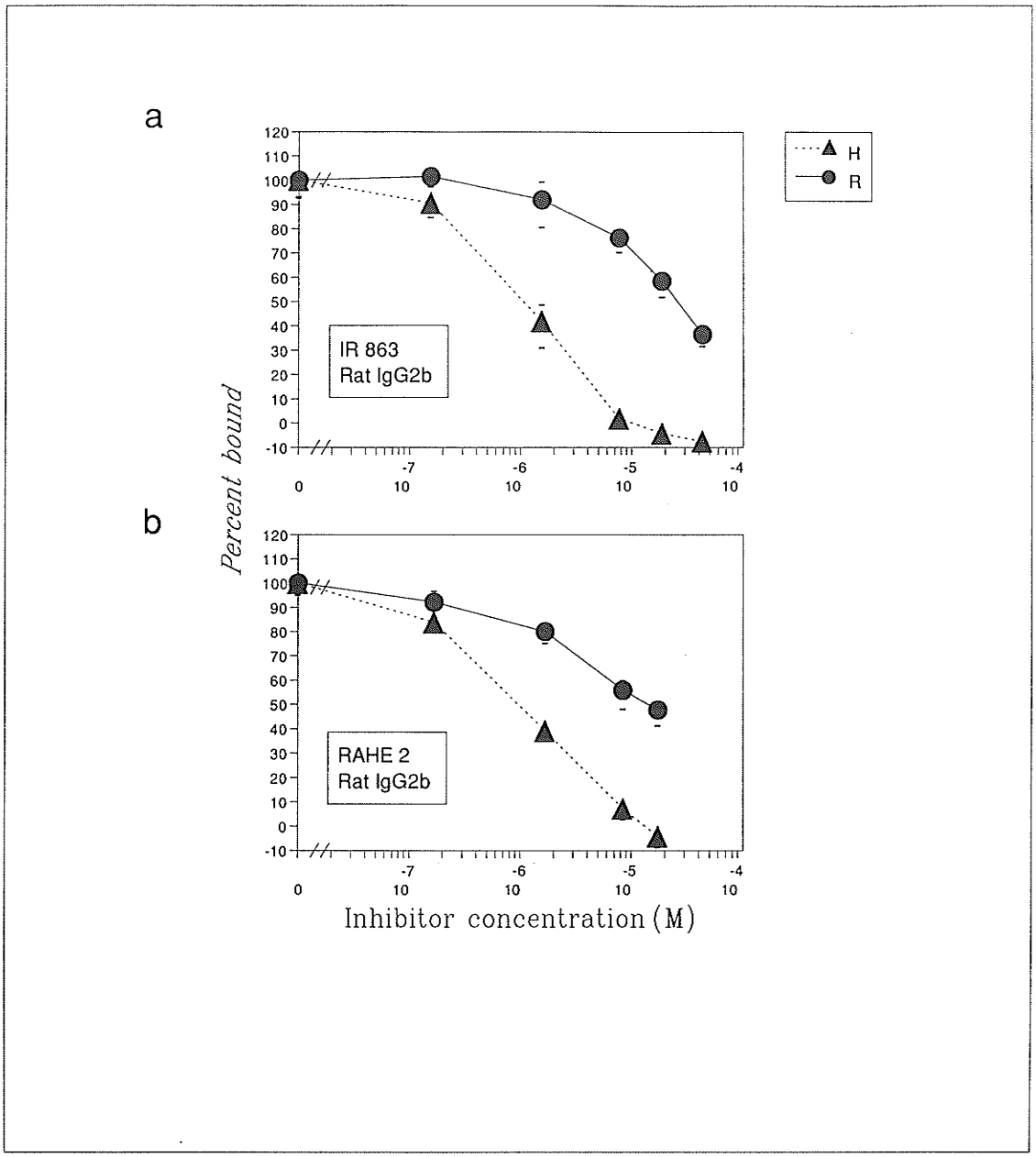


Figure 3.12 Inhibition of H and R binding to IgG-Sepharose by various concentrations of rat IgG_{2b}.

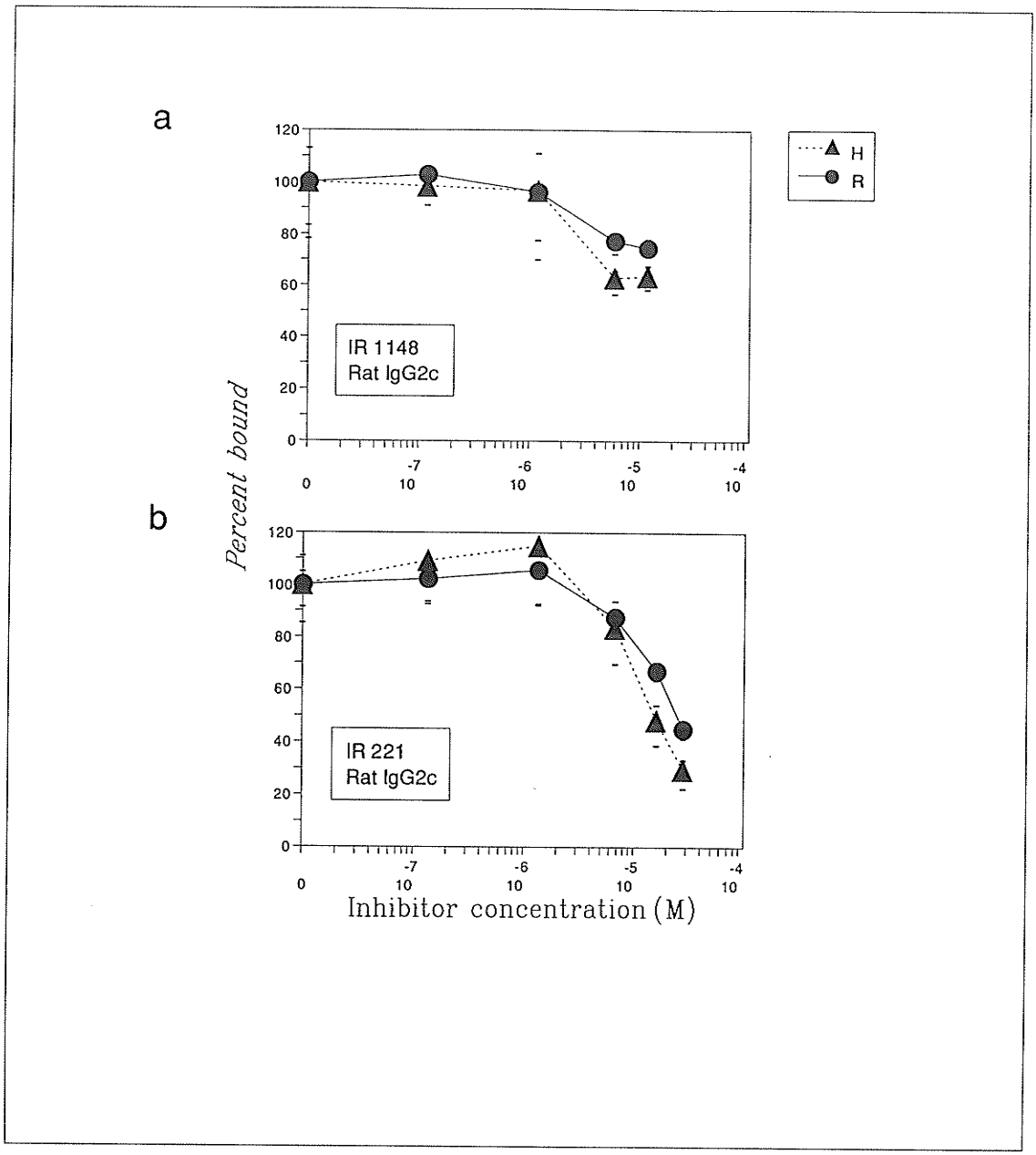


Figure 3.13 Inhibition of H and R binding to IgG-Sepharose by various concentrations of rat IgG_{2c}.

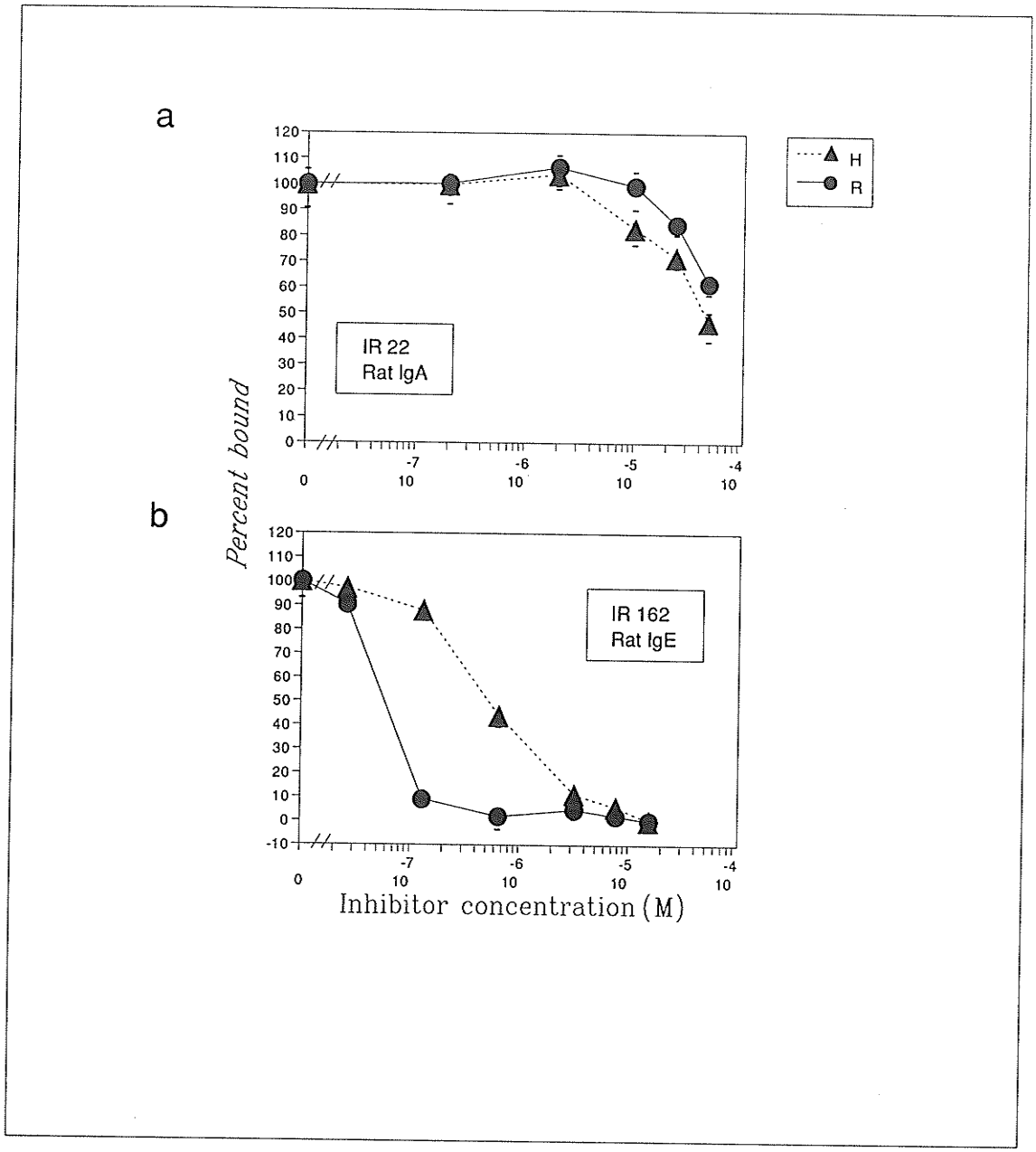


Figure 3.14 Inhibition of H and R binding to IgG-Sepharose by various concentrations of rat IgA or IgE.

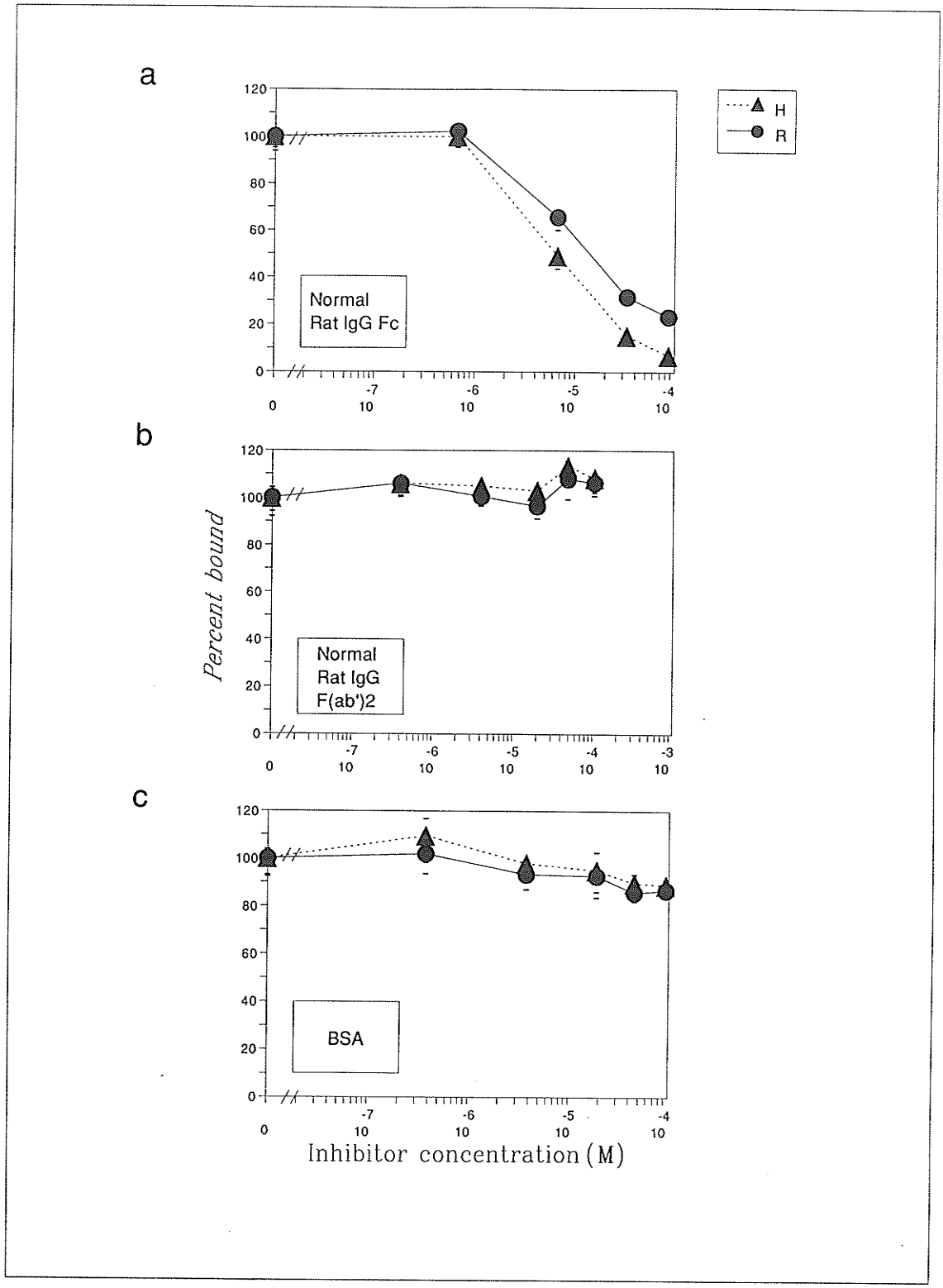


Figure 3.15 Inhibition of H and R binding to IgG-Sepharose by various Fc, $F(ab)'_2$ or BSA concentrations.

Comparing different panels within each figure, the graphs show that, generally, the pattern of binding inhibition is very similar for different proteins of the same subclass . The only obvious exceptions to this trend are the IgG_{2a} preparations (presented in Figure 3.11), and this discrepancy is discussed below. It should also be pointed out that since it was not possible to assay all of the proteins on the same day with the same cell extract, a normal rat IgG_{2a} inhibition series was run in each experiment in order to allow for comparison of results obtained with cell extracts prepared on different days. Although the data are not presented, repeated uses of this same protein gave very similar inhibition results in each assay.

It is apparent from a comparison of Figures 3.10-3.12 that the general pattern of IgG_{2a} inhibition (Fig 3.11) differed significantly from those of IgG_1 and IgG_{2b} (Figs. 3.10 and 3.12 respectively). Thus, IgG_{2a} can be seen to have inhibited the binding of R more strongly than it inhibited H binding, while the situation was reversed with the other two subclasses. Normal IgG_{2a} (Figure 3.11a) appeared to differentiate between the H and R proteins considerably more than its myeloma counterparts (Figure 3.11, panels (b) and (c)) did, but these discrepancies between the normal and myeloma preparations were likely attributable to contamination of the myeloma proteins with IgG_{2b} (see diffusion analysis above). As can be seen in Figure 3.12, IgG_{2b} showed a strong preference for the H protein, and any contamination of the IgG_{2a} preparations with this subclass would therefore tend to enhance the inhibition of H binding, decreasing the separation of the H and R inhibition curves.

The results obtained with the IgG_{2c} proteins (Figure 3.13) were inconclusive. Diffusion analysis of the two preparations of this subclass indicated that the IR1148 protein was contaminated with IgG_{2a}, while the IR221 appeared to contain IgG_1 contaminants. It was also difficult to obtain high concentrations of these proteins, since they tended to aggregate and precipitate extremely easily. Thus, the IR1148 inhibition curves could not be extended to higher concentrations to confirm the trend suggested in Figure 3.13a. None the less, it would *appear* that IgG_{2c} interacts preferentially with the H protein, similar to the IgG_1 and IgG_{2b} subclasses. Contamination of the IR1148 preparation with IgG_{2a} would have tended to preferentially inhibit R binding, decreasing the apparent distinction between the H and R proteins. This would suggest that, in the absence of IgG_{2a} contamination, H binding would be substantially more strongly inhibited than R binding, *enhancing* the trend suggested in Figure 3.13a.

Inhibition curves for IR22 IgA and IR162 IgE are presented in Figure 3.14. As mentioned above, although the IgA preparation appeared relatively clean by SDS-PAGE analysis, it showed somewhat surprising reactivity with anti(IgG) antisera in double diffusion analysis. Interaction of the receptors with the IgA preparation at high protein concentrations (Figure 3.14a) may therefore be consistent with IgG contamination. These results with IgA should therefore be considered somewhat preliminary, requiring confirmation with more highly purified IgA preparations. In any case however, it can be concluded that the H and R receptor proteins have a *markedly* lower affinity for IgA than for IgG. This is in marked contrast to IR162 IgE (Figure 3.14b) which strongly inhibited both H and R binding at low

concentrations (as would be expected). It should be noted however that there was very little inhibition at an IgE concentration of $2.6x10^{-8}$ M. The IgE RIA data indicated that the maximum concentration of IgE due to contamination of the IgG preparations would only amount to approximately $6.7x10^{-10}$ M. As mentioned above, this makes it extremely unlikely that inhibition of the FcR/IgG-Sepharose interaction might be attributable to IgE contamination of the IgG preparations.

Figures 3.15a-c demonstrate the Fc region specificity of the IgG/receptor protein interaction. Although the subclass composition of the Fc preparation was unknown, the preparation strongly inhibited both H and R binding to the IgG-Sepharose matrix (Figure 3.15a). Neither $F(ab')_2$ fragments (Figure 3.15b) nor BSA (Figure 3.15c) were capable of any significant inhibition over the range of concentrations tested.

The concentrations of each preparation required to inhibit receptor binding by 50% were determined from the various inhibition curves and are listed in Table 3.3.

Table 3.3 Inhibition of H and R binding to IgG-Sepharose by various rat immunoglobulins

Inhibitor	Immunocytoma	Molar concentration for 50% inhibition ($\times 10^{-6}$) H	R
IgG_1	IR27	2.7	12.4
IgG_1	IR401	4.0	15.0
IgG_1	IR595	1.4	13.0
IgG_{2a}	(Normal)	31.1 ± 12	9.8 ± 1.2
IgG_{2a}	IR33	7.8	6.8
IgG_{2a}	IR418	7.8	6.2
IgG_{2b}	IR863	1.2	28.3
IgG_{2b}	RAHE-2	1.2	14.6
IgG_{2c}	IR1148	> 12.0	> 12.0
IgG_{2c}	IR221	15.6	25.4
IgA	IR22	44.7	> 48.2
IgE	IR162	0.583	0.078
Fc	---	6.7	18.8
F(ab')2	---	N.I.[1]	N.I.
BSA	---	N.I.	N.I.

1. No significant inhibition

Average results and standard deviations (n=9) are listed for normal rat IgG_{2a}, based on its repeated use as a control in each experiment. Based on the data presented in the Table, it is possible to rank the subclasses in order of their affinities for each of the receptors. The higher the concentration required to induce 50% inhibition of binding, the lower the affinity of the interaction. Thus, for H the binding affinities were of the order:

$$IgE > IgG_{2b} > IgG_{1} > IgG_{2a}.$$

IgG_{2c} is not ranked due to uncertainties about the results obtained with both preparations of this subclass (see above). Note that, although the IgE/H interaction is stronger than any of the IgG/H interactions, it is not *vastly* greater, particularly in comparison to the affinity of the interaction between the H protein and IgG_{2b}. For R, the affinities were of the order:

$$IgE >> IgG_{2a} > IgG_{1} > IgG_{2b}.$$

In this case, the affinity of the IgE/R interaction *is* markedly greater than any of the IgG/R interactions (by about 2 orders of magnitude). It should be noted that, while IgG_{2a} binds to R more strongly than any of the other subclasses, the differences in affinities of the various subclasses are not as marked as they were in the case of the IgG/H interaction, where IgG_{2b} had a substantially greater affinity for H than normal IgG_{2a} had.

Discussion

The studies presented in the previous chapter indicated that the solubilized H and R proteins of RBL cells *both* bound to normal rat IgG-Sepharose. The work presented in this chapter has therefore extended these previous studies in an attempt to define the *subclasses* of IgG involved in this interaction. Unfortunately, purified rat IgG subclass preparations were not readily available, and considerable time and effort were required in their production.

A major problem lay in the fact that most of the subclass proteins purified thus far have been obtained from a variety of immunocytomas. In fact, as pointed out in Chapter 1, thorough characterization of many of these proteins has depended on their almost exclusive production in vast quantity by animals bearing various plasmacytoma or myeloma tumors. The problem is that the physicochemical characteristics of these proteins are not necessarily the same as those of the majority of proteins of the same subclass present in normal sera. For example, normal rat IgG_{2a} is readily prepared in high purity on DEAE cellulose by elution of a crude immunoglobulin preparation under starting conditions with a very low ionic strength buffer (0.005 *M* PO_4^{3-}, pH 8.0). It is worth noting that attempts to use DEAE Sephadex under similar conditions failed. However, even under *exactly* the same conditions it was not possible to purify IR462 IgG_{2a} on DEAE cellulose. The IR462 protein bound to the ion exchange column under these conditions, only eluting at higher ionic strength with other subclasses (results not shown). It is possible that the gene producing

the IR462 protein had undergone some mutation giving rise to the altered physicochemical properties of the protein, or perhaps glycosylation differences caused the protein to behave differently than "normal" IgG_{2a}. Regardless of the reasons underlying the differences, these results provide an indication of the difficulties involved in purification of the various subclasses, and serve as a reminder that myeloma proteins are not necessarily representative of the normal subclass proteins. On the other hand, normal IgG_{2a} is heterogeneous (likely as a result of variations in glycosylation and differences in the hypervariable region), and a substantial amount of this protein elutes from DEAE cellulose with other Ig (sub)classes as the ionic strength of the buffer is increased. So the "normal" preparation also is not *necessarily* representative of the *majority* of proteins of this subclass either.

It should be noted that, while these studies were in progress, a method was published for the purification of all of the subclasses of rat IgG from normal sera (131). Unfortunately, after several attempts, the procedure failed to produce clean subclass preparations when assayed for contamination at total protein concentrations of approximately 2 mg/ml. Re-examination of the paper showed that the authors had failed to indicate the concentration of the preparations used to check for contaminating subclasses. It would now appear that the protein concentration of these samples was simply too low to pick up the contamination present. None the less, the method may still prove useful in subclass *enrichment.*

Bazin and co-workers have been responsible for much of the characterization of the rat immunoglobulins, based on their studies of rat immunocytomas (7). In fact, without the

collaboration of the Louvain group, who provided immunocytoma ascitic fluid, sera and purified subclass proteins, the scope of the work presented in this chapter would have been much more restricted. The group has recently developed a rather elegant method for the purification of rat immunoglobulins from serum or ascitic fluid based on allotypic differences between the Ig light chains of various immunocytomas and the host animals (132, see also Materials and Methods). The IR27 IgG_1 was purified by this method, and it was therefore rather surprising to find very low (but detectable) IgE contamination of this preparation. As indicated in the results section, this contamination was too low to have made any substantial contribution to the inhibition of receptor binding that was seen with the IR27 protein. However, it does indicate that, although the method has great potential for the rapid purification of subclass proteins, it still has certain limitations which should be kept in mind.

The original intention had been to use a Sepharose solid phase bearing a single well defined IgG subclass. IgG_{2a} was chosen in view of its relatively high concentration and simple purification from normal rat serum. Furthermore, earlier studies by Stechschulte and Morse (111, 113) which were discussed in the introductory chapter, and our own preliminary studies, suggested that IgG_{2a} did indeed interact with the rat mast cell Fc receptors. Unfortunately, IgG_{2a}-Sepharose was found to bind R predominantly, binding very little of the H protein. In autoradiographs produced following SDS-PAGE analysis of the material eluted from the IgG_{2a}-Sepharose, this small amount of H appeared as a faint band, poorly separated from the predominant R band, making it virtually impossible to resolve the H and

R bands by densitometery. The problem was only aggravated by any further reduction of H binding in the inhibition assay. In view of these problems, the decision was made to revert to using normal rat IgG-Sepharose as in the previous chapter.

Unfortunately, as shown in the results, the use of IgG-Sepharose led to co-isolation of the transferrin receptor (TfR) due to transferrin contamination of the IgG preparation that was coupled to the Sepharose solid phase. However, the identity of the TfR band was established relatively quickly on the basis of the protein's strong interaction with an IgG preparation that was heavily contaminated with transferrin. Transferrin contamination of the IgG preparation was suspected due to the noticeable reddish color of the solution, and was further supported by comparison of the preparation on SDS-PAGE with a purified transferrin preparation. The high molecular weight band on the inhibition assay autoradiographs was conclusively identified as the TfR when it was shown that its binding was completely and specifically inhibitable with purified transferrin.

The development of the microassay represented a significant improvement over the methods used in earlier work. The use of SDS-PAGE tube gels in the previous studies had severely limited the number of samples which could be run simultaneously. Comparative studies, requiring several SDS-PAGE runs, were therefore subject to variations from run to run which were difficult to control for. Consequently, the reproducibility of the results suffered. Slicing the gels was time consuming, labor intensive, and limited the resolution of the separation to 2 mm. Furthermore, the radioactivity of each gel fraction was very low,

requiring counting times in a gamma counter which were prohibitive in large experiments. This last point also precluded any reduction in the volumes of reagents from the 0.1 ml IgG-Sepharose and $2x10^6$ cells per sample then in use.

In contrast, the slab gel apparatus used in the microassay is capable of handling 4 gels at a time, each carrying 20 samples. Autoradiography was found to be a sensitive enough method of detection to allow a 10x reduction in the volume of the reaction mixture, reducing the IgG-Sepharose requirement to only 0.010 ml per sample. As a result of this decrease in the reaction volume, even with the extremely limited supply of inhibitor proteins, triplicate samples could be run at each inhibitor concentration, and only $2x10^5$ cells were required per sample. Furthermore, the requirement for time in a gamma counter was almost completely eliminated. Similarly, the time and work involved in data acquisition was drastically reduced, with autoradiographs typically ready for scanning in just 24 hours.

Analysis of the autoradiographs by scanning densitometry further reduced the work involved in data analysis by eliminating the plotting of cpm *vs.* SDS-PAGE gel fraction number. The technique also took full advantage of the improved resolution of the autoradiographs. In comparison with the 2 mm resolution of the previous method, the resolution usually used on the scans was 0.3 mm. The use of an integrating plotter vastly simplified the data analysis, which was finally performed on a spreadsheet using a microcomputer. Needless to say, direct acquisition of data by the computer from the scanner, using systems such as those developed for HPLC data analysis, would have further simplified the assay.

^{22}Na was employed in the samples as a volume marker, in an effort to minimize differences between samples resulting from variations in the percentage of the total sample run on SDS-PAGE. Thus, determinations of ^{22}Na cpm in the sample before and after removing an aliquot for SDS-PAGE analysis allowed the determination of the fraction of the total sample that was actually analyzed. Data were then corrected to be representative of the total sample, as previously mentioned.

Unfortunately, determinations of ^{125}I cpm in the sample before and after removing the SDS-PAGE aliquot failed to provide an accurate indication of the fraction analyzed. This appeared to be the result of nonspecific binding of the ^{125}I labelled material by the Sepharose solid phase and the tube walls. On the other hand, ^{22}Na has been successfully used as a volume marker in a variety of other radioimmunoassays (140). It is freely diffusible, and has no interactions that we are aware of with either the proteins of interest, the Sepharose solid phase, or the tube material. Its energy spectrum makes it easily distinguishable from ^{125}I. On SDS-PAGE, it migrates toward the cathode (the opposite direction from the proteins, which migrate toward the anode on SDS gels), so it does not interfere with autoradiography.

Initial attempts to include a labelled protein as an internal standard indicated that it would be difficult to find a protein which is readily available, homogeneous, devoid of nonspecific interactions, and which ran in a position completely removed from any bands of interest.

None the less, in future studies, if such a protein can be found, this type of internal standard would still be preferable, since it would most accurately reflect the fraction of the total sample analyzed.

The inclusion of BSA in the reaction mixture and washing buffers was found to reduce nonspecific binding of radiolabelled material to the Sepharose and tube walls. This is a relatively common practice in radioimmunoassays. However, other agents such as 0.05 *M* EDTA, 0.5% Tween 20, and 1.0 *M* NaCl all produced a marked reduction in receptor binding to the IgG-Sepharose.

As previously mentioned, if 6000 cpm were applied in the SDS-PAGE sample from the uninhibited control, an autoradiographic exposure of approximately 24 hours seemed to be optimal. However, as shown in Figure 3.8, components other than the receptor proteins accounted for a relatively large percentage of this sample. In fact, previous studies have indicated that only about 26±9% of total sample counts are receptor related (126). This implies that *total* counts in the receptor bands of the *uninhibited* control samples only amount to about 1500 cpm. This should allow for some comparison of the micro assay with other methods employing gamma counters.

As mentioned above, earlier studies had indicated that IgG_{2a} interacted with RBL cells (118, our own preliminary results) and appeared to play a role in triggering mediator release from

rat mast cells (114, 117). Bach, Bloch and Austin's studies (117) and those of Halper and Metzger (118) suggested that IgG_{2a} actually interacted with the cell through the same receptor as IgE. The results presented in this chapter show that IgG_{2a} does indeed interact with the same receptor proteins as IgE, although with a considerably lower affinity. Furthermore, IgG_{2a} bound R (the high affinity Fc_ϵ receptor) in preference to H and showed the highest affinity for the R protein of any of the IgG subclasses. In fact, IgE and IgG_{2a} were the only two proteins to show this preferential interaction with R, and this *pattern* may be related to the IgG_{2a} subclass' reported ability to induce histamine release. On the other hand, the two monoclonal IgG_{2a} preparations (IR33 and IR418) did not show as marked a preference for R as the normal IgG_{2a} did. This may have been at least partially attributable to IgG_{2b} contamination of these preparations. IgG_{2b} contamination of both the IR33 and IR418 preparations was remarkably similar, and would have tended to enhance the inhibition of H binding. However the differences between the normal and immunocytoma IgG_{2a}'s may not be entirely the result of such contamination. Others have also noticed differences in the properties of rat myeloma proteins of the same subclass (141), and quite recently mouse IgG subclass preparations derived from different hybridomas have been shown to differ in their ability to bind to RBL cells (142). As mentioned above, the physicochemical properties of the proteins obtained from immunocytomas tended to differ from normal proteins, and these differences might have been due to variations in glycosylation. These differences in the affinity of the FcR/ligand interaction among various monoclonal members of a subclass therefore suggest that glycosylation may play a role in ligand binding. In fact, earlier studies by Nose and Wigzell (143) have shown that inhibiting

the glycosylation of a monoclonal antibody inhibits the interaction of the antibody with Fc receptors. This raises the intriguing possibility of similarities between the carbohydrate binding lectins and the various Fc receptors. In fact, it has been shown that the low affinity receptor for IgE on human B cells has a primary structure which exhibits homology with hepatic lectin and asialoglycoprotein receptors (144, 145).

The results reported here support the roles of rat IgE and IgG_{2a} in mediator release; in as much as both react preferentially with R, and this is the receptor that has been implicated in histamine release (146). However, they show that both IgG_1 and IgG_{2b} also bind R, although with a somewhat lower affinity and in a considerably different ratio relative to H (Table 3.3). The question is, are these other subclasses also capable of inducing mediator release. Early results of Stechschulte (111), Morse (114) and Bach (117), all working with Austen and Bloch, would suggest that only IgG_{2a} was involved in triggering release, and that the other IgG subclasses may have actually inhibited histamine release. However further discussion of this question and the possible biological function of the H protein will be postponed until the general discussion (Chapter 5) in order to include the results to be presented in the following chapter.

The IgG_{2c} proteins were difficult to work with, largely due to their spontaneous aggregation. This tendency made them difficult to prepare in concentrations high enough to run in the inhibition assay. Concentrating the preparations resulted in large losses of the IgG_{2c} protein due to precipitation, while tending to increase the relative concentrations of contaminant

proteins which did not precipitate. The spontaneous aggregation also made it difficult to be certain of the actual concentration of IgG_{2c} *monomers* in the reaction mixtures, despite deaggregation immediately before use. Lubeck *et al.* (29) found that aggregation of the analogous mouse IgG_3 protein occurred during normal storage at 4°C or as a result of freezing, and they avoided these problems by working with proteins of this subclass at room temperature. It would be worth while trying a similar approach in any further studies with rat IgG_{2c} proteins.

Fc fragments prepared from normal rat IgG strongly inhibited the interaction of IgG-Sepharose with both H and R (Figure 3.15a). Results presented in the previous chapter had shown that the $F(ab')_2$ preparation used in those studies produced significantly less inhibition of the receptor/solid phase interaction than undigested IgG was capable of, but unfortunately the preparation was contaminated with intact IgG. The $F(ab')_2$ preparation used in the present study was considerably purer than that used previously, and completely failed to inhibit the interaction of either receptor with the IgG-Sepharose (Figure 3.15b), providing conclusive support for the Fc specificity of the interaction. Since, thus far, the preparation of rat IgE Fc fragments has proven exceedingly difficult (if not impossible), it has not been possible to unequivocally establish the Fc specificity of the RBL IgE receptor proteins. These results therefore are the first *direct* demonstration of the Fc specificity of this receptor/ligand interaction that we are aware of.

Although it is a relatively minor point, the results obtained for the IgA/receptor interaction may actually prove to be quite valid. Although the IgA preparation was not as extensively purified as any of the other proteins, SDS-PAGE analysis failed to show any IgG contamination. It was quite surprising therefore that double diffusion analysis indicated substantial IgG contamination. However, the preparation was tested for contamination at quite a high total protein concentration, and it is possible that the precipitin bands that formed were actually a result of crossreactivity between the antisera and IgA.

Successful use of the microassay to demonstrate an interaction between the R protein and IgG fragments suggests that the same system might be generally useful for studying the interaction of this high affinity receptor with various other peptides, low affinity ligands or pharmacological agents. Such studies might help to reveal the structure of the receptor's Fc binding site. In view of the major role that the receptor plays in allergen induced mediator release from mast cells and basophils, such an assay might be quite useful in the study and treatment of allergic disease. For example, one strategy for the treatment of allergy is to interfere with the IgE/receptor interaction, preventing the sensitization of the mast cell population. This assay could easily be used to screen for such inhibitors. In a more general sense, this type of assay system might be useful in studies of a variety of other low affinity receptor/ligand interactions.

Chapter 4

The interaction of the Fc_ϵ receptors of the RBL cell with immunoglobulins of other species.

Introduction

A considerable amount of work has been done on immunoglobulin Fc receptors in the mouse and human systems, and it would be nice to be able to relate these studies to the rat system in the hope of being able to draw some conclusions about these receptors which might be generally true across species barriers. This after all is the rationale for using animal models in the hope of being able to extend our findings to the human system. Fortunately mouse IgG subclass preparations and IgG preparations from a variety of other commonly used mammalian species are readily available. Therefore, with the use of the microassay which had been developed to study the interaction of the RBL cell Fc receptors with the low affinity IgG ligands (Chapter 3), it was an extremely simple matter to extend the inhibition studies described in the previous chapters to include these heterologous proteins. This work is the subject of this relatively brief chapter.

Materials and Methods

All methods for the preparation of rat IgG_{2a}, the micro inhibition assay, and the analysis of results have been described in the previous chapter (Chapter 3).

Mouse immunoglobulins

MOPC 21 IgG_1, UPC 10 and RPC 5 IgG_{2a}, MOPC 195 and MOPC 141 IgG_{2b}, and J 606 IgG_3 were purchased from Bionetics (Charleston, SC), and were used without further purification.

Immunoglobulins from other species

Equine, goat, rabbit and sheep IgG's were purchased from Pelfreeze (Rogers, AK) and were used without further purification. A second preparation of rabbit IgG obtained from Sigma was further purified by Protein A-Sepharose chromatography to remove transferrin, and was the kind gift of Dr. M.S. Lao (this department).

Results

Only 3 of the 4 murine IgG subclasses showed significant interaction with the H and R proteins, and, of these 3, all interacted considerably more strongly with H than with R (see

Figure 4.1 and Table 4.1). The affinities of the H protein for the IgG_1, IgG_{2a} and IgG_{2b} subclasses were not markedly different, but they were highly consistent within a subclass and based on the data in Table 4.1 they could be ranked:

$$IgG_1 > IgG_{2b} > IgG_{2a}.$$

Similarly, the affinities of the R/subclass interactions were of the order:

$$IgG_1 > IgG_{2a} > IgG_{2b}.$$

IgG_3 failed to show significant inhibition of either H or R binding over the range of concentrations tested. However as indicated in the previous chapter, murine IgG_3 is analogous to rat IgG_{2c} and is subject to spontaneous aggregation. This made it difficult to test the J 606 protein at as high a concentration as the other subclasses, and raised the possibility that the concentration of IgG_3 actually *in solution* may have been rather different from that determined by Lowry assay of the inhibitor preparation. Thus, the actual soluble protein concentrations may have been even lower than those indicated in Figure 4.1f.

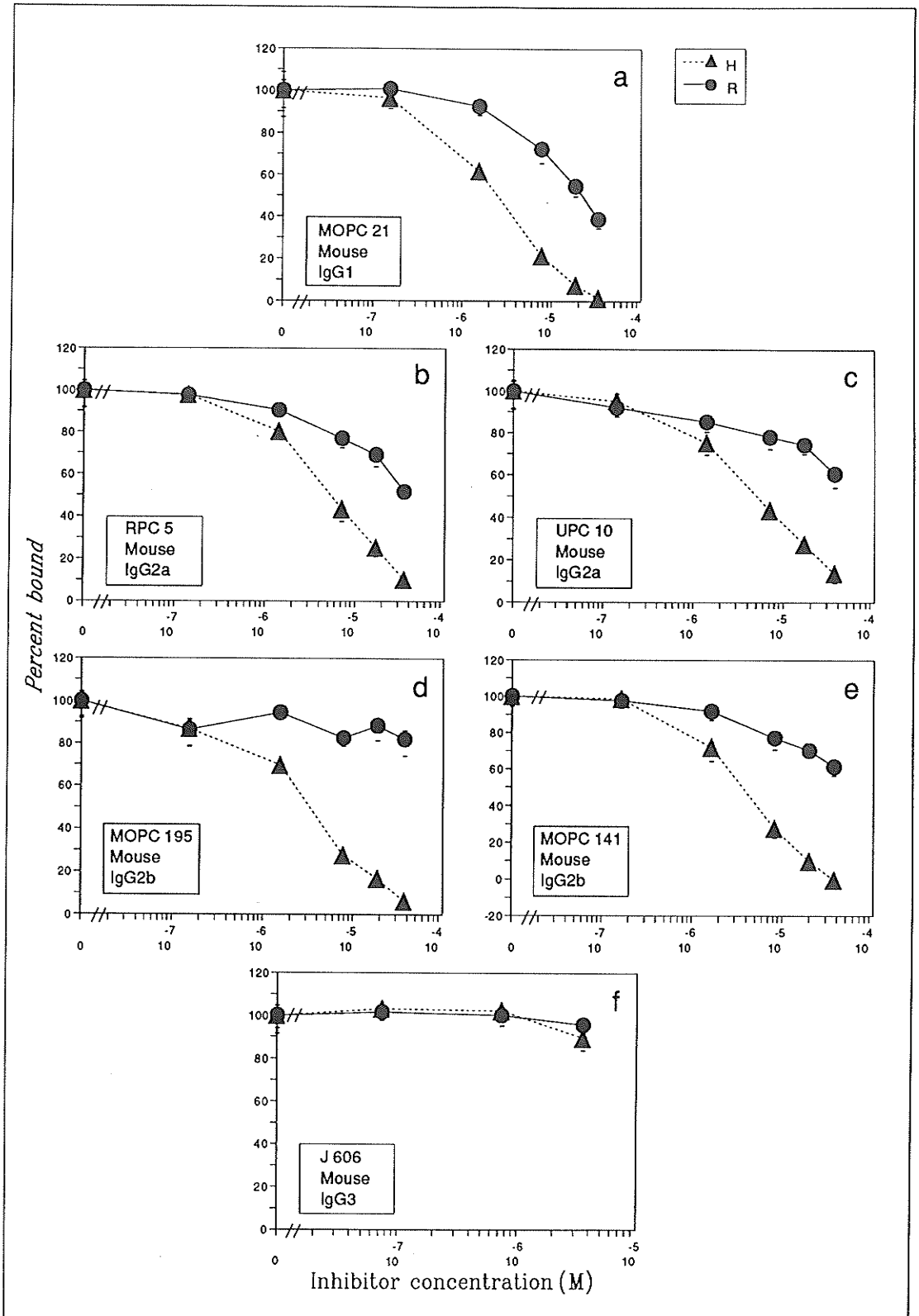


Figure 4.1 Inhibition of H and R binding to rat IgG-Sepharose by various mouse IgG subclass proteins.

Table 4.1 Inhibition of H and R binding to IgG-Sepharose by various mouse immunoglobulins

Inhibitor	Immunocytoma	Molar concentration for 50% inhibition ($\times 10^{-6}$) H	R
IgG_1	MOPC 21	3.3	22.9
IgG_{2a}	RPC 5	6.2	35.8
IgG_{2a}	UPC 10	6.0	> 36.7
IgG_{2b}	MOPC 195	4.6	>> 37.3
IgG_{2b}	MOPC 141	4.9	> 37.3
IgG_3	J 606	>> 3.6	>> 3.6

None of the murine proteins were assayed for IgE contamination. Mouse IgE has been shown to bind the high affinity rat Fc_ϵ receptor (R) with about the same affinity as rat IgE does (147, and our own unpublished observations). However, the fact that none of the proteins interacted *strongly* with R, and in no case interacted with R more strongly than with H, would tend to argue against any significant IgE contamination.

A number of heterologous IgG's of undefined subclass were tested at a single concentration of $1x10^{-5}$ *M* for interaction with the H and R proteins. The results of this study are presented in Figure 4.2. Only the H protein interacted significantly with these IgG preparations. The sheep protein showed little, if any, interaction with the receptor at the concentration tested. Goat IgG showed substantial interaction with H, reducing binding to about 35% of the control. Although the figure indicates that equine IgG completely inhibited the H/IgG-Sepharose interaction, this is not an accurate reflection of the situation in this case. H binding was *markedly* inhibited, but not completely. Unfortunately, the H and R protein bands could not be resolved by the scanning densitometer. The H band therefore contributed to the band recognized as R protein, producing an apparent *increase* in the amount of R bound to the IgG-Sepharose in the presence of horse IgG. Rabbit IgG was remarkable in that it *did* completely inhibit H binding at this concentration, while actually enhancing R binding. This enhancement might possibly have been attributable to an increased availability of binding sites on the IgG-Sepharose due to the complete inhibition of H binding.

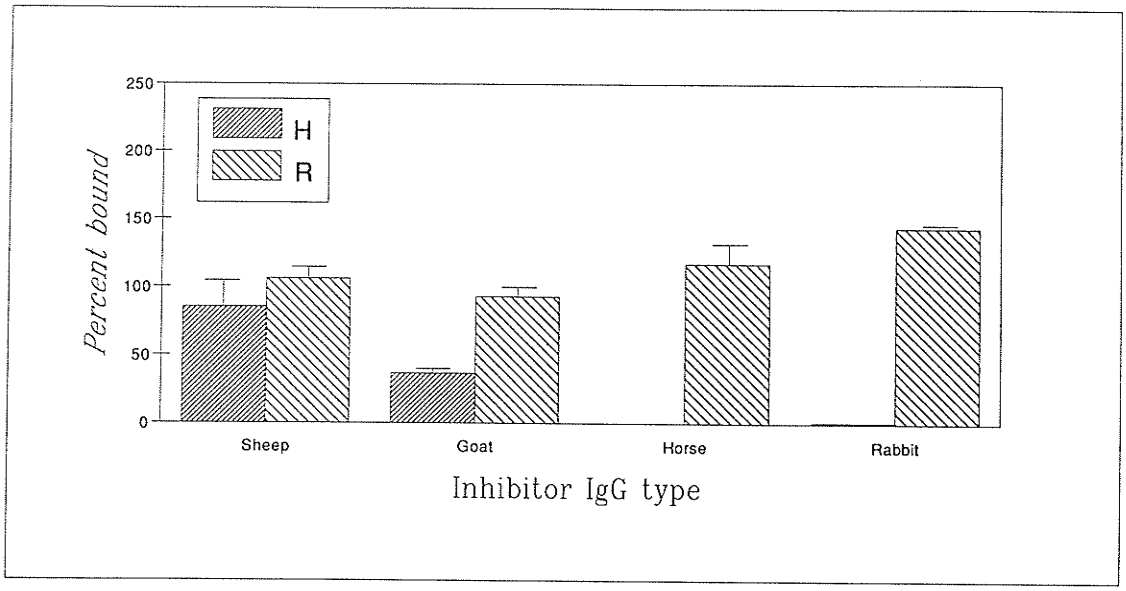


Figure 4.2 The effects of various heterologous IgG preparations on H and R binding to rat IgG-Sepharose. All preparations were tested for their abilities to inhibit this interaction at the same concentration (1×10^{-5} M). The apparent enhancement of R binding and complete inhibition of H binding induced by horse IgG was artifactual (see text).

To summarize therefore, only the H protein showed significant interaction with any of these heterologous IgG's, and the affinities of these interactions were of the order:

Rabbit > Horse > Goat > Sheep.

Discussion

Murine IgG_1 showed the strongest interaction with the R protein of any of the mouse subclasses, although the affinity of the interaction was still substantially lower than that of any of the *rat* IgG subclasses. This might tend to support recent findings of Kinet *et al.* (83) which, based on sequence data from an R receptor cDNA clone, suggested a 32% overall homology in the amino acid sequences of the R protein and the mouse macrophage $IgG_{1/2b}$ FcR alpha, previously cloned by Ravetch *et al.* (148). On the other hand, the mouse $IgG_{1/2b}$ FcR has previously been reported to interact most strongly with the mouse IgG_{2b} subclass (149). The fact that this is not the case for the mouse IgG/R interactions suggests that the functional homology with the $IgG_{1/2b}$ FcR is not too extensive. Similarly, one could argue that the H and R proteins might share some structural similarity based on the fact that they both bind a particular IgG preparation (be it rat or mouse). In fact, although there may indeed be some similarities between the two receptor proteins, they have not been apparent so far in structural analyses such as peptide mapping experiments (58, 86). Ultimately, of course, the nucleotide or amino acid sequence data which is now becoming available will provide the best comparisons of receptors both across species barriers and

within a single species. In the mean time however, comparison of the RBL and mouse macrophage Fc receptors might be possible by looking at the interaction of H and R with the monoclonal 2.4G2 anti(mouse macrophage $IgG_{1/2b}$ FcR) antibody (150). Although previous studies have failed to detect 2.4G2 interaction with FcR bearing cells in the rat (149), the assay system used in the studies described here would be ideally suited to detect any low affinity interaction of the anti(receptor) antibody with the H or R proteins, provided that such an interaction affected receptor/ligand binding. However, it should be pointed out that, because they recognize a *single* determinant, monoclonal antibodies, such as 2.4G2, may not be suitable for the detection of minor homologies between receptors.

Murine IgG_1 is analogous to rat IgG_{2a}, in that it has been implicated in the induction of mediator release from *murine* mast cells (110, 151). It would be interesting to test mouse IgG_1 for its ability to induce release from *rat* mast cells. In fact, such studies have been done earlier by other groups, and in the majority of these studies the mouse protein has failed to induce histamine release from rat mast cells, despite the fact that, on the basis of results obtained here for MOPC 21, the subclass appears to bind R (see Figure 4.1a). On the other hand, Mota & Perini (152) have suggested that it might be possible to subdivide the IgG_1 subclass of the mouse into IgG_{1a} and IgG_{1b} subgroups, based on their abilities to trigger mediator release from rat mast cells. They have found an IgG_1 subpopulation (IgG_{1a}) in hyperimmune mouse sera, having little or no affinity for Protein A-Sepharose at pH 7.2, which is able to induce passive cutaneous anaphylaxis (PCA) in both rats and mice. The more traditional IgG_1 (IgG_{1b}) is strongly bound by Protein A under the above

conditions, induces PCA in mice, but fails to similarly sensitize rats. Since there is no evidence for IgG_1 subgroups in the immunoglobulin heavy chain constant region genes of the mouse, these subgroupings may reflect carbohydrate, light chain or perhaps variable region differences. It would be interesting to examine the interaction of each of these IgG_1 subpopulations with the H and R proteins in our system. For reasons which will become clear later in the general discussion, it would be particularly interesting if the IgG_{1a} proteins which induce mediator release were found to interact with R more strongly than with H, in a manner similar to rat IgG_{2a}.

As with the rat IgG_{2c} proteins, the mouse IgG_3 data were inconclusive, due to spontaneous aggregation and precipitation of the protein. As mentioned in the previous chapter, in studies of the human monocyte FcR/mouse IgG_3 interaction, Lubeck *et al.* (29) found that aggregation of the IgG_3 proteins occured during normal storage at 4°C or as a result of freezing. The authors avoided these problems by working with this subclass at room temperature, and, as with rat IgG_{2c}, these methods would be worth employing in any future studies with mouse IgG_3. It should be noted that Lubeck reported a marked decrease in the affinity of the IgG_3/human monocyte FcR interaction on aggregation of the IgG_3. This was somewhat surprising, since most previous studies have actually *required* aggregation of the ligand to demonstrate various IgG/FcR interactions. None the less, on the basis of these findings, if the RBL receptors also exhibit decreased affinity for IgG_3 aggregates (relative to monomers) this would make any interaction with this subclass even more difficult to detect.

The results presented in this chapter confirm and extend those of Hall & Rittenberg (142), which have demonstrated an interaction between Fc receptors on intact RBL cells and some preparations of deaggregated monoclonal mouse $IgG_{1, 2a}$ and $_{2b}$ proteins. Unfortunately, the authors were not able to distinguish *which* receptor(s) were involved in binding. Actually, their results were somewhat surprising. Despite very gentle washing procedures, Segal *et al.* (127) had previously failed to detect binding of radiolabelled *rabbit* IgG monomers to RBL cells, and results presented here indicated that these rabbit proteins interact more strongly with the H protein than any of the mouse proteins do. In fact, although Hall and Rittenberg were able to demonstrate that monomeric *mouse* IgG interacted with the Fc receptors on intact RBL cells using a *rosetting assay*, they *were not* able to clearly demonstrate binding of *radiolabelled* proteins either, suggesting that the rosetting assay was considerably more sensitive than the radioligand assay. These authors further found that only certain monoclonals bound in the monomeric form, and they were unable to show binding of a polyclonal IgG preparation, suggesting that the monoclonals that bound may not have been representative of the majority of the murine IgG population. The work presented in this chapter on the other hand indicated that all of the mouse proteins assayed showed considerable interaction with at least the H protein, although a much smaller number of proteins was screened (5, compared to 23 in Hall and Rittenberg's study). It would be interesting to study the proteins used by these other authors in our system in an attempt to further quantitate the Fc receptor affinity differences of the various preparations.

There remains virtually no doubt that the H protein corresponds to the RBL IgG Fc receptor described by Segal *et al.* (127), and our findings with rabbit IgG help to clarify some of their previous observations, which were discussed in Chapter 2. It was actually quite fortuitous that these authors chose to work with rabbit antibodies. Since rabbit IgG is so selective in its interaction with the H protein, there would have been a more clear cut distinction between the H and R receptor interactions with rabbit IgG and rat IgE in the group's inhibition studies, making the interpretation of results somewhat more definitive than they might have been had say mouse IgG been used. It is also important to note that Segal was unable to induce histamine release with rabbit IgG, which would suggest that the H protein does not act as a release trigger. Furthermore, rabbit IgG failed to significantly *inhibit* IgE mediated histamine release. Both of these points will be returned to in the general discussion of the final chapter.

Collaborative studies (reviewed by Froese, 42) have shown that virtually all of the RBL cell lines presently in use by various groups bear both the H and R proteins, but their molecular weights vary somewhat and the two occasionally overlap on SDS-PAGE analysis. This overlap partially explains why some groups have failed to detect the presence of both receptors in previous studies. The strong interaction of rabbit IgG with the H protein might also partially explain why those groups who pre-clear RBL cell lysates on rabbit IgG-Sepharose (60) fail to see the H protein.

The results obtained with the other heterologous IgG preparations bear consideration in attempts to prepare anti(receptor) antibodies. The indications are that nonspecific interaction with the receptor through the Fc portion of the antibody could be minimized by raising these antibodies in sheep, since sheep IgG showed the lowest affinity for either of the receptors of any of the preparations tested.

In summary, rat IgE and IgG_{2a} appear to be unique in their preferential interaction with the R protein relative to the H. All of the other proteins assayed thus far have preferentially bound H, and the rabbit IgG/H interaction has been unique in both its strength and selectivity, binding H exclusively over R. These characteristics may make rabbit IgG a valuable tool in determining the biological function of the H protein. Although there are extensive crossreactivities across species barriers, the patterns of reactivity differ for a particular panel of IgG proteins. The relative affinities for the mouse IgG subclasses for instance vary from one receptor to another, within a species or across species barriers. Thus, on the basis of these studies, Fc receptor structure would not appear to be extremely highly conserved from one species to the next, and it would be difficult to say that the H or R proteins were *exactly* analogous with any of the mouse Fc receptors. Even the high affinity IgE FcR of mouse mast cells might not be exactly comparable to the rat mast cell Fc_ϵ receptor. In the rat, the lack of histamine release in response to rabbit IgG sensitization (127) suggests that H is not involved in triggering release. IgG_{2a} mediated release must therefore be triggered through the high affinity IgE FcR (R protein). In the mouse on the other hand, IgG mediated histamine release appears to be triggered by

interaction of IgG_1 subclass proteins with some receptor other than the high affinity IgE Fc receptor (151, 129). It would be interesting to assess the ability of rat IgG_{2a} to induce mediator release from mouse mast cells. Such studies would help to clarify the relationship of the rat and mouse high affinity Fc_ϵ receptors.

In view of the similar structure of the immunoglobulins themselves in all mammalian species, and in view of the similar basic functions of Fc receptors in these species, the observed crossreactivities are not surprising. As pointed out by Unkeless, Scigliano and Freedman in a recent review (153), the Fc receptor family probably evolved in parallel with the immunoglobulins. In fact as the genetic messages encoding these proteins are cloned and sequenced, it is becoming apparent that the various Fc receptors share certain structural similarities which suggest that they form a subgroup of the immunoglobulin supergene family (153, 37, 26). Unfortunately, on the basis of the studies reported here and the data available on the Fc receptors of the mouse, it is not yet possible to draw many broad conclusions about the similarities of mouse and rat receptor proteins.

Chapter 5

General discussion

Previous studies by the Ishizakas' group have shown that crosslinkage of R with anti(receptor) antisera induced histamine release from normal rat mast cells (reviewed in 44). The studies presented in the previous chapter indicated that rabbit IgG primarily bound the H protein. However, in earlier work Segal *et al.* (127) were not able to induce histamine release from either rat peritoneal mast cells or a histamine releasing RBL variant (2H3) with rabbit IgG oligomers, suggesting that crosslinking of the H protein does not trigger degranulation. This conclusion is further supported by the work of Conrad et al. (74), who were unable to induce mediator release by receptor crosslinking with an anti(H) antiserum, raising the question of just what functional role the H receptor *does* play.

Some possibilities for the biological function of H are suggested by the early work of Austen's group viewed in the context of the results that have been presented here. Re-

call from the introduction that Stechschulte, Austen and Bloch (111) found that antisera from hyperimmune rats could be used to sensitize rat peritoneal cells such that subsequent exposure of these cells to antigen resulted in leukotriene release, but failed to induce the release of significant amounts of histamine. In the course of further studies to define the Ig class mediating this release of leukotrienes, Morse, Austen and Bloch (114) found that, whereas whole antisera and crude IgG preparations induced leukotriene (but not histamine) release, purified IgG2a induced the release of **both** leukotrienes and histamine. This suggests that some component of the crude IgG preparations was somehow acting to inhibit histamine release. This conclusion was further supported by subsequent work of Bach *et al.* (117) showing that, although IgG_{2a} induced histamine release was enhanced slightly by up to 1% normal or decomplemented rat serum, release was *inhibited* by serum concentrations above 1%. Unfortunately the effects of serum on leukotriene release were not reported. On the basis particularly of the results obtained with crude IgG (purified by ion exchange on DEAE cellulose), it could be suggested that, whereas IgG_{2a} is capable of inducing histamine release, the other IgG subclasses may actually *inhibit* such release.

The results presented in Chapter 3 showed that IgG_{2a} interacted quite strongly with the R protein. Based on the studies mentioned above, it is reasonable to conclude that IgG_{2a}-induced histamine release is probably triggered by crosslinking of R in the same way as IgE mediated release. The problem is that *all* of the rat IgG subclasses showed significant interaction with R, and all subclasses might therefore be expected to be capable of inducing

release, but thus far only IgG_{2a} has been formally implicated in such a role (except see below).

Actually, although the IgG_{2a} protein showed the highest affinity for R of all the IgG subclasses, on the average, this affinity was only slightly greater than that of the IgG_1 or IgG_{2b}/R interactions. As shown in Table 3.3, the average protein concentration required to inhibit R binding by 50% was only about 1.4 fold greater for IgG_1 than for normal rat IgG_{2a}. The average IgG_{2b} concentration required was only about 2.1 fold greater than for IgG_{2a}. As discussed in Chapter 3, the results obtained with the immunocytoma IgG_{2a}'s may have reflected IgG_{2b} contamination, and they are therefore excluded from this discussion. On the other hand, normal rat IgG_{2a} required *11* and *26* fold higher concentrations than IgG_1 or IgG_{2b} respectively, to produce 50% inhibition of H binding. On this basis, there would appear to be much more variation in the H/subclass interaction than in the R/subclass interaction. It is possible therefore that interaction with the H protein might result in the transmission of a negative regulatory signal which inhibits histamine release by IgG_1 or IgG_{2b}, since both of these subclasses interact with R *about* as well as IgG_{2a} does, but they apparently fail to induce histamine release. Thus, it is the relative affinities of H and R for a particular subclass which might determine the functional consequences of binding. IgG_{2a} might induce mediator release not so much by its interaction with the high affinity IgE receptor (the R protein) as by its *failure* to bind strongly to the H protein. Similarly, an inhibitory signal from the interaction of $IgG_{1/2b}$ and H would explain the failure of semipurified IgG to induce release and the inhibition of IgG_{2a} mediated release by nor-

mal serum, mentioned above. It might also help to explain the finding that the only anti(Fc receptor) antiserum shown to induce mediator release thus far has also been the only antibody preparation free of anti(H) antibodies (42).

This would suggest that a reagent *specific* for the H protein alone might inhibit histamine release by IgG_{2a}, if not by IgE. Rabbit IgG would appear to be just such a reagent. As indicated in Chapter 4, at the concentration tested, the selectivity of the rabbit IgG/H interaction was exceptional in that it completely inhibited H binding to the IgG-Sepharose solid phase, while having virtually *no* apparent effect on R binding. Although Segal *et al.* were not able to detect any inhibition of IgE mediated histamine release by rabbit IgG (127), it would be interesting to see what effects rabbit IgG might have on *IgG2a* induced mediator release. With regard to its inability to inhibit *IgE* induced release, it is possible that the heterologous rabbit immunoglobulin might have bound strongly to the H protein but for some reason failed to induce any signal. However, it should be kept in mind that IgE binds to H more strongly than *any* of the IgG proteins, and it still induces mediator release. If interaction with H does induce a negative regulatory signal, then, as pointed out above, it is probably the *relative* affinities of the ligand for the H and R proteins that determines whether release is triggered. Stronger interaction with R might induce release, while stronger interaction with H might inhibit release. In the case of IgE, its doubtful that any combination of H binding ligands could induce a strong enough signal to inhibit release, since none of the ligands studied thus far interact with H more strongly than IgE does and the IgE/R interaction still induces release. This would explain the findings of Segal *et al.*

On the other hand, rabbit IgG might be capable of inhibiting *IgG2a* induced release, since the affinity of the rabbit protein for H appears to be greater than that of IgG_{2a}.

Similarly, it would be interesting to assess the histamine release capabilities of IR33 and IR418 IgG_{2a} relative to normal rat IgG_{2a}. On the basis of the decreased R/H binding ratio of the immunocytomas (see Figure 3.11), one would predict that these proteins might trigger somewhat less release than normal IgG_{2a}. Likewise, as mentioned in Chapter 4, it would be interesting to compare the R/H binding ratios of those mouse IgG_1 preparations which induce histamine release from rat mast cells (IgG_{1a}) (152) with those that fail to induce such release (IgG_{1b}). If the hypothesis is correct, the IgG_{1a} proteins should bind R more strongly than the IgG_{1b} preparations. On this basis, the MOPC 21 protein would appear to be an IgG_{1b} and would not be expected to induce release (see Figure 4.1).

It was pointed out above that rat IgG_{2a} is the only subclass to have been *formally* implicated in triggering histamine release. However, preliminary results have been presented by McGivney *et al.* (154) which suggested that *all* rat subclasses induce release. Unfortunately, these results are only available in an abstract, making them difficult to discuss in view of the lack of data and details of the methods employed. However the results were generally consistent with the findings described in this work. Thus, radiolabelled oligomers of IgG_1, IgG_{2a} and IgG_{2b} were shown to bind to a histamine releasing RBL-2H3 subline. IgE inhibited all IgG binding. IgG_1 binding could be inhibited by itself and IgG_{2b}, but was only

partially inhibited by high concentrations of IgG_{2a}. This supports our finding that IgG_1 and IgG_{2b} both bound H considerably more strongly than IgG_{2a} did. IgG_{2a} would therefore be expected to have a hard time inhibiting the IgG_1/H interaction. On the other hand, IgG_{2a} oligomer binding was found to be inhibitible by itself, IgG_1 and IgG_{2b}. Again, this would be expected based on our finding that IgG_1 and IgG_{2b} both have affinities for R comparable to that of IgG_{2a} and higher affinities than IgG_{2a} for H. However, their finding that IgG_{2b} binding was inhibited by itself, IgG_1 and IgG_{2a} is inconsistent with the results reported here. Based on the finding that the IgG_{2b} proteins had the highest affinities for H of all the subclasses, one would expect to have seen an inhibition pattern in the results of McGivney *et al.* similar to that for IgG_1 (see above), with an even weaker inhibition by IgG_{2a}.

These authors found that rabbit IgG inhibited binding of the homologous rat IgG_1 and IgG_{2b} proteins, but only partially inhibited IgG_{2a} binding. Again, this supports our results in that, at the concentration tested, rabbit IgG bound H virtually exclusively and would be expected to most strongly inhibit the binding of those subclasses which interacted preferentially with H (IgG_1 and IgG_{2b}). The IgG_{2a} subclass on the other hand binds R preferentially, and this binding therefore would not be expected to be as strongly inhibited by rabbit IgG as the other subclasses. The *complete* inhibition of $IgG_{1/2b}$ binding reported by McGivney *et al.* suggests that, at the concentration of rabbit IgG used, there was some interaction of the rabbit protein with the R receptor. Unfortunately, the concentration of rabbit IgG is unknown, but one would predict that it was substantially higher than that used in the studies described in Chapter 4. Based on their inhibition studies, these authors

suggested that there are 3 receptors, as shown in Table 5.1. We, on the other hand, see no evidence for any more than the *two* receptors H and R. Actually, the receptors that McGivney *et al.* proposed would not have been consistent with their findings either. Based on the scheme in Table 5.1, the binding of IgG_{2b} to receptor 3 should not have been completely inhibitible by IgG_{2a}. Since the inhibition of IgG_{2b} binding by IgG_{2a} is the only point inconsistent with our results, this would tend to suggest that these inconsistancies might be cleared up if the work of this other group is eventually published. This also indicates how difficult it is to characterize a system like this using the "black box" approach to study binding to intact cells.

If, in fact, the results of McGivney *et al.* are reproducible and all subclasses *do* induce mediator release, this still would not rule out the suggested regulatory role of H binding. For instance, it might be found that the *relative* ability of each subclass to induce histamine release is related to the extent of interaction with the H protein. In any case, the effects of interaction with the H receptor warrant further study both at the level of mediator release and at the level of the underlying biochemical consequences of such interaction.

Table 5.1 RBL-2H3 Fc receptors proposed by McGivney *et al.*

Receptor	Ligands
1.	IgE
2.	IgE, IgG_1, IgG_{2a}, IgG_{2b}
3.	IgE, IgG_1, IgG_{2b}

As indicated in Chapter 4, one would ultimately like to be able to extend results obtained with animal models to the human system. In humans, IgE/IgG Fc receptor crossreactivity is of interest in the treatment of allergy. The rationale for the desensitization of individuals by injection with progressively larger doses of allergen has been to raise "blocking antibodies", which it is hoped will interfere with the IgE/allergen/mast cell (basophil) interaction and histamine release. The blocking antibodies are of the IgG isotype, and there are several levels at which they are thought to interfere with the IgE mediated reaction. For instance, they might act to simply sequester the allergen, making it unavailable to IgE. On the other hand, the Ishizakas have shown that human basophils seem to carry two types of Fc receptors; distinct IgG and IgE FcR (128). Although the authors were not able to show crossreactivity of the two receptors, this may have been due to intermediate washing steps. Or this may have been due to the subclass composition of the IgG preparation (recall from Chapter 1 that there are four human IgG subclasses). Or a combination of the two explanations is possible. In any case, the human system would appear to have at least several *potential* similarities with the rat system. Recently obtained sequence data for the high affinity Fc_ϵ receptor of a human mast cell line indicates structural similarities with the R protein and an overall homology of 45% at the amino acid level (37). The possibility exists that any inhibitory role of the blocking antibodies may be somewhat more complex than simply sequestering allergen. Depending on the extent of *functional* similarities with the rat system, it is even conceivable that a human IgG subclass may *induce* mediator release. In order to effectively treat allergic individuals therefore, and to prevent any adverse effects of desensitization therapy, it becomes important to understand the human

system more thoroughly. It may be possible to adapt the system described here to similar studies of human Fc receptor crossreactivity and low affinity ligand binding. As an alternative, the interaction of human IgG subclasses with the Fc receptors of the RBL cell should be investigated. An important point in these studies however should be the analysis of the *rat IgG/human FcR* interaction, to fully clarify the relationship between the rat and human systems. Similarly, in order to be able to relate the rat and *mouse* systems, one should examine the crossreactivity of mouse mast cell Fc receptors with rat immunoglobulins. The assay system described in this work might be ideally suited to studies of such low affinity interactions with mouse or human Fc receptors.

Finally, to re-emphasize a point made in an earlier chapter, the assay system described here would be well suited to studies of the interaction between Fc receptors and various pharmacological agents. It could be used to screen rather large numbers of compounds for such interaction, and thus may be of use in the development of drugs for the treatment of allergy.

Michael Kepron

Appendix A *Instrument settings - Scanning densitometry*

Helena Quick Scan R&D

Voltage	= 350
Gain	= 385
Zero	= 542 (typical value, no sample)
Scan speed	= 050
Slit	= 0.3x3 mm
Wavelength	= 520 nm

Hewlett-Packard 3390A Reporting Integrator

Run parameters

Zero	= 0
Attenuation	= 9
Chart speed	= 5.5
Peak width	= 0.04
Threshold	= 8
Area reject	= 0

Report options

Response factor (uncalibrated peaks)	= 0
Multiplication factor	= 1
Peak height mode	= No

Extend retention time	= No
Report uncalibrated peaks	= No

Time table

0.00	Intg # = 4	(Disable auto solvent testing and tangent skimming)
0.00	Intg # = 1	(Set baseline at next valley point)
0.00	Thrsh = 8	(Set threshold = 8)
0.05	Intg # = 5	(Extend baseline horiz. from last baseline set pt.)

Calibration table

Empty

Appendix B *Diffusion analysis data for rat IgG subclass preparations*

Sample: IR401 AcA44 060284 Fr.5-6
Concentration: 25.00 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c
4	6.2500	+--	+	+-	-
8	3.1250	+--	+	+--	-
16	1.5625	+--	+-	-	-
32	0.7813	+-	+---	-	-
64	0.3906	+	-	-	-
128	0.1953	+	-	-	-
256	0.0977	*	-	-	-
512	0.0488	+-	-	-	-
1024	0.0244	+---	-	-	-
2048	0.0122	-	-	-	-
4096	0.0061	-	-	-	-

Sample: IR595 Conc. 150884
Concentration: 21.30 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c
10	2.1300	+--	-	-	-
20	1.0650	+-	-	-	-
40	0.5325	+	-	-	-
80	0.2663	+	-	-	-
160	0.1331	*(1)	-	-	-
320	0.0666	+-	-	-	-
640	0.0333	+---	-	-	-
1280	0.0166	-	-	-	-
2560	0.0083	-	-	-	-
5120	0.0042	-	-	-	-

Sample: IR27 3.9 mg/ml 200784
Concentration: 3.90 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c
1	3.9000	+-	-	-	-
2	1.9500	+-	-	-	-
4	0.9750	+-	-	-	-
8	0.4875	+	-	-	-
16	0.2438	+	-	-	-
32	0.1219	*	-	-	-
64	0.0609	+	-	-	-
128	0.0305	+-	-	-	-
256	0.0152	-	-	-	-
512	0.0076	-	-	-	-

Sample: DE2A 290785
Concentration: 24.00 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c
10	2.4000	-	+-	-	-
20	1.2000	-	+	-	-
40	0.6000	-	+	-	-
80	0.3000	-	*	-	-
160	0.1500	-	+	-	-
320	0.0750	-	+-	-	-
640	0.0375	-	-	-	-
1280	0.0188	-	-	-	-
2560	0.0094	-	-	-	-
5120	0.0047	-	-	-	-

1. * denotes approximate equivalence point

Appendix B. IgG subclass diffusion analysis data

Sample: IR33 141283 (301085)/020885
Concentration: 20.00 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c
10	2.0000	-	+--	+--	-
20	1.0000	-	+-	-	-
40	0.5000	-	+	-	-
80	0.2500	-	+	-	-
160	0.1250	-	*	-	-
320	0.0625	-	+	-	-
640	0.0313	-	-	-	-
1280	0.0156	-	-	-	-
2560	0.0078	-	-	-	-
5120	0.0039	-	-	-	-

Sample: IR418 Conc. 090785
Concentration: 36.40 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c
4	9.1000	-	+--	+	-
8	4.5500	-	+--	+-	-
16	2.2750	-	+-	+--	-
32	1.1375	-	+	-	-
64	0.5688	-	+	-	-
128	0.2844	-	+	-	-
256	0.1422	-	*	-	-
512	0.0711	-	+--	-	-
1024	0.0355	-	-	-	-
2048	0.0178	-	-	-	-
4096	0.0089	-	-	-	-

Sample: IR863 Conc. 110785
Concentration: 25.71 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c
4	6.4264	-	+-	+-	-
8	3.2132	-	+	+-	-
16	1.6066	-	+	+	-
32	0.8033	-	*	+	-
64	0.4017	-	+	+	-
128	0.2008	-	+--	+	-
256	0.1004	-	-	*	-
512	0.0502	-	-	+--	-
1024	0.0251	-	-	-	-
2048	0.0126	-	-	-	-
4096	0.0063	-	-	-	-

Sample: RAHE-2
Concentration: 5.00 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c	IgG
prior to conc.		-	+-	+	-	+
10	0.5000	-	+-	+	-	+
20	0.2500	-	+--	*	-	
40	0.1250	-	-	+-	-	
80	0.0625	-	-	+--	-	
160	0.0313	-	-	-	-	
320	0.0156	-	-	-	-	
640	0.0078	-	-	-	-	
1280	0.0039	-	-	-	-	

Appendix B. IgG subclass diffusion analysis data

Sample: IR1148 160885
Concentration: 7.18 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c	IgG
	2.6000	-	+-	-	+	++
13.33	0.5387	-	+-	-	++	+
27	0.2693	-	+--	-	*	
53	0.1347	-	-	-	+-	
107	0.0673	-	-	-	-	
213	0.0337	-	-	-	-	
427	0.0168	-	-	-	-	
853	0.0084	-	-	-	-	
1706	0.0042	-	-	-	-	
3412	0.0021	-	-	-	-	

Sample: IR221 conc. 260785
Concentration: 16.87 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c	IgG
4	4.2166	++	+-	+-	++	++
8	2.1083	+	-	-	*	
16	1.0541	*	-	-	+	
32	0.5271	+	-	-	+-	
64	0.2635	+--	-	-	-	
128	0.1318	-	-	-	-	
256	0.0659	-	-	-	-	
512	0.0329	-	-	-	-	

Sample: IR22 281283
Concentration: 53.00 mg/ml

Sample Dilution	Sample Protein Conc. (mg/ml)	Precipitin band density Antiserum specificity IgG1	IgG2a	IgG2b	IgG2c	IgG	
2	26.5000	+	*	+	-	+++ mb	(1)
4	13.2500	+	+	+-	-	+++ mb	
8	6.6250	*	+	+--	-	+++ mb	
16	3.3125	+	+	+--	-	++ mb	
32	1.6563	+-	+-	-	-	+ mb	
64	0.8281	+--	-	-	-	+ mb	
128	0.4141	-	-	-	-	NA	(2)
256	0.2070	-	-	-	-	NA	
512	0.1035	-	-	-	-	NA	
1024	0.0518	-	-	-	-	NA	

1. mb denotes multiple bands
2. Not assayed

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