THE INHIBITION OF COMPLEMENT MEDIATED PHENOMENA BY IgA

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ABSTRACT

The work presented in this thesis was designed to investigate the possibility that IgA has a role as a "blocking" antibody. Two molecular species, a (13S) trimer and a reduced and alkylated (7S) monomer of the di- and trinitrophenyl-specific MOPC-315 IgA myeloma protein were prepared and tested for their ability to inhibit a number of antibody- and complement-dependent reactions *in vitro* and *in vivo*. The reactions examined included complement-dependent haemolysis, complement activation, cutaneous anaphylaxis, the Arthus reaction and *in vitro* polymorphonuclear leukocyte chemotaxis.

Both 7S and 13S IgA were found to inhibit the complement-dependent lysis of trinitrophenylated-sheep erythrocytes sensitized with dinitrophenyl-specific IgG antibodies. On a molar basis, polymeric (13S) IgA was 100 times more efficient as an inhibitor than monomeric (7S) IgA. The inhibition was predominantly antigen-specific and could be counteracted by increasing the level of sensitizing IgG. It was concluded that polymeric IgA may inhibit the lysis of sensitized cells by sterically interfering with the capacity of cell-bound IgG "dimers" to fix complement. However, when the antibodies were mixed before sensitizing the cells, IgA molecules were able to compete with IgG for antigenic sites and interfere with the formation of complement-fixing IgG dimers.

IgA was also tested for its capacity to block the activation of complement by dinitrophenylated-bovine serum albumin and by antigen-antibody complexes. Effective blockage was achieved at low molar ratios (5:1 or 0.1 to 1) of IgA to DNP-BSA or of IgA to IgG antibody when the two were mixed prior to incubation with antigen. The inhibitory effects of the IgA molecules were considered to result from competition for antigenic sites, occlusion of potential complement activating sites, or steric inhibition of C1 fixation.

The addition of low levels of 7S or 13S IgA to antigen, prior to its injection into sensitized mice, specifically inhibited the induction of an active cutaneous anaphylactic reaction. The presence of IgA antibodies in sensitized mice was also associated with marked reductions in both active cutaneous and Arthus reactions to the sensitizing antigens. The possible mechanisms by which these two reactions were inhibited are discussed.

The chemotaxis of polymorphonuclear leukocytes to both antigen-antibody complexes and to bacterial culture fluids was inhibited when the cells were incubated with either form of IgA. In contrast, chemotaxis to antigen-antibody complexes was inhibited by IgA in an antigen-specific manner when the IgA was present during the formation of the complexes. However the addition of IgA to bacterial culture fluid did not inhibit chemotaxis.

It was concluded that IgA may have a definite role in moderating or controlling the reactions studied *in vivo*.

STATEMENT

The material in this thesis has not been previously submitted for a degree in any University, and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text.

(G. J. RUSSELL-JONES)

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CHAPTER 1

Introduction and Literature survey

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INTRODUCTION

1.1 Preamble

The results of research carried out in recent years have established that the ability of the vertebrates to resist a variety of infections depends, in most instances, upon co-operation between the cellular (e.g. lymphocytes, macrophages) and humoral elements (e.g. antibody, complement) of the immune system (Nossal, 1969). With few exceptions (e.g. cytotoxic "killer" T cells; activation of the alternate pathway of complement), virtually all host-protective immune reactions require the presence of antibody for the recognition event. In many instances the nature of reaction which ensues (e.g. cytolosis, phagocytosis, hypersensitivity), is largely determined by the class of antibody produced in response to antigenic challenge.

The work in this thesis is concerned with IgA, a class of antibody which predominates in secretions. This immunoglobulin does not elicit a number of immune reactions induced by other classes of antibody, such as complement fixation, opsonization and hypersensitization. The review which follows will cover reactions mediated by antibody, the properties of IgA immunoglobulin and finally the apparent role of IgA in immunity.

1.2 Reactions mediated by antibody

1.2.1 Inactivation of toxic or enzymically-active antigens

The binding of antibody *per se* to biologically-active antigens

(such as viruses, bacterial flagellae, pili or endotoxins) is sometimes sufficient to inactivate or neutralize the activity(ies) of that antigen. Viruses shown to be inactivated by antibody include poliovirus, influenza virus, rhinovirus, respiratory syncytial virus and echovirus (Hodes, 1964; Kasel *et al.*, 1971; Douglas *et al.*, 1967; Ogra & Karzon, 1969a; Ogra, 1970; Scott & Gardner, 1974; Shvartsman, 1977). Antibodies have also been shown to inactivate toxins such as those released from cholera, shigella, salmonella, tetanus and clostridium (Kaur *et al.*, 1971; Wigley *et al.*, 1969; Batty & Bellen, 1961; Britton, 1969; Burrows *et al.*, 1971; Cash *et al.*, 1974). There is evidence to suggest that the binding of antibody to structures such as bacterial flagellae or pili can prevent attachment of these bacteria to epithelial cells of the oral cavity and gut. Bacterial adherence to these cells is considered to be necessary for colonization (McClelland *et al.*, 1972; Genco *et al.*, 1974; Gibbons, 1974; Svanborg-Eden & Svennerholm, 1978).

1.2.2 Binding of antibody to cells via Fc receptors

Binding of some classes of antibody to relevant cells (via Fc receptors) has been shown to be important in opsonization and anaphylaxis. Phagocytosis, of antigen-IgG antibody complexes by macrophages and polymorphonuclear leukocytes (PMN), can be effectively initiated by binding of the complex to Fc receptors present on the cell surface, or by binding of free antigen to cytophilic antibody already attached (through the Fc region) to the cell surface (Huber *et al.*, 1968; Ehlenberger & Nussenzweig, 1977; Bar-Sharit *et al.*, 1979).

In a similar fashion, anaphylactic reactions are initiated when antigens bind to homocytotrophic (cytophilic) antibody, usually of the IgE class or of a particular IgG subclass (Ovary *et al.*, 1963; 1970), which is attached (through its Fc region) to the surface of mast cells or basophil leukocytes. These cells are then triggered to release histamine and other anaphylactic mediators (Cohen & Porter, 1964; Souza *et al.*, 1974; Lehrer & Vaughn, 1976; Lehrer, 1977; Watanabe & Ovary, 1977).

1.2.3 Complement activation

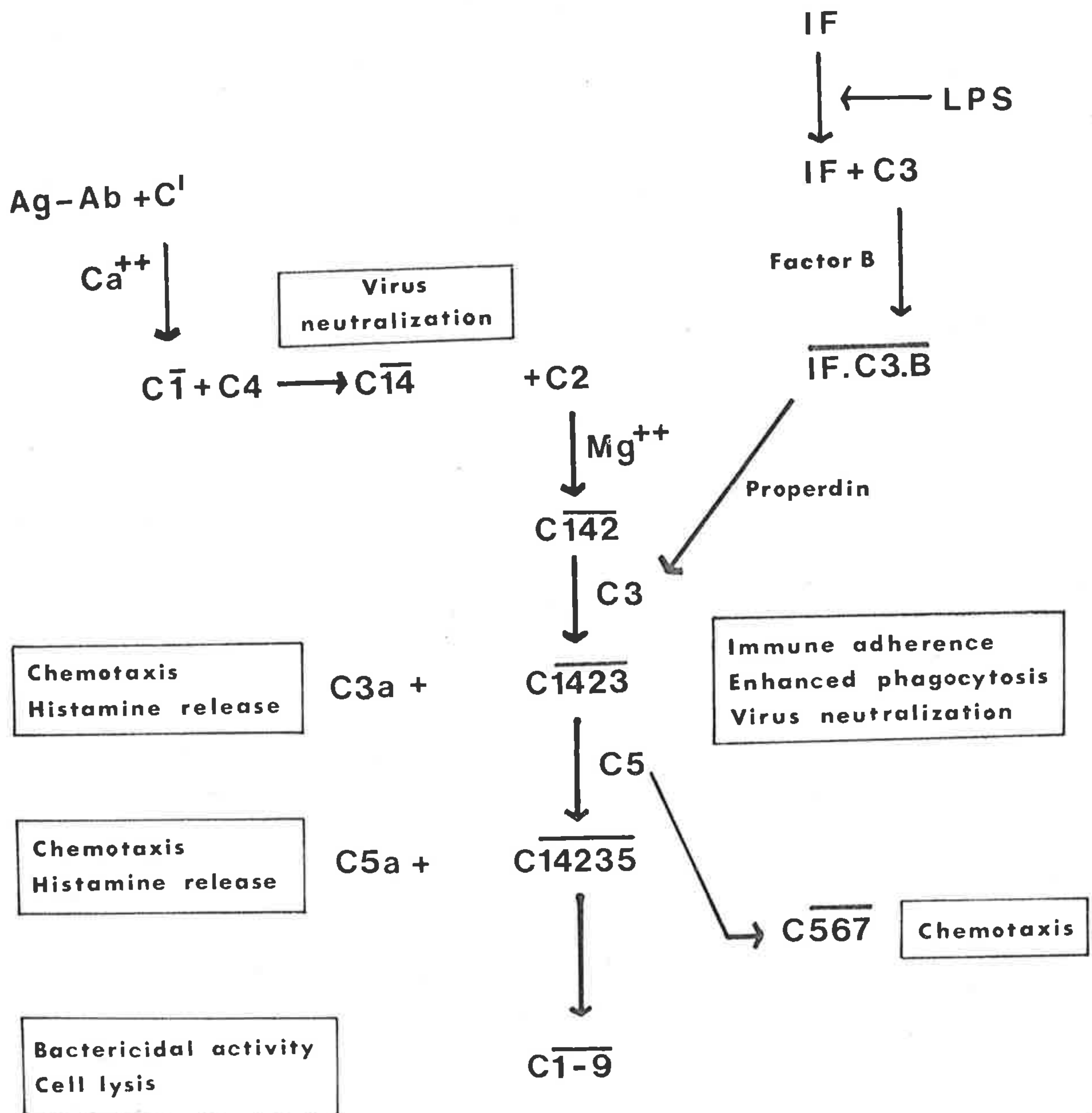
The activation of complement (C) by antigen-antibody complexes triggers a series of reactions (outlined in Fig. 1.1) in which a number of inflammatory and chemotactic mediators are released. These reactions may ultimately lead to phagocytosis or cytolysis. Complement may be activated through two pathways (see Fig. 1.1). The classical pathway can be activated by antigen-antibody complexes, lipopolysaccharide and DNP-substituted proteins (Müller-Eberhard, 1975; Loos & König, 1977; Loos & Thiesen, 1978). Activation of the alternate (or properdin) pathway, can be initiated by aggregated immunoglobulin and a number of other substances including lipopolysaccharide, zymosan, endotoxin and inulin (Sorber, 1978; Müller-Eberhard, 1975; Huber & Wigzell, 1976). The alternate pathway may contribute to immunity against certain organisms in the absence of antibody (Sorber, 1978).

FIGURE 1.1

Activation of the complement sequence by the classical and alternate pathways

Details of the sequence are modified from Colten (1976). Complement components are designated by the symbols C^1, C1-C9. The classical pathway is shown as being initiated by an antigen-antibody (Ag-Ab) complex. The alternate pathway in this instance has been activated by the interaction of lipopolysaccharide (LPS) with the initiating factor (IF).

Complement-dependent biological activities (within boxes) are listed alongside the relevant complement components.


IF
LPS
IF + C3
Ag-Ab + C'
Ca++
Factor B
Virus neutralization
IF.C3.B
C1 + C4
C14
+C2
Mg++
Properdin
C142
C3
Chemotaxis
Histamine release
C3a +
C1423
Immune adherence
Enhanced phagocytosis
Virus neutralization
C5
Chemotaxis
Histamine release
C5a +
C14235
C567
Chemotaxis
Bactericidal activity
Cell lysis
C1-9

1.2.4 Biological activities of complement

a) Opsonisation

Macrophages, and other types of phagocytic cells (i.e. PMN), (Ehlenberger & Nussenzweig, 1977), possess surface receptors for the C3b fragment of complement (C). Activation of C3 and adsorption of antigen-antibody associated C3b to these receptors is sufficient to mediate phagocytosis (Müller-Eberhard, 1968; Miller & Nussenzweig, 1974; Okada & Okada, 1975; Dierich & Reisfeld, 1975; Bar-Shavit *et al.*, 1979).

b) Cytolysis

Lysis of bacteria or other cell types by C has been shown to require the activation of the later components (C5-C9) of the C cascade (Müller-Eberhard, 1975). This may occur through activation of the classical or alternate pathways, resulting in cleavage of C3 and in turn C5. The C5b fragment which binds to the cell surface acts as a substrate for the formation of the C5b-7 complex. Lysis is effected through the subsequent binding of C8 and C9 (Müller-Eberhard, 1975; Hammer *et al.*, 1976). Although C-mediated bacteriolysis is particularl effective against rough, gram negative strains of bacteria, smooth, gram negative strains, bearing large quantities of lipopolysaccharide on their surface, are resistant to killing by complement (Reynolds *et al.*, 1975; Reynolds & Pruul, 1971).

c) Inflammatory reactions

Two important peptides, C3a and C5a, are generated during the activation of C (Müller-Eberhard, 1975). These molecules have

been recognized as anaphylatoxins, since they cause mast cell degranulation and histamine release. C3a and C5a also appear to act directly on blood vessels, increasing vascular permeability (Weigle, 1961; Dias Da Silva & Lepow, 1967; Müller-Eberhard, 1968; Johnson *et al.*, 1975).

Activation of C by antigen-antibody complexes leads to the production of C3a, C5a and the $C\overline{567}$ complex which are chemotactic for polymorphonuclear leukocytes (PMN) (Becker, 1977; Tack *et al.*, 1974), and may lead to PMN infiltration. The Arthus reaction represents an *in vivo* example of this reaction and occurs when C is fixed by antigen-antibody precipitates at sites surrounding capillary blood vessels. The ensuing inflammation results from mediators released from infiltrating PMN (Ward *et al.*, 1965, 1966; Keller & Sorkin, 1967; Tack *et al.*, 1974).

d) Virus neutralization

In some circumstances, antibodies are ineffective in neutralization of viruses unless complement is fixed. The effect of C seems to result from activation of C4 or C3 (Daniels *et al.*, 1970; Osler, 1961).

1.3.1 Antibody classes involved in immune reactions

Five classes of immunoglobulin are known (IgM, IgG, IgA, IgE, IgD in humans) and, all are capable of interacting with antigen. Activation of complement via the classical pathway has been demonstrated with IgM and by most subclasses of IgG. For

instance the C-fixing subclasses of IgG are IgG_1, IgG_2 and IgG_3 in man (Ishizaka *et al.*, 1970; Spiegelberg, 1974) and IgG_{2a}, IgG_{2b} (Miller & Nussenzweig, 1974) and a subclass of IgG_1 (Ey *et al.*, 1980) in the mouse. A number of aggregated myeloma proteins (e.g. human IgG_1, IgG_2, IgG_3 and IgM) have been reported to activate significant amounts of C via the alternate pathway (Augener *et al.*, 1971; Boackle *et al.*, 1974; Iida *et al.*, 1976; Soltis *et al.*, 1979).

Phagocytosis of antigen can be promoted by both IgM and C-fixing IgG through the binding of antigen-antibody complex-associated C3 to C3 receptors on phagocytes. IgG, but not IgM, can also promote phagocytosis of antigen after binding of the antigen-IgG complex to the Fc receptor present on phagocytic cells (Ehlenberger & Nussenzweig, 1977; Huber *et al.*, 1968; Bar-Sharit *et al.*, 1979).

Two classes of homocytotrophic (cytophilic) antibodies have been recognized, IgE (human, rat, mouse, guinea pig) and in some species a subclass of IgG (humans, IgG_4; mice, IgG_1) (Vaz & Ovary, 1968; Barth & Fahey, 1965).

1.3.2 Immune reactions involving IgA

IgA, like other antibodies, has the ability to neutralize biologically active antigens (e.g. see Section 1.2.a). Protection against viruses, particularly those infecting the mucosal surfaces (e.g. rhinovirus, poliovirus, influenza virus and echovirus), becomes solid after local immunization with

live or attenuated virions (Keller & Dwyer, 1968; Ogra 1968; Smith *et al.*, 1966; Ogra & Karzon, 1969; Ogra, 1970; Ganguly *et al.*, 1973; Waldman & Ganguly, 1974). Binding of secretory IgA to structures such as bacterial pili and flagellae is effective in preventing adherence of the bacteria to epithelial cells and subsequent penetration and colonization of the latter cells (Ellen & Gibbons, 1972; Freter, 1972; Genco *et al.*, 1974; Fubara & Freter, 1973; Gibbons, 1974; Svanborg-Eden & Svennerholm, 1978). The presence of secretory IgA antibodies specific for bacterial antigens or toxins, has been shown to be effective in protecting the host against infection by the relevant bacteria (Hodes, 1964; Ogra & Karzon, 1969; Murray *et al.*, 1973; Scott & Gardner, 1974; Rogers & Synge, 1978). One mechanism postulated to be involved in protection by secretory IgA is that the antibodies may agglutinate the bacteria, and facilitate their removal by cilia which line the mucosal surfaces (McClelland *et al.*, 1972; Reynolds & Thompson, 1973). The appearance in saliva of secretory IgA antibodies to one serotype of oral streptococci has been correlated with a change in the predominant serotype inhabiting the oral cavity (Genco *et al.*, 1974; Bratthall & Gibbons, 1975).

a) Complement fixation

Many workers have tested "native" serum (7S) IgA and/or secretory (11S) IgA antibodies for their capacity to activate C, both in the presence or absence of antigen. Although some reports have suggested that IgA can activate C (Kaplan *et al.*,

1972; Adinolfi *et al.*, 1966; Hill & Porter, 1974), it is now generally accepted that unaggregated 7S serum IgA or secretory IgA is unable to activate either the classical or by-pass pathways of C (Knop *et al.*, 1971; Eddie *et al.*, 1971; Boacke *et al.*, 1974; Colten & Bienenstock, 1974; Hill & Porter 1974). In contrast, preparations of IgA (mainly myeloma proteins) aggregated by various means (freeze-dried, bis-diazotized, heat aggregated) have been shown to fix C (via the classical pathway) in the absence of antigen (Iida *et al.*, 1976). It is important to note, however, that aggregated preparations of IgA fix C far less efficiently than preparations of aggregated IgG (one fifth to one tenth the amount of C consumed per unit weight). Preparations of aggregated IgA $F(ab^1)_2$ fragments were found to activate C with an efficiency similar to that of the aggregated intact molecules (Boackle *et al.*, 1974). It is therefore believed that the Fab rather than the Fc region, is responsible for the C-fixing activity of aggregated, intact IgA (Boackle *et al.*, 1974; Iida *et al.*, 1976; Robertson *et al.*, 1976). It therefore seems unlikely that the activation of complement by aggregated IgA is of physiological significance. The procedures used to aggregate IgA are likely to cause partial denaturation of the molecules and so it seems probable that the C-fixing ability of aggregated IgA is simply due to the effect of denaturation rather than the aggregation of IgA molecules, since the manipulation of other non-immunoglobulin proteins has been found to confer similar C-fixing abilities on these molecules. For example, the extensive dinotrophenylation of human serum albumin or excessive trinitrophenylation of erythrocytes confers considerab

C-fixing properties on these otherwise non C-reactive conjugates (Loos *et al.*, 1974; Loos & Bitter-Suermann, 1976; Loos & Konig, 1977; Loos & Thesen, 1978).

b) Opsonic properties

Antigen-antibody complexes can be bound to phagocytes via either the Fc or C3 receptors present on the cell surface (Ehlenberger & Nussenzweig, 1977; Huber *et al.*, 1968). IgA antibodies have been shown to be inactive as opsonins for their specific antigens (Eddie *et al.*, 1971; Huber *et al.*, 1971; Wilson, 1972; Steele *et al.*, 1974). This observation is consistent with the finding that IgA is neither C-fixing (Knop *et al.*, 1971; Eddie *et al.*, 1971; Boackle *et al.*, 1974; Colten & Bienenstock, 1974; Hill & Porter, 1974), nor is it capable of binding to the Fc receptors of human macrophages, monocytes, basophils or eosinophils (Lawrence *et al.*, 1975; Spiegelborg, 1974; Dickler, 1976).

It is interesting to note that IgA immunoglobulin has been reported to bind to receptors on human polymorphonuclear (PMN) leukocytes (Lawrence *et al.*, 1975). High levels of IgA appear, in fact, to block or inhibit the phagocytic capacity of these cells for antigen-IgG antibody complexes. Only polymeric or aggregated forms of IgA were able to inhibit phagocytosis, thus suggesting that the inhibition may have been due to steric blockage of the Fc receptors for IgG, on these cells (Van Epps & Williams, 1976; Van Epps *et al.*, 1978; Wilton, 1978; Magnussen *et al.*, 1979).

Since IgA cannot fix complement, and it is ineffective as an opsonin, one may ask what special role(s) it has in situ. Although there is no clear evidence that IgA has a role in immunity within the tissues, it is clear, from the discussion presented below, that it is an important factor in the local immunity associated with the gastrointestinal and respiratory tracts.

1.4 Local immunity (historical)

The first worker to infer the existence of a local immune system was Besredka in 1919 (cited in Tomasi & Bienenstock, 1968) following his experiments of oral infections with enterobacteriaceae and skin infections with *Bacillus anthracis*. Davies (1922), who observed agglutinins specific for shigella in the stools of patients with bacillary dysentery, suggested that the agglutinins may have an important role in protection of the host against these infections. Burrows *et al.*, (1947) examined faecal extracts from guinea-pigs experimentally infected with cholera and were able to correlate the titres of faecal antibody (coproantibody), with host protection. There was little correlation however, between serum antibody titres and protection against oral cholera challenge. "Copro-antibodies", therefore, seemed particularly effective against *V. cholerae* since loss of cholera symptoms coincided with the appearance of antibodies in duodenal fluid, rather than their appearance in serum (Freter *et al.*, 1964).

A striking example of the stimulation of a local antibody response and its importance in protection comes from the work

of Ogra and colleagues (Ogra & Karzon, 1969a; Ogra 1968), who injected polio vaccine into the distal colon of children with double barrelled colostomies. IgA antibodies appeared predominantly in the immunized distal segment, very little being observed in the proximal colon and none in the nasopharyngeal secretions. Following a subsequent oral challenge, virus replication was observed in the nasopharynx but not in the colon, suggesting that the antibody produced locally in the immunized colon was involved in protection against the virus.

Local immune responses have also been elicited in a number of other tissues. Murray *et al.*, (1973) found that conjuctival infection of guinea pigs with live guinea pig inclusion organisms (Chlamydia) stimulated good immunity to a subsequent conjuctival infection. Intraperitoneal priming, on the other hand, did not elicit conjuctival immunity. Local immunity has been observed in other tissues including the lachrymal glands, the mucosa of the nares, middle ear and bronchi, the salivary gland, the urinary and genital tracts, and the lactating mammary gland (Smith *et al.*, 1966; Douglas *et al.*, 1967; Scott & Gardner, 1974; Hodes *et al.*,1964; Ogra & Karzon, 1969b; Bellanti *et al.*, 1969; Ehrenkrantz, 1966).

1.4.1 IgA; the predominant immunoglobulin in secretions

In 1963, Tomasi and Ziegelbaum reported that the predominant immunoglobulin found in the secretions of the lachrymal and

salivary glands of humans was IgA, a protein first detected in human serum by Grabar and Williams (1953) and named Bx-globulin, on the basis of its electrophoretic properties. IgA was subsequently found to be the major immunoglobulin in human colostrum, saliva, tears, sweat and urine and to predominate in the secretions of many other species, including the dog, rabbit, mouse, rat, guinea pig, hedgehog and pig (Vaerman & Heremans, 1970, 1972; Cebra & Robbins, 1966; Porter, 1969; Crabbé *et al.*, 1970; Tomasi & Grey, 1972). Ruminants differ from these species in that IgG, rather than IgA, is found to be the predominant secretory immunoglobulin (Sullivan & Tomasi, 1964; Pierce & Feinstein, 1965; Mackenzie & Lascelles, 1968; Heimer *et al.*, 1969).

1.4.2 Function of locally synthesized IgA

IgA antibodies produced at local sites seem to be effective in protecting secretory surfaces against viral or bacterial invasion, either through neutralization of virions or bacterial toxins, or by preventing the adherence of bacteria to adjacent mucosal surfaces (see above, Ogra *et al.*, 1968; Ellen & Gibbons, 1972; Freter, 1972; Ganguly *et al.*, 1973; Waldman & Ganguly, 1974; Genco *et al.*, 1974; Gibbons, 1974; Svanborg-Eden & Svennerholm, 1978). The concentration of IgA around these sites is quite high (5-14 times of that found in serum; Vaerman *et al.*, 1973) and the antibodies have been shown to function in reducing the absorption of a number of antigens by the gut and respiratory tract (Stokes *et al.*, 1974; André *et al.*, 1974; Heremans & Vaerman, 1971). This may minimize the induction of

other classes of antibody which could induce tissue reactions to the absorbed antigen (Beale *et al.*, 1971; Bull & Tomasi, 1968; Scott *et al.*, 1977). For example, Walker *et al.*, (1977) have demonstrated that oral immunization with protein antigens could induce the production of IgA antibodies and reduce absorption of the antigen by the gut. Preincubation of human serum albumin with immune intestinal juice resulted in a 50% reduction in the uptake of this antigen by the intestine (André *et al.*, 1974). The importance of IgA in preventing the induction of serum antibodies can be seen from a study of IgA deficient patients, half of whom showed a significantly higher level of circulating antibodies to milk proteins compared with "normal" patients (Buckley & Dees, 1969; Ammann & Hong, 1971; Tomasi & Katz, 1971).

1.5 Properties of IgA

1.5.1 Structure

Blood and lymph contains two major species of IgA, which are characterized by sedimentation coefficients. They are: 7S (molecular weight 160,000 daltons) and 9S (380,000 daltons). The latter species consists of two disulfide-linked 7S molecules, and a small polypeptide (termed the J chain), which is covalently attached to the IgA dimer immediately prior to secretion from the plasma cell (Halpern & Koshland, 1970; Mestecky *et al.*, 1971; 1972; Corte & Parkhouse, 1973; Parkhouse & Corte, 1976). Secretory IgA (11S, S-IgA) is formed when the 9S molecule is complexed to the secretory component, a protein

present on the surface of the cells of the outer crypt epithelial mucosa. Binding of the 9S dimer to the secretory component occurs firstly through non-covalent interactions and is followed by the formation of disulfide bonds (Brandtzaeg, 1978; Crago *et al.*, 1978). The secretory component does not bind to 7S IgA and will not bind to IgA dimers unless the J chain is present (Halpern & Koshland, 1970; Mestecky *et al.*, 1972; Eskeland & Brandtzaeg, 1974).

1.5.2 Synthesis

IgA has been shown to be synthesized in a number of tissues; the bone marrow, spleen, peripheral lymph nodes, intestinal mucosa, and in various submucal areas, such as the lachrymal and salivary glands and the submucosa lining the respiratory tract (Freter *et al.*, 1964; Smith *et al.*, 1966; Brandtzaeg *et al.*, 1967; Douglas *et al.*, 1967; Crabbé *et al.*, 1969; Tourville *et al.*, 1969; Vaerman & Heremans, 1970; Murray *et al.*, 1973; Radl *et al.*, 1974; Scott & Gardner, 1974). The IgA found in the secretions of these tissues comes predominantly from local synthesis.

The IgA found in the bile of a number of species (rat, dog, guineapig, mouse) is not synthesized locally but is produced predominantly in the intestinal mucosa. It enters the blood supply via the mesenteric and thoracic duct lymph vessels which have much higher levels of IgA than serum (viz: 14-, 3- and 4.5-fold higher in the rat, dog and guineapig respectively; Kaartinen *et al.*, 1978; Vaerman *et al.*, 1969, 1973; Lemaître-Coelho *et al.*, 1978a&b; Orlans *et al.*, 1978;

Birbeck *et al.*, 1979). The IgA is actively transported from the blood into bile and in the rat an estimated 10-15 mg of S-IgA enters the intestine per day, via the bile (Lemaître-Coelho *et al.*, 1978a&b). Vaerman and co-workers (Vaerman & Heremans, 1970; Vaerman *et al.*, 1969) have estimated that the intestinal mucosa of the dog is capable of producing all of the IgA found in serum. Similar results have been found in the guineapig and rat (Kaartinen *et al.*,1978; Lemaître-Coelho *et al.*, 1978a&b; Hall *et al.*, 1978; Birbeck *et al.*, 1979). In these species, the IgA in serum is predominantly 9S, most of which (80%) is derived from intestinal (mucosal) synthesis (Vaerman & Heremans, 1970, 1973). Human serum IgA, however, is derived mainly from plasmacytes in the bone marrow, spleen and peripheral lymph nodes. The intestinal mucosa contributes very little IgA (2-3%) to the serum pool (Hijmans *et al.*, 1971; Radl *et al.*, 1974). Furthermore, in humans, the level of IgA in the mesenteric or thoracic duct lymph does not exceed that of serum (Radl *et al.*, 1974; Kaartinen *et al.*, 1978).

In man, the amount of IgA synthesized per day equals that of IgG and is approximately 30 mg/kg (Heremans, 1974). However, the metabolic half-life of IgA is much shorter (5-6 days) than that of IgG (3 weeks) (Walmann & Strober, 1969; Spiegelberg, 1974).

1.5.3 Distribution and secretion

The IgA present in human serum constitutes 10-15% of the total immunoglobulin pool (0.5-2.2 mg/ml) and is comprised

predominantly of one species, although up to 20% may be polymeric (10-18S) (Tomasi & Grey, 1972). However, in the secretions (tears, saliva, and those of the respiratory and gastrointestinal tract), IgA is the predominant immunoglobulin (Tomasi & Grey, 1972), and is composed mainly (80%) of 9S molecules coupled to secretory component (Newcomb & De Vald, 1969). Many animal species differ from man, in that more than 50% of serum IgA is polymeric. The total concentration of IgA in serum, however, is less than that found in man. For example, in the dog and cat, the concentration of IgA in serum ranges from 0.1 - 0.4 mg/ml and is composed to a large extent (80%) by dimers (Vaerman & Heremans, 1969; Reynolds & Johnson, 1971; Kaartinen *et al.*, 1978).

Secretion of IgA into areas such as the lumen of the gut and into the ducts of the lachrymal and salivary glands, occurs after dimeric IgA becomes complexed to the secretory piece on the surface of the submucosal epithelial cells. The IgA is then "selectively" pinocytosed, transported through the cytoplasm of the cell and secreted (Brandtzaeg, 1978). In a number of animal species (viz:- dog, cat, rat, mouse), as much as 50% of the IgA produced in the submucosa of the gut, is believed to drain via the thoracic and mesenteric duct lymph into the blood. Up to 80% of the IgA in the serum of these animals, is thought to originate in areas drained by these vessels (Vaerman & Heremans, 1970; Vaerman *et al.*, 1973; Hall *et al.*, 1978; Kaartinen *et al.*, 1978). During the course of this thesis, it became apparent that much of the polymeric, IgA present in serum could be transported into the

bile (Lemaître-Coelho *et al.*, 1978a&b; Orlans *et al.*, 1978; Birbeck *et al.*, 1979). Orlans and co-workers (1978) reported that 90% of radiolabelled IgA, injected into the blood of rats, was cleared within 3 hours, and up to 40% appeared in the bile. In another study, rats showed a 10 to 20 fold increase in the concentration of serum IgA within 24 hours of bile duct ligation. Upon removal of the ligature the level of serum IgA rapidly returned to normal (Lemaître-Coelho *et al.*, 1978a&b).

1.5.4 Resistance of S-IgA to proteolysis

S-IgA has been found to be significantly more resistant than 7S IgA or IgG to proteolysis by pepsin, papain, trypsin or chymotrypsin (Brown *et al.*, 1969, 1970; Kenny *et al.*, 1967; Tomasi & Calvanico, 1968; Shuster, 1971). For instance, treatment of S-IgA with trypsin or chymotrypsin (38°, 8 hours) caused less than a 20% reduction in the molecular weight of the intact molecules, whereas similar treatment of IgG or 7S IgA caused the loss of the Fc portion of these molecules (Tomasi & Calvanico, 1968; Brown *et al.*, 1970). Treatment of mouse IgG and IgM with intestinal fluid caused a 97% reduction in vibriocidal titre but less than a 50% reduction in the ability of each antibody to protect baby mice against vibrio infection (Horsfall, 1977). In comparison, S-IgA appeared resistant to such treatment, as judged by Sephadex G-200 chromatography and by the baby mouse protection test.

1.6.1 Functions of IgA

Secretory IgA clearly seems to be involved in protecting secretory surfaces against bacterial and viral infections (see Section 1.3.b) and possibly in preventing antigen uptake across these secretory surfaces. While the IgA occurring in lymph and serum has a definite function when it is secreted, it is as yet unclear as to whether it has any other function within the body. Moreover, there is a lack of information concerning the effect which the presence of IgA antibodies might have on the activities of other classes of antibody. Data obtained from experiments using polymeric and aggregated myeloma IgA proteins have suggested that IgA may, in fact, block the initiation of bacteriolytic, phagocytic and hypersensitivity reactions by other classes of antibody (see Section 1.6.b). One can therefore ask whether IgA may, under certain circumstances, function at local sites by reducing or inhibiting reactions such as those mentioned above. The magnitude of such reactions is normally limited by the short half-life of the activated mediators (e.g. C components), and by various inhibitors of the C cascade, but it may be that some control over the initiation of these reactions is also necessary and is governed by the relative levels of IgA and the other classes of antibody present.

1.6.2 Blockage of reactions by IgA

a) Bacteriolysis

The early work of Hall & Manion (1953) and Zinneman *et al.*,

(1959) demonstrated the presence in hyper-immune human serum of a factor which could inhibit the lysis of brucellae organisms by normal serum. Preliminary characterization of this factor showed it to co-purify with the IgA class of immunoglobulin (Glenchur *et al.*, 1961; Zinneman *et al.*, 1964). In another study, the bacteriolytic activity of IgG and IgM antibodies could be inhibited by IgA isolated from the sera of hyper-immune rabbits. The degree of inhibition was proportional to the ratio of IgA to IgG or of IgA to IgM used to sensitize the bacteria (Hall *et al.*, 1971). Griffiss (1975), who obtained similar results using serum fractions isolated from patients convalescing from meningococcal infection, found that the IgA antibodies were more efficient at blocking the sensitization of meningococci for lysis by IgG than by IgM antibodies. These investigators (Hall *et al.*, 1971; Griffiss, 1975), did not isolate the specific antibodies from each immunoglobulin class, nor did they determine the number of molecules of each antibody class binding to the bacterial surface during blocking. Griffiss did, however, postulate that IgA antibodies compete with IgG or IgM molecules for specific antigenic sites on the bacterial surface thus decreasing the amount of C fixed. The degree of reduction would be proportional to the ratio of IgA to IgG or IgA to IgM antibodies competing for antigenic sites on the cell surface

b) Phagocytosis

While there is evidence (see Section 1.3.a(ii)) to show that IgA does not promote phagocytosis of an antigen, other data

suggest that IgA may in fact inhibit phagocytosis of antigen-antibody complexes by polymorphonuclear (PMN) leukocytes. For example, Wilton (1978) found that human IgA myeloma proteins and normal human serum or colostral IgA could inhibit the IgG antibody-dependent phagocytosis of *Candida albicans* by PMN leukocytes. The IgA apparently prevented the attachment of IgG molecules, bound to the yeast, to Fc receptors on the polymorphs. In another study, IgA paraproteins were found to prevent PMN cells from killing *E.coli* and *Staphlococcus avreus* opsonized with IgG (Van Epps *et al.*, 1978). Inhibition of phagocytosis was observed only with polymeric forms of IgA. Similarly, treatment of polymorphs with IgA inhibited the phagocytosis of IgG coated latex particles.

c) Hypersensitivity in allergic individuals

Many allergic individuals, typically hay fever sufferers showing sensitivity to allergens such as timothy-grass pollen, ragweed allergen, the house dust mite or *Dermatophagoides farinae*, show reduced sensitivity to allergen following desensitization with the appropriate antigen (Blair *et al.*, 1975; Melam *et al.*, 1971; Sobotka *et al.*, 1976; Deuschl *et al.*, 1977; Zeiss *et al.*, 1977; Wilie *et al.*, 1978). Examination of the serum of other patients undergoing desensitization revealed that the levels of IgG antibody increased during treatment (from 238 to 3142 ng of antigen bound/ml) concomitant with an increase in the ratio of IgG to IgE antibody (from 19-290:1 to 1167:1) (Lichtenstein *et al.*, 1968; Melam *et al.*, 1971; Sobotka *et al.*, 1976; Skou & Norn, 1977; Zeiss *et al.*, 1977). Increases in the titre of serum IgA antibody have also

been reported to occur during treatment (Wilkie *et al.*, 1978; Stokes *et al.*, 1974; Deuschl *et al.*, 1977), but for lasting desensitization a persistent reduction in the level of reagin is required (Schumacher & Jeffery, 1979).

Desensitization is thought to result from an increase in titre of 'blocking' antibodies which bind to allergens, present in the respiratory tract mucous or extracellular tissue fluid, and thus prevent these allergens from binding to cell-bound reaginic antibody in the underlying mucosa (Giessen *et al.*, 1976; Schumacher & Jeffery, 1979). The class(es) of antibody responsible for the postulated blocking activity in these areas have not been clearly defined. Since IgA predominates at most secretory surfaces and because it has been shown to increase during desensitization, there is a high probability that this class of antibody is active in blockage and desensitization.

Other studies investigating the mechanisms of sensitization have shown the development of allergy in the first year of life following a transient deficiency of IgA during the first three months of age (Taylor *et al.*, 1973; Stokes *et al.*, 1974). Such a deficiency may allow increased absorption of allergens in areas such as the respiratory tract leading to an over-stimulation of IgE antibody which then remains high throughout life. Thus, it appears that IgA antibodies may be important both in preventing the initial sensitization to allergen and in the desensitization of allergic individuals.

1.7 Aims of the thesis

The evidence discussed in the preceding sections suggests that IgA has an important role in the protection of secretory surfaces. There is a lack of information, however concerning the possible functions of IgA in local tissue sites. Since IgA does not elicit many of the reactions demonstrable with other immunoglobulins, it seems clear that if it does in fact fulfill some functional role within the tissues, then this role will be distinct from those of IgG and IgM. One possible role is suggested from the experiments, discussed earlier, in which IgA was found to block antibody-dependent reactions such as bacteriolysis, phagocytosis and hypersensitivity. It may be, therefore, that IgA has, under certain circumstances, a role as a blocking antibody, inhibiting or controlling the reactions of other antibody classes.

The idea that IgA has such a role seems to have received little consideration in the literature. It was therefore deemed worthwhile to investigate the capacity of IgA to block a number of antibody-dependent reactions, with a view to assessing the probability that IgA functions in such a manner. The work presented in this thesis has examined the capacity, particularly on a quantitative basis of the DNP-specific IgA myeloma protein (isolated from the mouse plasmacytoma, MOPC-315), to block a number of antibody-dependent reactions, including cytolysis (Chapter 3), complement fixation (Chapter 4), immediate-type hypersensitivity (Chapter 5), and PMN leukocyte chemotaxis (Chapter 6).

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CHAPTER 2

A. Materials and Methods

2.A.1 Gel filtration media

Sephacryl S-200, Sephadex G-200, Sepharose 6B and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Sydney). The gels were poured into columns to give, in each case, a bed of dimensions 80 x 2.5 cm.

2.A.2 Buffers

a) Isotonic Veronal Buffer (VB)

A 5x concentrated stock solution of VB (Hoffmann & Mayer, 1977), prepared according to the following formula, was stored at 4°.

Na Cl	83.0 gm
Sodium 5.5^{1} dimethyl barbiturate	10.19 gm
Na N_3	5.0 gm

The solution was adjusted to pH 7.35 with 1.0M HCl and made up to 2 litres with distilled water. The stock solution was diluted with sterile distilled water to make a working solution.

b) Isotonic Tris-EDTA-Saline-Azide (TESA)

An isotonic buffer for use in column chromatography was prepared as a 5-fold concentrated stock solution which was

stored at 4°. The concentrated solution contained:-

	gm/2 litre
Na Cl	77.43
Na N_3	5.0
Trizma base	30.28
EDTA $Na_2.2H_2O$	0.375

The pH of the solution was adjusted to 8.0 with 1.0 M HCl (approx. 151.1 ml).

c) Phosphate Buffered Saline (PBS)

This buffer was prepared from a 10x concentrated phosphate buffer (19 parts 0.04 M NaH_2PO_4 plus 81 parts 0.04 M Na_2HPO_4; containing 40 mM NaN_3). The final pH was 7.4.

2.A.3 Protein A-Sepharose affinity chromatography

Staphlococcal protein A, covalently linked to Sepharose CL-4B (protein A-Sepharose), was obtained from Pharmacia Fine Chemicals (Sydney). The gel was swollen in PBS and packed into a 10 ml glass pipette. The column was stored and used in a 4° coldroom. Eluant buffer solutions consisted of 0.14 M sodium phosphate (pH 8.2) or 0.1 M sodium citrate-citric acid (pH range 3-6), all containing 8 mM NaN_3. Fractions were neutralised with 1 M Tris.HCI (pH 9) immediately after collection. The column was regenerated by washing at pH 3.0 before fractionation.

2.A.4 Antigens

a) Dinitrophenylated bovine serum albumin (DNP-BSA)

DNP-BSA was prepared by reacting 1-fluoro-2,4-dinitrobenzene (BDH Chemicals Ltd.) with BSA (Cohn fraction V, Commonwealth Serum Laboratories, Melbourne) according to Porter (1950). The degree of substitution of the BSA by DNP was calculated by the method of Little & Eisen (1967), assuming a molar extinction coefficient at 360 nm for DNP of 17,530. The weight of DNP (calculated from $O.D_{360}$) was deducted from the dry weight of each DNP-BSA preparation, and the molar concentration of BSA thus determined. Two preparations were used, one containing 20 ($DNP_{20}BSA$) and the other containing 46 ($DNP_{46}BSA$) moles of DNP per mole of BSA.

b) Dinitrophenylated *E. coli* (DNP-*E.coli*)

DNP-*E.coli* were prepared by reacting 1 gm of lyophilised *E.coli* K12 (kindly supplied by Dr. P. Reeves of this department) with 1 gm of sodium 2,4-dinitro-benzenesulphonate (BDH Chemicals Ltd.) in 50 ml of 0.2 M K_2CO_3 for 20 hr at room temperature (vigorous stirring, protected from light) (Furuichi & Koyama, 1975). The labelled cells were washed five times with distilled water and dialysed against water overnight at 4°.

c) Dinitrophenylated bovine gamma globulin (DNP-BGG)

BGG, containing 16.6 moles of DNP per mole, was prepared using a modification of the method of Little & Eisen (1967). Briefly,

1.0 gm of BGG was reacted with 50.8 mg of sodium dinitrobenzenesulphonate in 50 ml water containing 1.0 gm of Na_2CO_3 for 48 hrs at room temperature (sheltered from light). The solution was dialysed exhaustively against cold water. The degree of substitution was then determined (Sec. A.4(a)).

d) Trinitrophenylated keyhole-limpet haemocyanin (TNP-KLH

TNP-KLH was kindly donated by Dr. P.L. Ey of this department. It was prepared by reacting 2,4,6-trinitrobenzenesulphonate (BDH Chemicals Ltd.) with keyhole-limpet haemocyanin (Calbiochem.) and contained 24 moles of TNP per 100,000 gm.

e) Sheep red blood cells (SRBC)

SRBC were collected from a merino sheep kept at the University animal facility. Blood was drawn aseptically into sterile 3.8% trisodium citrate and stored at 4°. The cells were washed in saline immediately before use.

f) Trinitrophenylated sheep red blood cells (TNP-SRBC)

TNP-SRBC were prepared by reacting 1 ml of packed SRBC with 12 mg of sodium 2,4,6-trinitrobenzenesulphonate (BDH Chemicals Ltd.) for 20 min at room temperature in 9 ml PBS (Rittenburg & Pratt, 1969). Labelled cells were washed sequentially with 20 ml volumes of ice-cold VB, VB containing glycine (20 mg/ 20 ml) and then twice more with VB. The cells were then resuspended to a final concentration of 2% (v/v).

2.A.5 Purification of DNP-specific antibodies

Sera containing DNP-specific antibodies were passaged at room temperature through a 25 ml column of DNP-Sepharose. After the column had been washed with PBS, adsorbed antibodies were eluted with 5 ml of 0.1 M DNP-glycine. DNP-glycine was separated from the antibodies in the eluate by adsorption to DOWEX 1X8 anion exchange resin (Sigma) according to Goetzl & Metzger (1970). The DNP-Sepharose and DNP-glycine were prepared as follows:

a) DNP-Sepharose

Aminohexyl Sepharose 4B was prepared by reacting 1,6-diaminohexane with CNBr-activated Sepharose 4B according to Cambiaso *et al.*, (1975). Sixty ml of 50% aminohexyl Sepharose (in 0.2 M Na_2CO_3) was reacted in the dark with 0.4 gm 2,4-dinitrobenzenesulphonate (13 hr, 37°) and then washed extensively with distilled water before equilibration with PBS plus 15 mm NaN_3.

b) DNP-glycine

N-DNP-glycine was prepared by the method of Porter (1950). Briefly, glycine (3.8 gm in 140 ml of ethanol containing 11 gm of $NaHCO_3$) was reacted (O/N; 22°) with 11 gm of 1-fluoro-2,4-dinitrobenzene (FDNB) in ethanol (280 ml). The ethanol was removed on a rotary evaporator and unreacted FDNB was extracted by washing the residue several times with ether. The DNP-glycine was precipitated with 1M HCl, filtered

and air-dried after recrystallization from aqueous methanol. A 0.1 M solution was prepared in distilled water containing 15 mM NaN_3. The pH of this solution was approximately 8.4.

2.A.6 Production of antisera and isolation of antibodies

a) DNP-specific rabbit IgG

Rabbits were given multiple subcutaneous injections of $DNP_{20}BSA$ (1.5 mg in Freund's incomplete adjuvant) on days 0 and 14. Blood was collected on day 28. The antibodies were isolated from the serum by adsorption to DNP-Sepharose. Following their elution with DNP-glycine, IgG antibodies were adsorbed to protein A-Sepharose at pH 8 (Goding, 1978) and subsequently eluted at pH 3.0. The eluate was neutralised and applied to a column of Sephacryl S-200. The IgG antibodies eluted in a single peak. The relevant fractions were pooled, concentrated using a PM-10 ultrafiltration membrane (Amicon Corp., Mass., U.S.A.) and finally dialysed against saline containing 15 mM NaN_3.

b) Mouse IgG_{2a} immunoglobulin containing TNP-specific antibody

This immunoglobulin was provided by Dr. P.L. Ey. The preparation and analysis is described in detail by Ey *et al.*, (1980). Briefly, LACA mice were immunized subcutaneously with trinitrophenylated human gamma globulin (0.1 mg) in Freund's complete adjuvant (day 0), boosted on days 34, 57 and 71 using incomplete adjuvant and bled on day 91. The

pooled serum was chromatographed on Sephadex G-200 and fractions containing IgG antibody were passaged through protein A-Sepharose at pH 8. After eluting IgG_1 at pH 5.8, the IgG_{2a} immunoglobulins were eluted at pH 4.5, neutralised and dialysed against saline-containing 15 mM NaN_3.

c) Mouse IgG_{2a} immunoglobulin containing SRBC-specific antibody

This immunoglobulin (see Ey *et al.*, 1980) was generously supplied by Dr. P.L. Ey. An antiserum was raised in LACA mice which had each been injected intravenously with 0.2 ml of 1% SRBC in saline on days 0,17,42,64,80,94 and 112. They were bled on day 127. The pooled serum was fractionated as described in the previous section.

2.A.7 Preparation of an anti-μ chain immunoadsorbent column

Commercial anti-μ chain serum (1.5 mls; Meloy Laboratories, Springfield, U.S.A.) was coupled to 10 ml of CNBr-activated Sepharose 4B (March *et al.*, 1974) by reaction for 60 min at room temperature followed by further reaction for 48 hours at 4°. The gel was poured into a column and washed extensively with TESA pH 8.0. After use as an immunoadsorbent, the column was regenerated with 1 M HCl and then re-equilibrated with TESA.

2.A.8 Radioiodination

Immunoglobulins were radiolabelled by a modification of the method of Greenwood *et al.* (1963). Briefly, 30 μl of immuno-

globulin (0.5-1.0 mg/ml in PBS, pH 8.0), 1 µl of ^{125}I (IMS 30, The Radiochemical Centre, Amersham) and 30 µl of chloramine-T (0.25 mg/ml in PBS) were mixed and the reaction terminated after 5 min by the addition of 30 µl of $Na_2S_2O_5$ (0.5 mg/ml in PBS). The reaction solution was mixed with 20 µl of 0.1 M KI and with 400 µl of 10% normal rabbit serum in PBS before being dialysed exhaustively against TESA. The reaction was carried out at room temperature. Dialysis and storage of the radiolabelled material was performed at 4°.

2.A.9 Quantitation of mouse immunoglobulins

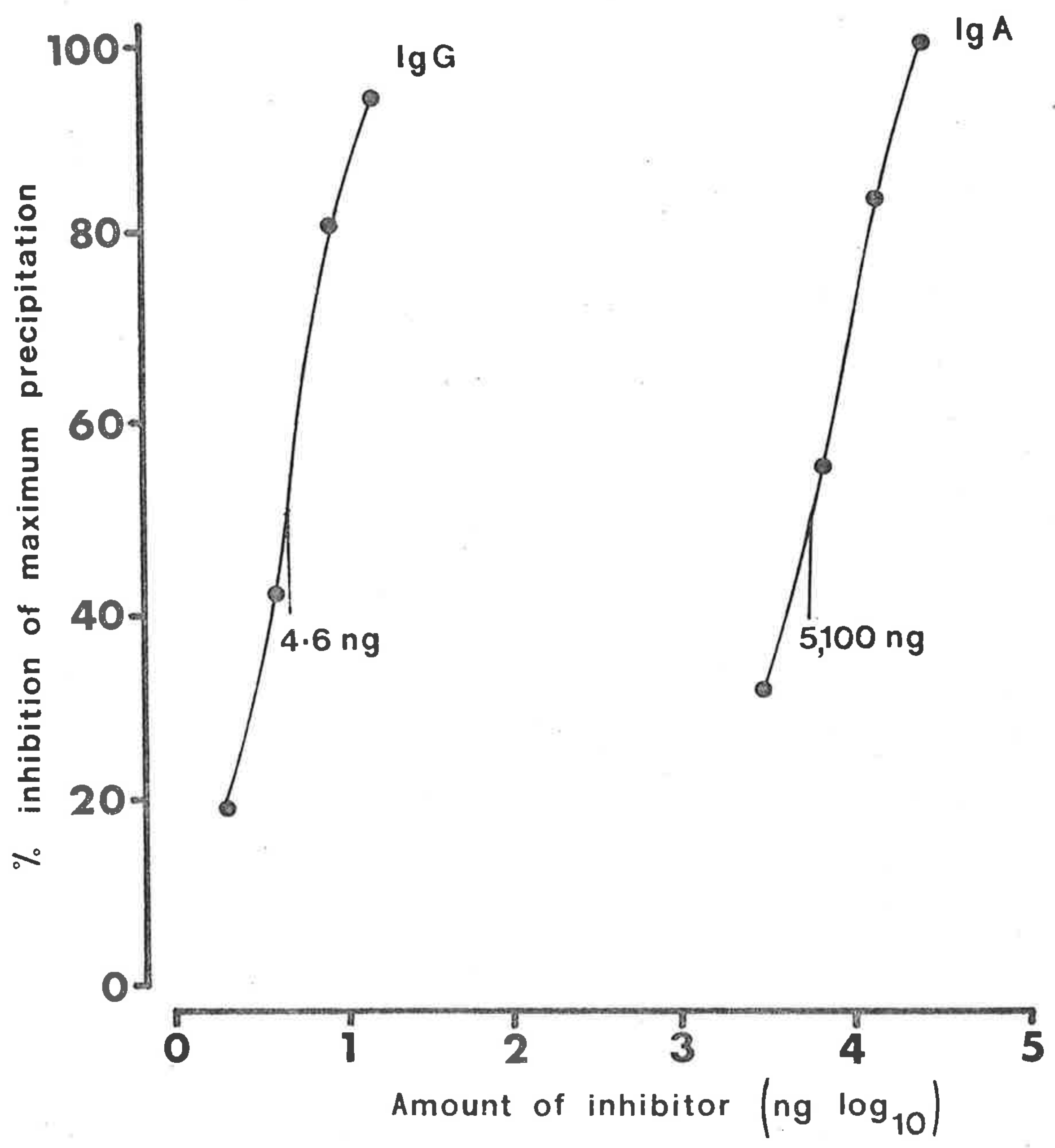
a) Single radial immunodiffusion

The immunoglobulin content of antibody preparations was determined using a modification of the method of Mancini *et al.* (1975). One millimetre thick layers of agarose (A grade, Sigma Chem. Co.) in saline containing a predetermined amount of mouse H chain-specific goat antiserum (γ_1, γ_{2a}, γ_{2b}, µ or α; Meloy Laboratories, Springfield, U.S.A.) were formed on glass slides, which had been cleaned in detergent and coated with 0.1% agarose. Plates were poured by pipetting 5 ml of 1% agarose, which had been mixed at 56° with anti-serum, onto warmed slides. After the layers had set, a series of wells were cut in the gel using a brass punch. Each well was filled with 3 µl of sample. Plates were incubated in an humidified atmosphere at 37° for 18-24 hours. They were then washed and stained with 0.5% Coomassie brilliant

FIGURE 2.1

Ability of unlabelled IgG_1 or IgA to inhibit the precipitation of ^{125}I-labelled IgG_1 by anti-γ_1 chain serum

Inhibition curves showing the ability of unlabelled IgG_1 or IgA to inhibit the precipitation of IgG_1 (5 ng) by a fixed amount of anti-γ_1 chain serum. Details are described in Section 2.A.9(b).


IgG
IgA
4·6 ng
5,100 ng
100
80
60
40
20
0
0
1
2
3
4
5
% inhibition of maximum precipitation
Amount of inhibitor (ng log_{10})

blue R-250 according to the procedure described by Weeke (1973). The diameter of the precipitin rings was measured using an eye gauge.

b) Radioimmunoassay

The immunoglobulin composition of antibody preparations was alternatively determined by a radioimmunoassay technique (Herzenberg & Herzenberg, 1973; Ey *et al.*, 1978). Dilutions of each sample were tested for their ability to inhibit precipitation of 5 ng of ^{125}I-labelled mouse immunoglobulin by a fixed amount of antiserum specific for the corresponding isotype. The amount of immunoglobulin necessary to achieve a 50% inhibitory end point was determined for each isotype using a reference serum containing known concentrations of each isotype. A typical inhibition curve is shown (Fig. 2.1) in which 4.6 ng of reference IgG_1, or 5,100 ng of purified 13S IgA was needed to inhibit (by 50%), the precipitation of ^{125}I-labelled IgG_1 by an antiserum specific for γ_1 chains. Thus the IgA preparation contained 0.09% IgG_1.

2.A.10 Haemagglutination

Haemagglutinating antibodies were titrated by mixing serial 2-fold dilutions of antibody (0.2 ml) with 0.2 ml of 1% SRBC or TNP-SRBC. The diluent was VB^{2+} (see 2.A.11). Agglutination was assessed after the cells had settled for several hours at room temperature.

2.A.11 Haemolysis

All reagents were diluted in isotonic veronal buffer (pH 7.4) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (VB^{2+}) (Hoffmann & Mayer, 1977), supplemented with 0.2 mg/ml BSA and 8 mM NaN_3 (VB^{2+}-BSA). For C, fresh guinea pig serum was adsorbed with TNP-SRBC (1.5 ml of packed cells/20 ml serum, 30 min, 0°) or with SRBC and DNP-Sepharose. The serum was stored at -20° in 0.25 ml aliquots. These were thawed at room temperature as required and used immediately. With the exception of Fig. 3.4, haemolytic assays involved sensitisation of TNP-SRBC (0.2 ml, 2.5%) with 0.4 ml of antibody at 37° (period of incubation indicated) prior to addition of C (0.6 ml, diluted 1/50) and incubation for a further 30 min. The tubes were immediately centrifuged and the $O.D._{541}$ of each supernatant was measured. Controls consisting of cells incubated with saline, C or antibody, or lysed with water, were routinely included. Experimental details are given in the Figure legends.

2.A.12 Complement consumption

Incubation of antigen-antibody complexes with complement (C) causes activation and consumption of complement components. The extent of C consumption is proportional to the amount of complex present. The amount of C consumed can be determined in an haemolytic assay by titrating the residual C for its ability to lyse haemolysin-sensitised SRBC.

a) Sensitization of sheep red blood cells (SRBC)

SRBC (1 ml of packed cells) were sensitized with antibody by suspension in 10 ml of a $^{1}/100$ dilution of haemolysin (Commonwealth Serum Labs., Melb.) in saline. After incubation at 37° for 20 min, the cells were washed 3X in saline and suspended in saline at 2% (v/v).

b) Assay

The following procedure, modified from Mayer (1961) was employed in all complement consumption assays. Replicate samples to be tested (0.2 ml) were mixed with 0.4 ml of guinea-pig serum (diluted to give 14 CH_{50} units per 0.4 ml) and incubated at 37° for 60 min. After this time, 0.2 ml of saline was added and the entire contents of each tube were diluted in serial, 2-fold steps with 0.8 ml aliquots of saline. Sensitized SRBC (0.2 ml of 2%) were then added to each tube. The contents of the tubes were mixed, incubated for 30 min at 37° and unlysed cells were immediately pelleted by centrifugation. The absorbance of each supernatant at 541 nm was determined. The amount of C consumed was calculated from the CH_{50} titre (the dilution required to lyse exactly 50% of the cells) of each sample by comparison with the titre of serum incubated with saline (Johnson *et al.*, 1975).

2.A.13 Skin tests

a) Active cutaneous anaphylaxis (A.C.A.)

The procedure adopted for the development of A.C.A. reactions

is that of Coulson (1976). The hair was clipped from the backs of sensitized male (BALB/C x C57 Bl) F_1 mice (Section 2.A.13 (d)), which were then injected intravenously with 0.2 ml of 1% Evans Blue dye (Eastman Kodak, Rockester, New York) in saline. The mice were anaesthetised with diethyl ether and injected intradermally (26 gauge needle, bevel up) with 0.05 ml of antigen (as indicated) at a number of sites (up to six) on either side of the midline. When IgA antibody was used to block the reaction, the IgA and antigen were mixed and incubated at 37° for 60 min prior to intradermal injection.

Reactions were allowed to develop for 30 min, after which time a sample of blood (approx. 0.4 ml) was taken from each mouse. The mice were then killed by cervical dislocation, the skin of the back was reflexed and the intensity/diameter of the blueing reaction at each site was determined.

b) Passive cutaneous anaphylaxis (P.C.A.)

This technique was based on that described by Ovary (1975). Antibody (0.05 ml) was injected intradermally into a number of sites on the shaved backs of unimmunized mice. One hour after the injections, the mice were challenged intravenously with antigen (0.5 mg of $DNP_{20}BSA$, dissolved in saline containing 1% Evans blue). After 30 min the mice were killed and the intensity of the reactions determined.

c) The Arthus reaction

This technique was adapted from that of Cochrane (1976). Mice immunized with DNP-*E.coli* (see Section 2.A.13 (d)), were given an intravenous injection of 1% Evans blue (0.2 ml). They were immediately injected intradermally with 0.05 ml of antigen (10 µg) at each of a number of dorsal sites. Five hours later, the mice were bled from the eye, killed, and the intensity of reaction at each site was determined.

d) Sensitization of mice

i) Immunization with DNP-BSA

Male (BALB/C x C57 Bl) F_1 mice were injected intraperitoneally with 1.0 mg of $DNP_{20}BSA$ in Freund's incomplete adjuvant. Four weeks later, a number of animals were tested for cutaneous anaplylaxis by the intradermal injection of 20 µg of TNP-KLH at a number of sites. All gave strong positive reactions. The remaining mice were used in blocking experiments.

ii) Immunization with DNP-*E.coli*

Male F_1 mice received two intraperitoneal injections of DNP-*E.coli* (0.2 mg) in Freund's incomplete adjuvant on day 0 and day 60. On day 90, several mice were injected intra-peritoneally at a number of sites with antigen (20 µg of TNP-KLH) and tested for immediate (A.C.A.) or delayed (Arthus) reactions.

2.A.13 Chemotaxis

a) Preparation of polymorphonuclear leukocytes (PMN)

PMN were collected from rabbits by a modification of the method of Hirsch & Church (1960). Each rabbit was injected intraperitoneally with 150 ml of saline containing 150 mg of glycogen (Sigma Chemical Co.). Eighteen hours later, the animals were anaesthetised and injected with a further 150 ml of saline buffered with 10mM HEPES, pH 7.4 (Calbiochem., La Jolla, California) and containing 1 mM EDTA. The peritoneal exudate was immediately collected and the cells were centrifuged (5 min; 1600 rpm) and resuspended to $2x10^6$ cells/ml in saline/10 mM HEPES, pH 7.4. Normal or heat-inactivated rabbit serum was added to the cells at a final concentration of 4% prior to their addition to the chemotaxis chamber.

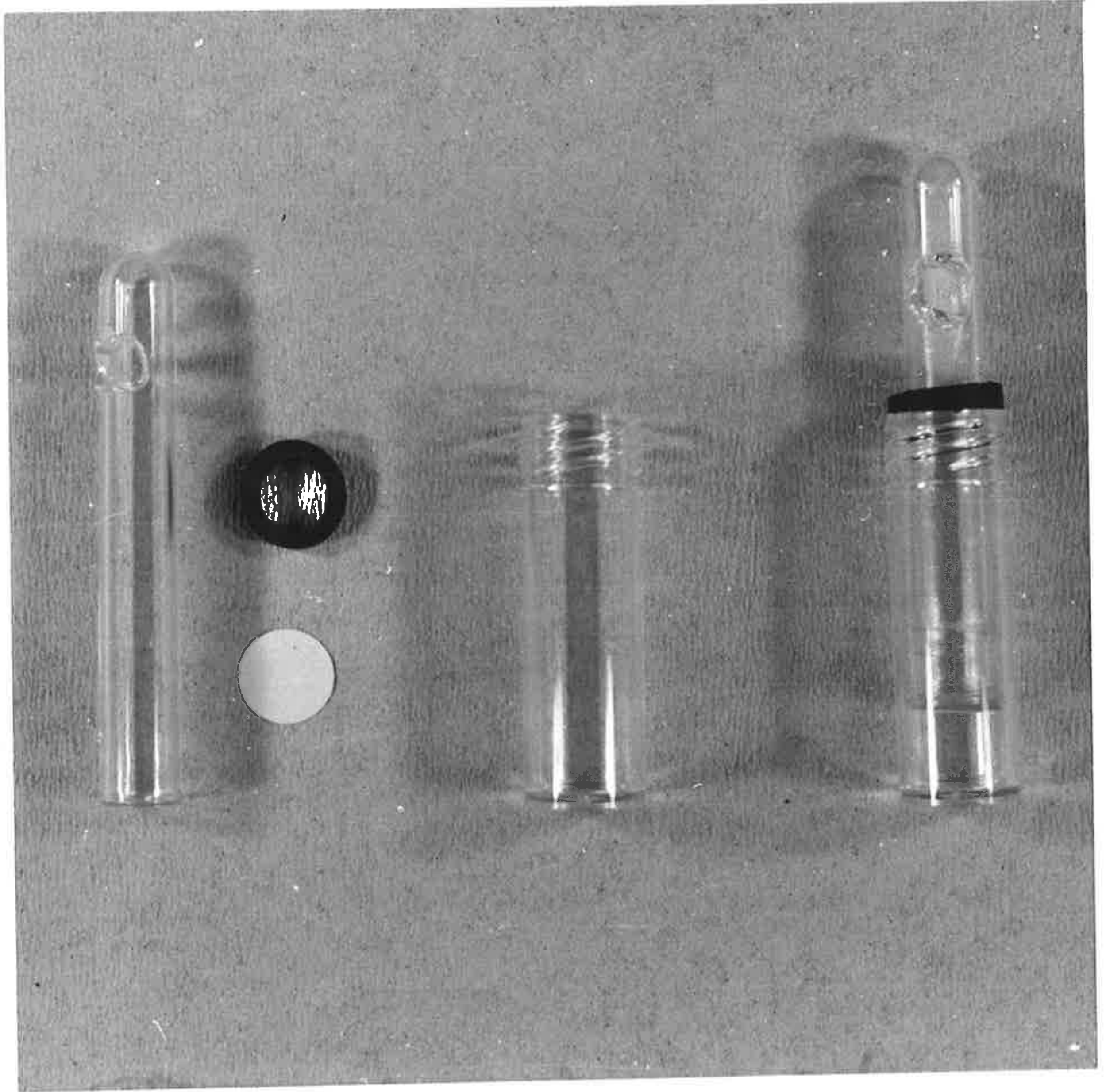
b) Chemotaxis chamber

Chemotaxis chambers were prepared by dipping the open end of a 5 ml plastic serology tube (Medical Plastics Pty. Ltd., S.A.) into molten paraffin wax and placing the waxed end onto a 1.3 cm diameter filter (SSWP01300; Millipore Corp., Bedford, Massachusetts). The cell suspension was introduced into the tube through a small hole made using a hot wire. A rubber grommet was placed around the tube, which was then suspended in a 5 ml plastic blood container (Medical Plastics Pty. Ltd., S.A.) containing 1.0 ml of the test solution. The position of the tube was adjusted to equalize the internal and external fluid levels (Fig. 2.2).

FIGURE 2.2

The chemotaxis chamber

The elements of the chamber comprised (from left to right) a 5 ml serology tube, rubber grommet and filter, and a 5 ml plastic blood container. An assembled chamber is shown on the right.



c) Chemotaxis assay

Test samples (0.2 ml) were incubated with 0.4 ml of rabbit serum for 60 min at 37°, and the volume then adjusted to 1.0 ml with saline/10mM HEPES, pH 7.4. When antigen-antibody complexes were used, they were allowed to form overnight at 37°. Following incubation with serum, samples were added to the lower compartment of the chamber. The upper compartment of each chamber was then loaded with cells ($1x10^6$), routinely suspended in 0.5 ml of 40% rabbit serum in saline/ 10 mM HEPES, pH 7.4. Each chamber was incubated in a 37° water bath for 2 hours. The filters were then removed and washed in saline. The cells were fixed and stained with haemotoxalin and the filters cleared in xylene (Boyden, 1961). The number of cells which had migrated to the bottom of each filter was then determined.

B. The MOPC-315 IgA myeloma protein

2.B.1 Properties

A number of studies have been carried out on the binding site specificity of the monomeric (7S) IgA formed by mild reduction and alkylation of the MOPC-315 myeloma protein (Potter, 1972; Jaffe *et al.*, 1969; Eisen *et al.*, 1968; Goetzl & Metzger, 1970). These investigations have shown that the myeloma protein has an affinity for phenol-based substances, particularly those containing nitrophenyl moieties. For instance, association constants of $1x10^7M^{-1}$ for N-ε-DNP lysine, $5x10^5M^{-1}$ for menadione and $1x10^5M^{-1}$ for 2,4-dinitronaphthol have been reported (Eisen *et al.*, 1968; Michaelides & Eisen, 1970). The protein can also bind a number of TNP-ligands (K_A not reported; Potter, 1972). The valency of the 7S monomer for E-DNP-lysine is apparently 2 (Potter, 1972).

The amino acid sequence of the MOPC-315 L chain has been determined and a 73% sequence homology with other mouse λ chains was found for the C-terminal 104 positions (Schulenberg *et al.*, 1971; Goetzl & Metzger, 1970).

The native IgA secreted by the MOPC-315 plasmacytoma has been reported to consist of a number of different oligomers (Eisen *et al.*, 1968). Electronmicroscopic examination of a dimeric form of the protein revealed that the two 7S subunits were joined via the tips of their

F_c regions (Munn *et al.*, 1971). In the presence of bi-functional haptens, the dimers formed a number of aggregates ranging in size from tetramers to larger cyclic structures, presumably linked via the Fab binding sites (Dourmashkin *et al.*, 1971; Potter, 1972; Munn *et al.*, 1971; Green *et al.*, 1971).

2.B.2 Propagation of the MOPC-315 plasmacytoma

The mouse plasmacytoma MOPC-315 (Potter, 1972) was obtained through the courtesy of Dr. A. Harris (The Walter and Eliza Hall Institute for Med. Res., Melbourne). It was passaged in (BALB/C x C57Bl) F_1 mice by intraperitoneal injection of $2x10^6$ viable tumour cells. A substantial ascites formed within three to four weeks, at which time the mice were bled from the retro-orbital plexus. They were then killed by cervical dislocation. A peritoneal washout was immediately performed by injecting 5 ml of saline (containing 1 mM EDTA) and collecting the exudate. On average, 8 ml of fluid was obtained from each mouse. The pooled ascitic fluid was centrifuged (400 g, 5 min) and the supernatant fluid containing the myeloma protein was removed. The cells were resuspended, washed in saline and injected into mice for maintenance of the tumour.

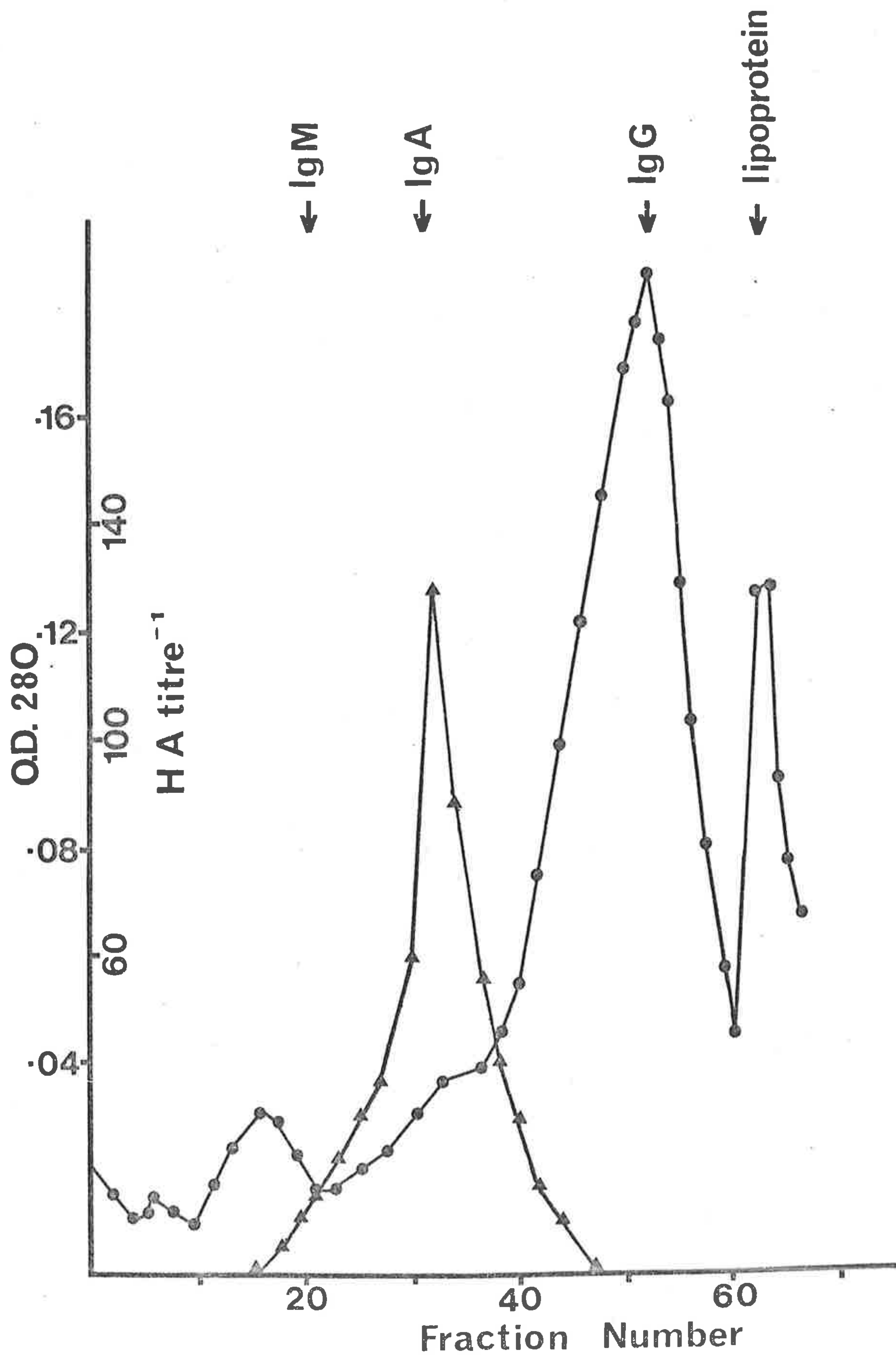
2.B.3 Purification and characterization of the myeloma protein

The myeloma protein present in the pooled sera and ascitic fluids was analysed initially by velocity sedimentation on sucrose gradients. The IgA sedimented as an apparently

FIGURE 2.3

Velocity sedimentation analysis of serum from F_1 mice bearing the IgA-secreting plasmacytoma MOPC-315

A sample (0.2 ml) of serum was mixed with 0.001 ml of rabbit haemolysin (Commonwealth Serum Laboratories, Melbourne) layered over a 12 ml sucrose density gradient (5-21% w/v; solvent : PBS) and centrifuged for 16 hr at 5° (International SW41 rotor; 40,000 rpm). Fractions (0.185 ml each) were collected from the bottom of the tube and diluted with saline before being analyzed for protein (OD_{280}) and for haemagglutinating antibody by titration against SRBC (haemolysin IgM, IgG and TNP-SRBC (IgA)). Sedimentation is from right to left. ●——● , $O.D._{280}$; ▲——▲ , TNP-specific haemagglutination titre. The positions of IgM and IgG are indicated.


IgM
IgA
IgG
lipoprotein
O.D. 280
H A titre⁻¹
·16
·12
·08
·04
140
100
60
20
40
60
Fraction Number

homogeneous molecule, as judged by haemagglutination, with an approximate sedimentation coefficient of 13S (Fig. 2.3).

The protein was purified from the pooled sera and ascitic fluid by adsorption to DNP-Sepharose and elution with DNP-glycine. Trace amounts of "natural" DNP-specific IgM antibody were removed by passage through an anti-μ chain immunoadsorbent (goat antibody (Meloy Laboratories) coupled to CNBr-activated Sepharose; Section 2.A.7). IgG antibodies were removed by exhaustive adsorption to protein A-Sepharose at pH 8.0 (Ey *et al.*, 1978). The resulting preparations were stored at 4° (in the presence of NaN_3) to minimise aggregation.

a) <u>Sepharose 6B chromatography</u>

A sample of purified IgA was chromatographed on Sepharose 6B. The IgA migrated as a single homogeneous peak, eluting between the IgM and IgG markers (Fig. 2.4). Using molecular weights of 150,000 and 900,000 for IgG and IgM respectively, the molecular weight of the IgA was estimated, by plotting log (molecular weight) versus elution volume (Marrink & Gruber, 1969), to be approximately 510,000.

b) <u>Radioimmunoassay analysis</u>

The purity of each IgA preparation was established by radioimmunoassay (Section 2.A.9(b)). The overall contamination of all preparations by IgM, IgG_1, IgG_{2a} and IgG_{2b} was determined to be less than 0.351% (e.g. Table 2.1).

FIGURE 2.4

Analysis of purified MOPC-315 IgA by chromatography on Sepharose 6B

Sepharose 6B chromatography of 13S MOPC-315 IgA. Column, 2.5x80, flow rate, 30 ml/hr; fractions, 10 ml; eluant, 0.132 M NaCl-25 mM Tris. HCl-0.1 mM EDTA-8 mM NaN_3 (pH 8.0); sample, 18 mg IgA in 6 ml eluant buffer, including 0.01 ml, ^{125}I-labelled mouse IgG and $Na^{125}I$ as intrinsic markers.

▲——▲ $O.D._{280}$(IgA); vertical bars, SRBC agglutination titre; ●——● radioactivity.

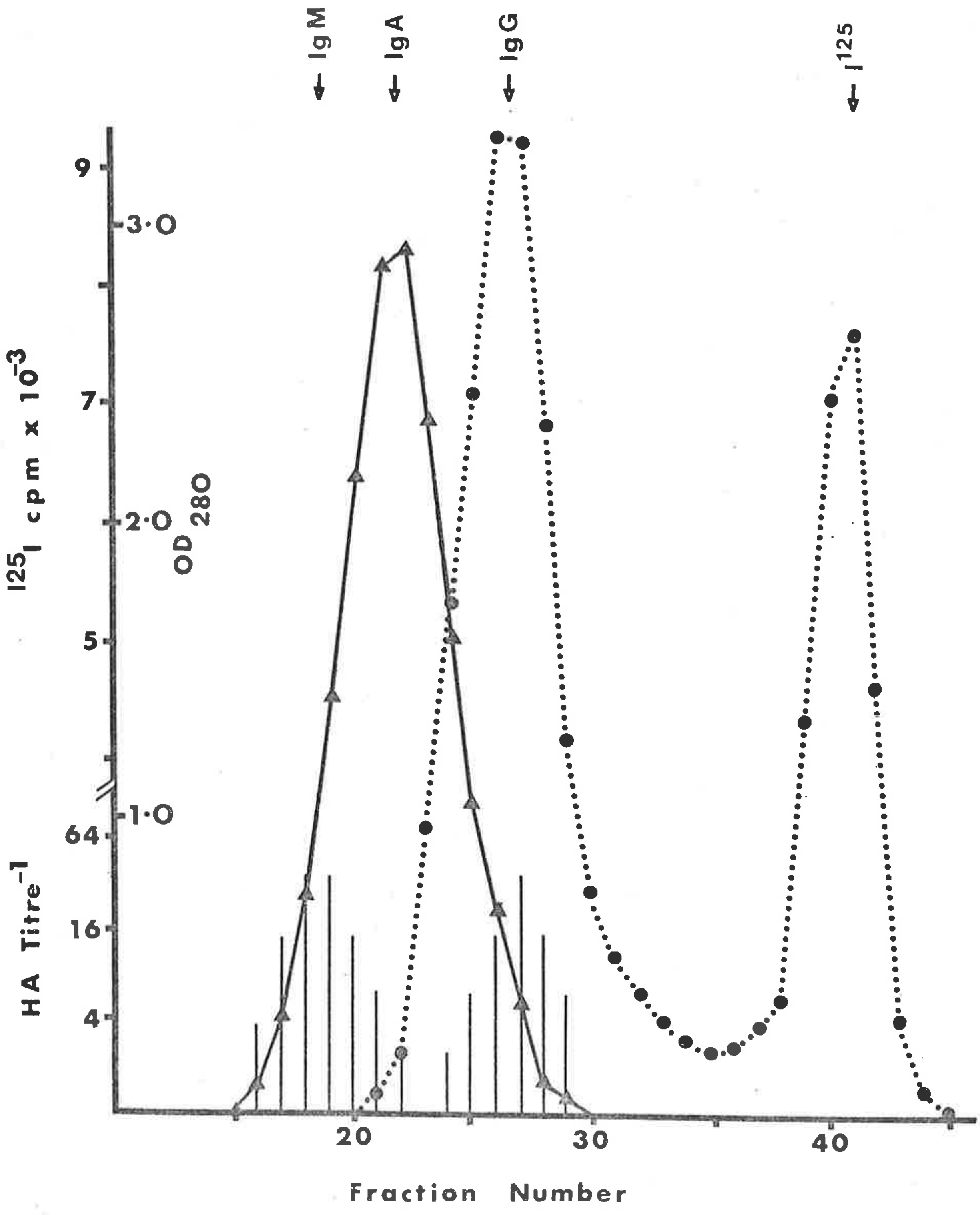

IgM
IgA
IgG
I125
125I cpm x 10-3
OD280
HA Titre-1
9
7
5
3·0
2·0
1·0
64
16
4
20
30
40
Fraction Number

TABLE 2.1

Radioimmunoassay of purified 13S MOPC-315 IgA[a]

	Estimated content		
	μg/ml	% w/w	
IgA	1930[b]	>99.65	
IgM	<1.138	< 0.068	<0.351% (total)
IgG_1	1.74	0.09	
IgG_{2a}	<3.54	< 0.178	
IgG_{2b}	<0.288	< 0.015	

a. See Section 2.A.9(b)

b. Protein concentration, calculated from $O.D._{280}$, was 1.97 mg/ml

c) S.D.S.-polyacrylamide gel electrophoresis

Electrophoresis of 2-mercaptoethanol reduced and native 13S IgA on polyacrylamide gels in the presence of sodium dodecylsulphate (Fairbanks *et al.*, 1971) showed the unreduced IgA to be heterogeneous with respect to interchain disulphide bond linkage. A number of bands were evident, ranging in molecular weight 22,000 to over 450,000. These bands probably corresponded to various covalently-linked aggregates of L and H chains since after full reduction, only L and H chains and two minor components (mol. wt. 65,000 and 87,000) were evident (Fig. 2.5).

2.B.4 Reduction of 13S IgA

IgA (13S) prepared as above (2.B.3(a)) was depolymerized by partial reduction (0.75 mg/ml IgA, 10 mM dithiothreitol (Sigma) in 0.1 M Tris. HCl, pH 8.0; 60 min, 20°, N_2) and alkylation with iodoacetamide, as described by Goetzl & Metzger (1970). Upon chromatography on Sepharose 6B, the reduced IgA migrated as a single homogeneous peak which eluted behind an ^{125}I-13S IgA marker (Fig. 2.6).

2.B.5 Haemagglutinating efficiency of 13S and 7S IgA

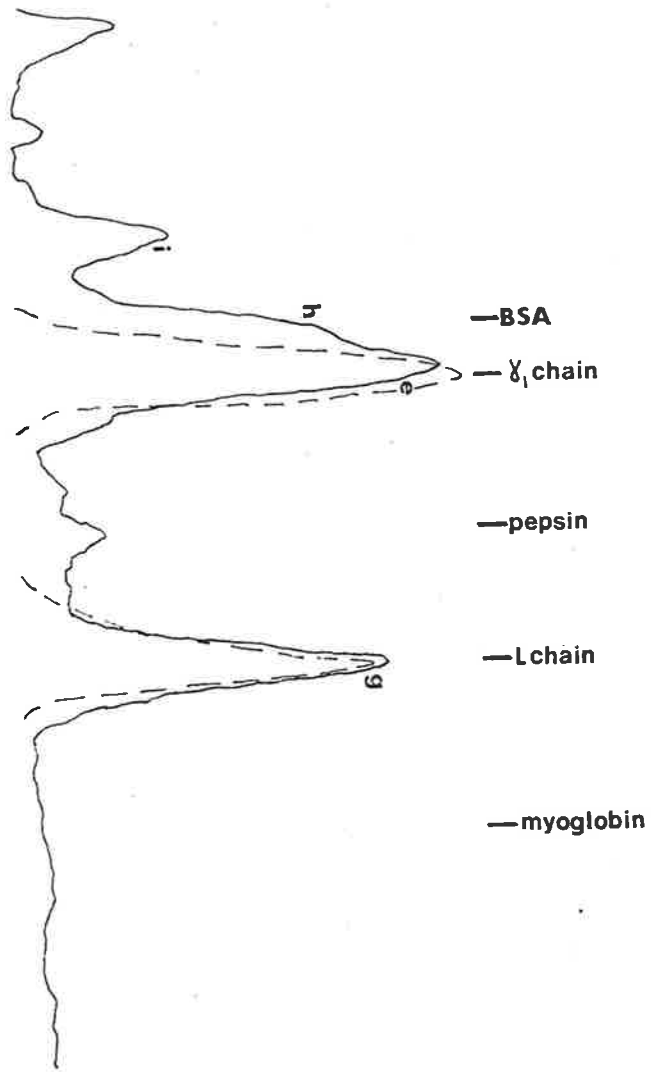
A 0.92 mg/ml solution of 13S IgA exhibited a haemagglutination titre of 1 in 16,000 against 1% TNP-SRBC (corresponding to an average of 240 IgA molecules per cell). In comparison, a 1.0 mg/ml solution of 7S IgA exhibited a titre of 1 in 500 (23,700 molecules per cell). Thus, 13S IgA was more than

FIGURE 2.5

Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis of MOPC-315 13S IgA

Top: Unreduced IgA, 5% polyacrylamide gel.
Bottom: Fully-reduced IgA, 8.5% polyacrylamide gel.
The molecular weight and probable identity of each component is indicated. The positions of mouse IgG_1 (as indicated by the dashed line), and of other markers are indicated.

a. ≥510,000 (trimer?)
b. 310,000 (dimer)
c. 158,000 (monomer)
d. 130,000
e. 58,000 (α chain)
f. 46,000 (L-chain dimer)
g. 23,000 (L-chain)
h. 65,000
i. 87,000


i
h
e
g
BSA
γ_1 chain
pepsin
L chain
myoglobin

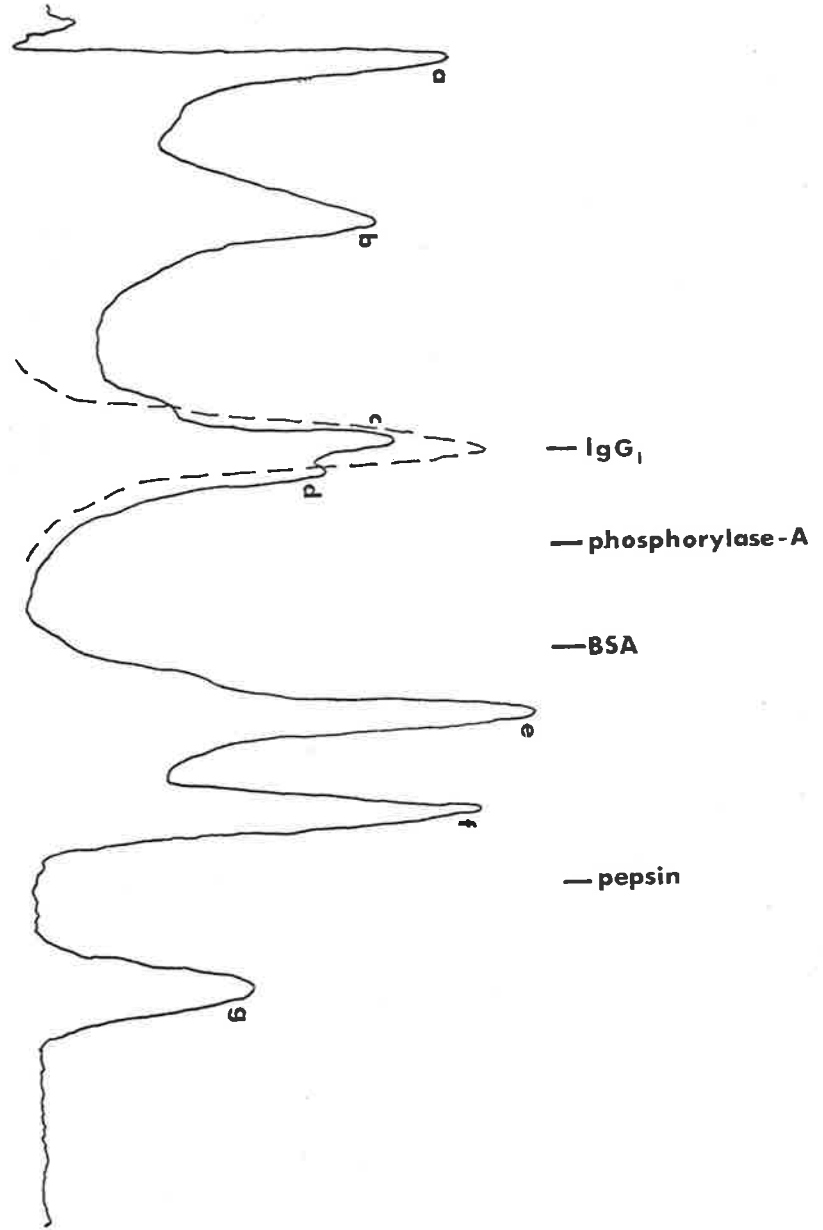
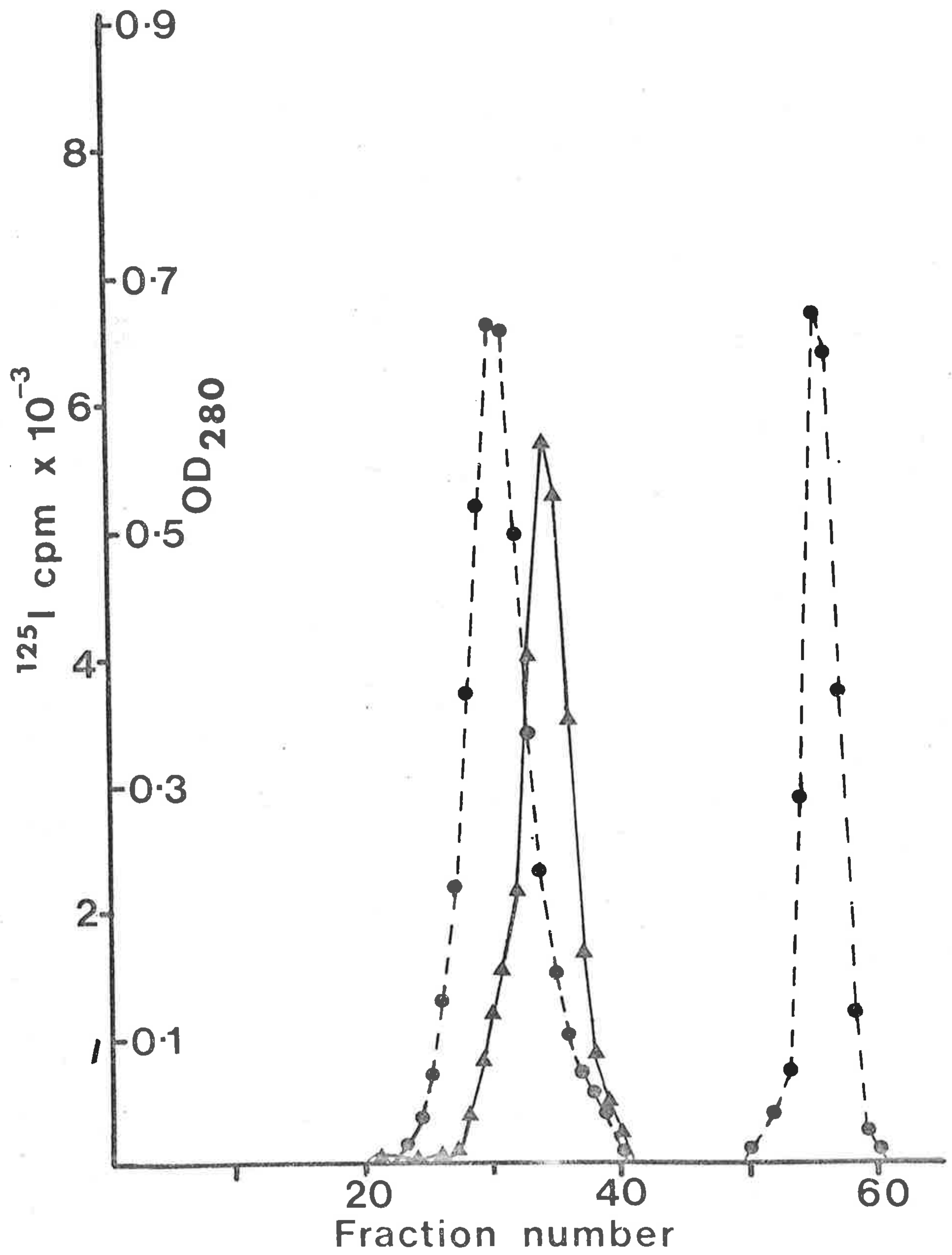

a
b
c
d
e
f
g
IgG_1
phosphorylase-A
BSA
pepsin

FIGURE 2.6

Chromatography of 13S MOPC-315 IgA and reduced 7S IgA on Sepharose 6B

Column, 2.5 x 80 cm; flow rate, 30 ml/hr; fractions, 6 ml; eluant, TESA (pH 8.0); sample, 6 mg 7S IgA in 3 ml eluant buffer including 0.05 ml ^{125}I-labelled 13S IgA and Na^{125}I as intrinsic markers

▲——▲ O.D.$_{280}$ (7S IgA); ●——● radioactivity.


0·9
8
0·7
6
0·5
4
0·3
2
0·1
^{125}I cpm x 10^{-3}
OD$_{280}$
20
40
60
Fraction number

100 times as efficient (on a molar basis) in agglutinating TNP-SRBC than 7S IgA. Neither form of IgA agglutinated SRBC, nor lysed TNP-SRBC in the presence of TNP-SRBC-adsorbed complement.

2.B.6 Binding affinity of 13S IgA

The concentration of N-ε-DNP-L-lysine required to halve the TNP-SRBC agglutinating titre of 13S IgA was described by performing replicate titrations in the presence of different concentrations of the hapten. A straight line was obtained by plotting the reciprocal antibody titres against log (DNP-lysine) and from this, the agglutinating titre was estimated to be halved in the presence of 60 μM DNP-lysine.

CHAPTER 3

The ability of IgA to inhibit the complement-mediated lysis of target red blood cells sensitized with IgG antibody

Contents

3.1 Preamble

3.2 Results

3.2.1 Lysis of TNP-SRBC sensitized with IgG/IgA mixtures

3.2.2 Sequential presentation of antibody: presensitization with IgA or IgG

3.2.3 Quantitation of cell-bound antibody

3.2.4 Specificity and efficiency of blockage by 7S and 13S IgA

3.3 Discussion

3.4 Summary

CHAPTER 3

3.1 Preamble

IgA antibodies have been reported to be unable to fix complement (C) (Eddie *et al.*, 1971; Colten & Bienenstock, 1974; Heddle & Rowley, 1975). It is therefore conceivable that IgA antibodies may, under certain circumstances, compete with C-fixing antibodies for common antigenic determinants and thereby prevent C activation. Several independent studies have in fact demonstrated that the sera of some patients with chronic bacterial infections can specifically decrease ('block') the bactericidal activity of normal serum (Hall & Manion, 1953; Zinneman *et al.*, 1959). A 'blocking' factor was first isolated from human serum by Zinneman *et al.* (1964) and identified as IgA. Rabbit secretory IgA antibodies to *S. typhimurium* were subsequently found to inhibit the *S. typhimurium*-specific bactericidal activity of IgG and IgM antibodies (Eddie *et al.*, 1971). More recently, Griffiss separated the different immunoglobulins from the sera of patients convalescing from meningococcal infections and observed that the IgA fractions, if remixed with IgG or IgM fractions prior to sensitisation of bacteria, could inhibit the C-dependent bacteriolytic activity of the IgG and IgM (Griffiss, 1975). Because the extent of inhibition depended on the ratio of IgM or IgG to IgA in the sensitising mixture, Griffiss suggested that IgA antibodies, which do not fix C (Eddie *et al.*, 1971; Colten & Bienenstock, 1974; Heddle &

Rowley, 1975), may compete with other antibodies for antigenic sites on the surface of the cells. Since neither the antibody specificities nor the number of antibody molecules involved were reported, however, the mechanism(s) underlying this phenomenon remain uncertain.

In an attempt to clarify these points, a haemolytic assay was chosen in which target cells bearing a defined haptenic group (TNP) were sensitised with IgG antibodies specific for either TNP or the target cell antigens. The mouse MOPC-315 IgA myeloma protein, which exhibits a binding specificity for DNP/TNP (Eisen *et al.*, 1968) was employed as a 'blocking' antibody. The use of the polymeric and monomeric forms of the MOPC-315 protein in this system presented an excellent opportunity to study the various features of the 'blocking' phenomenon.

3.2 Results

3.2.1 Lysis of TNP-SRBC sensitised with IgG/IgA mixtures

Rabbit IgG antibody was titrated for its ability to cause lysis of TNP-SRBC in the presence of C (Fig. 3.1). Using a 1/50 dilution of guinea pig serum, 50% haemolysis was effected by 0.75 μg of DNP-specific IgG (approximately 20,000 mol/cell). To determine whether IgA could inhibit haemolysis, various amounts of 13S IgA were mixed with 1.2, 2.3 or 9.3 μg of rabbit IgG and the mixtures were used to sensitise the target cells prior to the addition of C. The results depicted in Fig. 3.2 show that lysis was significantly inhibited at high

FIGURE 3.1

Haemolytic titration of DNP-specific rabbit IgG

TNP-SRBC sensitised for 90 min with dilutions of IgG were mixed with C and incubated for a further 30 min. The tubes were then centrifuged for measurement of the supernatant OD_{541}. The arrows indicate the dilutions (containing 1.2, 2.3 or 9.3 of μg IgG) used to assess inhibition of lysis by IgA in Fig. 3.2).

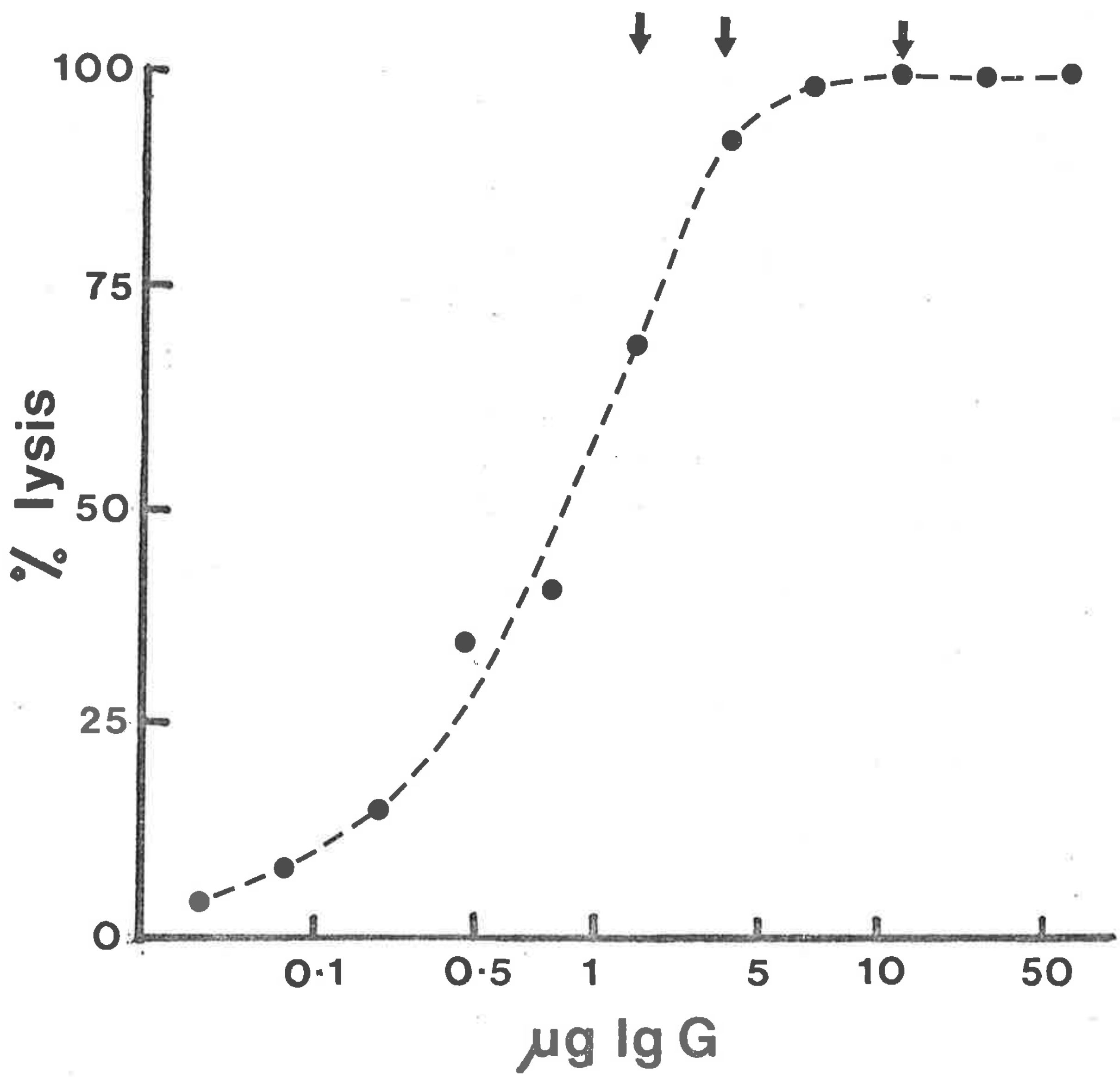
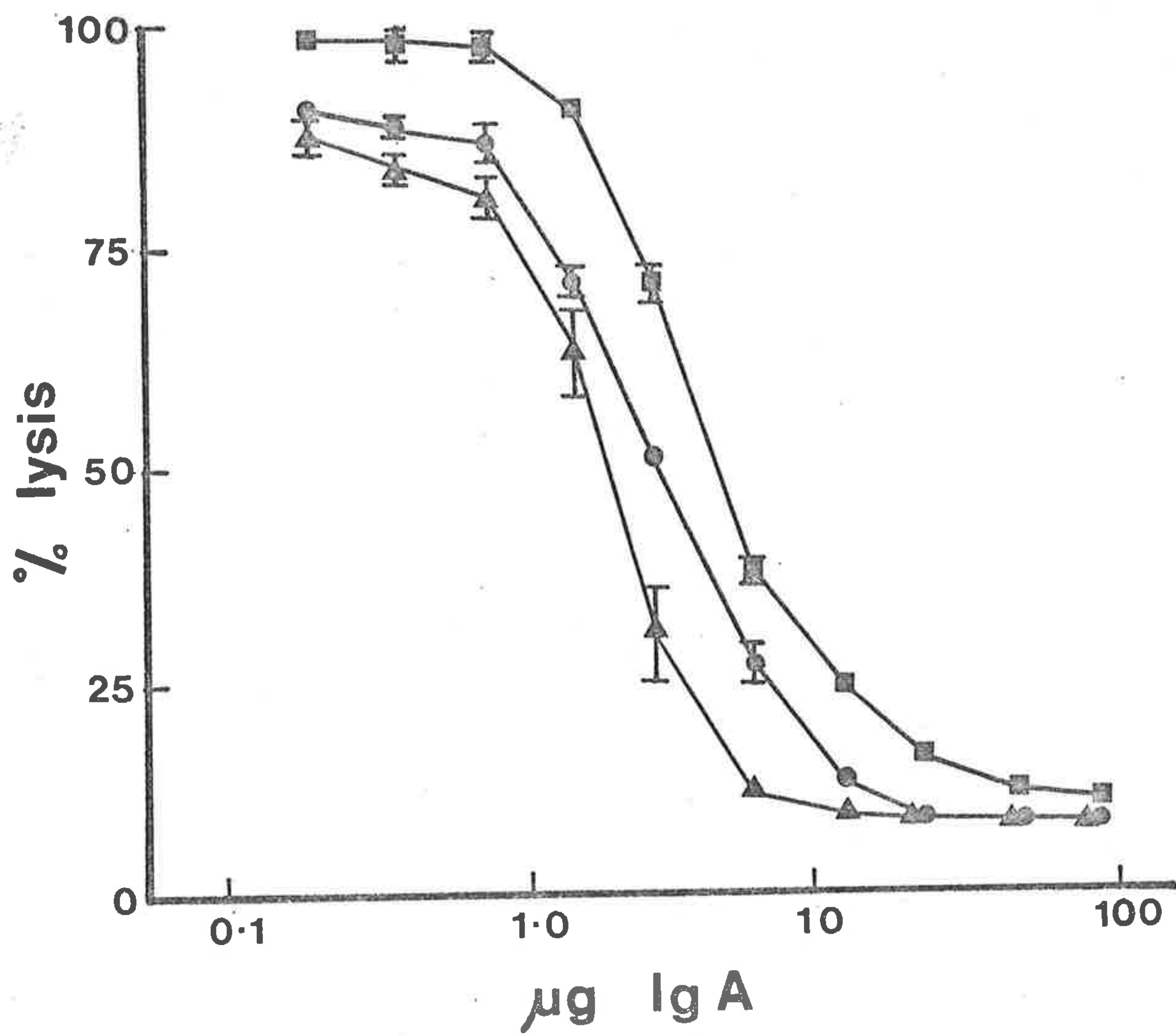

100
75
50
25
0
% lysis
0·1
0·5
1
5
10
50
µg Ig G

FIGURE 3.2

Lysis of TNP-SRBC sensitised with mixtures of IgG and IgA antibody

DNP-specific rabbit IgG (0.2 ml) mixed with dilutions of IgA (0.2 ml) was used to sensitise TNP-SRBC (60 min) before adding C and incubating for a further 30 min. ▲——▲, 1.2 μg IgG; ●——●, 2.3 μg IgG; ■——■, 9.3 μg IgG.



100
75
50
25
0
% lysis
0·1
1·0
10
100
µg IgA

IgA concentrations. The amount of IgA required to inhibit lysis depended on the amount of IgG antibody present, suggesting that the two types of antibody were competing for antigenic (TNP) sites on the cells. (Competition between IgG and IgA molecules for sites on the target cells, as evidenced by the above results, might be expected to occur since the antibodies share a specificity for DNP/TNP).

3.2.2 Sequential presentation of antibody: presensitisation with IgA or IgG

Provided that dissociation of bound molecules of either type from occupied sites was insignificant during the period of the lytic experiments, then under conditions of limiting antigenic sites it could be expected that sequential exposure of target cells to the two types of antibody would favour either lysis (IgG added first) or inhibition of lysis (IgA added first) compared with cells sensitised simultaneously with the two antibodies. This was indeed the case, as is evident from the results shown in Fig. 3.3 which demonstrate that the inhibition of lysis by any fixed amount of IgA was most pronounced when the cells were presensitised with IgA, less when the IgA and IgG were added together and least when IgA was added after sensitisation with IgG.

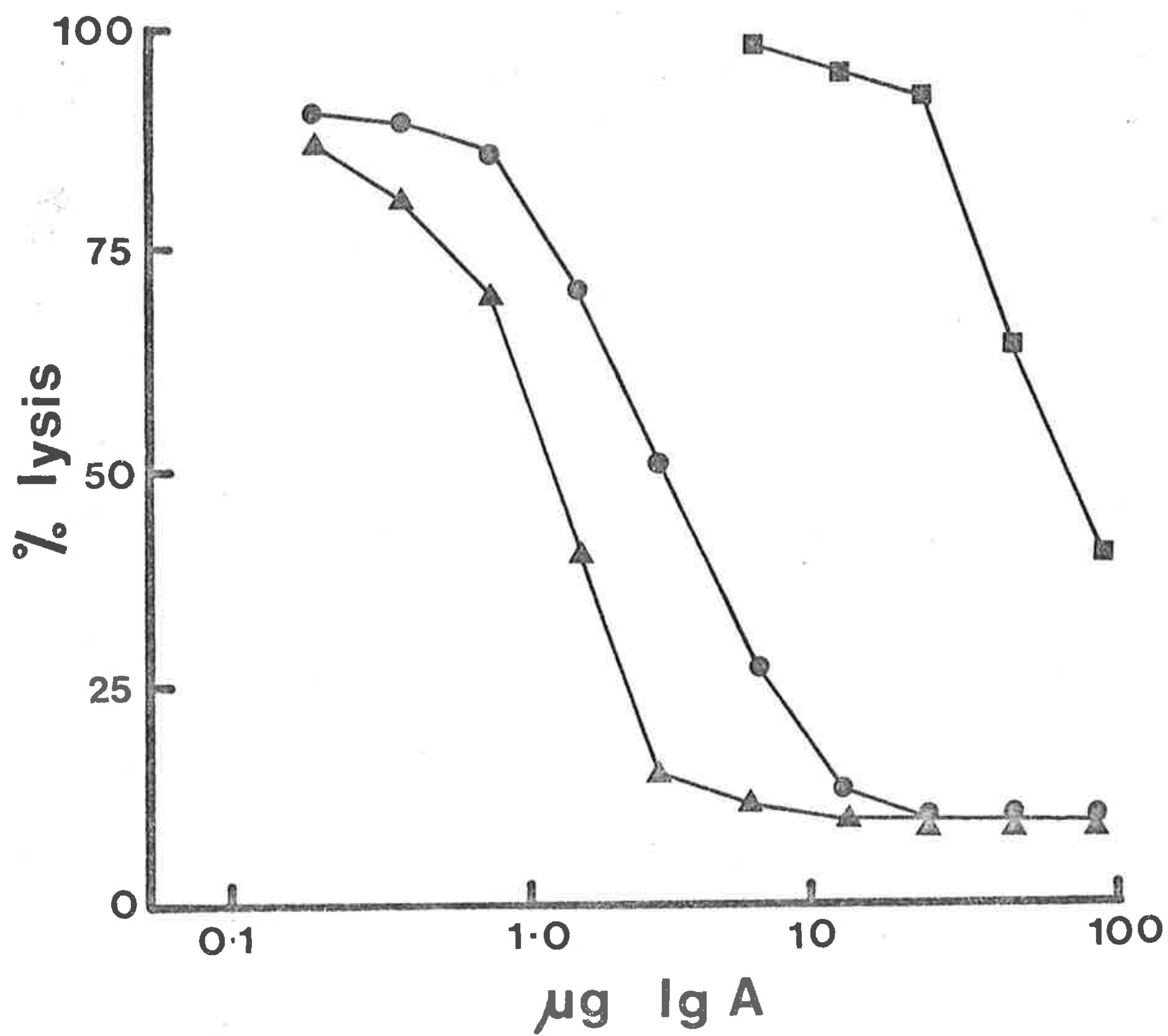
3.2.3 Quantitation of cell-bound antibody

The results presented above suggested that IgA molecules may have been replacing cell-bound IgG molecules. For such a

FIGURE 3.3

Lysis of TNP-SRBC sensitised sequentially with IgG and IgA antibody

TNP-SRBC were incubated (60 min) with IgA or 2.3 μg DNP-specific rabbit IgG prior to addition of the second antibody. The tubes were then incubated for a further 120 min before adding C. For comparison, tubes containing cells sensitised with mixtures of IgG and IgA were also included. ▲——▲, IgA, IgG; ■——■, IgG, IgA; ● —— ●, IgG + IgA.


100
75
50
25
0
% lysis
0·1
1·0
10
100
µg Ig A

mechanism to operate, the IgG molecules must dissociate from the cell and one would expect to observe decreased amounts of IgG antibody on IgG-presensitised cells incubated with IgA. Accordingly, the number of cell-bound IgG and IgA molecules was determined.

The sensitising antibodies, trace-labelled with ^{125}I, were incubated with the cells as described in the footnote to Table 3.1. The radioactivity associated with unlysed cells was measured and the extent of lysis occurring in the presence of C was determined on parallel sets of sensitised cells.

The addition of increasing amounts of IgA to IgG-pretreated TNP-SRBC resulted in an increased number of cell-bound IgA molecules (Table 3.1), although the incremental increases in bound IgA did not correspond proportionally with the extra IgA added. It may be that the higher levels of IgA (3-11 μg) were near-saturating amounts, restricting the availability of antigenic sites. No concurrent displacement of IgG was apparent, indicating that dissociation of cell-bound IgG molecules was insignificant during the period of the experiment. Despite this finding, the extent of lysis of IgG-presensitised cells was reduced by incubation with increasing amounts of IgA. Thus the IgA appeared, in this instance, to be interfering with either the initial activation of C by C-fixing IgG duplets present on the cell surface (Humphrey & Dourmashkin, 1969; Perelson & Wiegel, 1979), or with the subsequent steps leading to cytolysis through some mechanism(s) other than displacement of IgG molecules.

Table 3.1. Estimated average number of IgG and IgA molecules binding to target TNP-SRBC

Antibody used to sensitise TNP-SRBC (μg)[a]				Inhibition of lysis[b]	Antibody molecules bound per cell[c]	
A		B				
IgG	IgA	IgG	IgA	(%)	IgG	IgA
2.3	-	-	-	0	34,603	-
2.3	-	-	0.72	0	38,670	6,540
2.3	-	-	2.9	36	38,000	12,060
2.3	-	-	11.5	90	37,500	22,226
-	0.72	2.3	-	15	22,235	7,290
-	2.9	2.3	-	82	16,750	15,400
-	11.5	2.3	-	100	14,000	32,500
2.3 +	0.72	-	-	10	-	-
2.3 +	2.9	-	-	74	26,800	13,650
2.3 +	11.5	-	-	100	20,875	41,500

a Antibody A (0.2ml) was incubated for 1 hr at 37° with 0.2ml of 2.5% TNP-SRBC before addition of antibody B or diluent (0.2 ml) and incubation for a further 2 hr. Each sensitisation was performed in triplicate using (i) unlabelled IgG and IgA, (ii) ^{125}I-labelled IgG and unlabelled IgA, or (iii) unlabelled IgG and ^{125}I-labelled IgA. Set (i) was used to measure haemolysis during an additional 30 min incubation in the presence of C (0.6 ml, 1/50). The cells in sets (ii) and (iii) were washed 3X in chilled saline and the radioactivity associated with each cell pellet was determined.

b Relative to lysis of cells sensitised with IgG only.

c Calculated as = $\frac{\text{cell-associated cpm}}{\text{total cpm}} \times \frac{\text{number of molecules added}}{\text{number of TNP-SRBC}}$

In contrast to the results using IgG-presensitised cells, fewer IgG molecules became bound to cells which were sensitised with IgA or with both antibodies simultaneously (Table 3.1). Furthermore, a decrease in the number of cell-bound IgG molecules was observed as the cells were exposed to increasing amounts of IgA. For example, when cells were presensitised with 0.72 µg IgA, each cell bore an average of 22,235 molecules of IgG whereas using 11.5 µg IgA only 14,000 molecules of IgG were bound per cell. The number of antigenic sites accessible to IgG antibody on IgA presensitised cells therefore seemed to be restricted.

3.2.4 Specificity and efficiency of blockage by 7S and 13S IgA

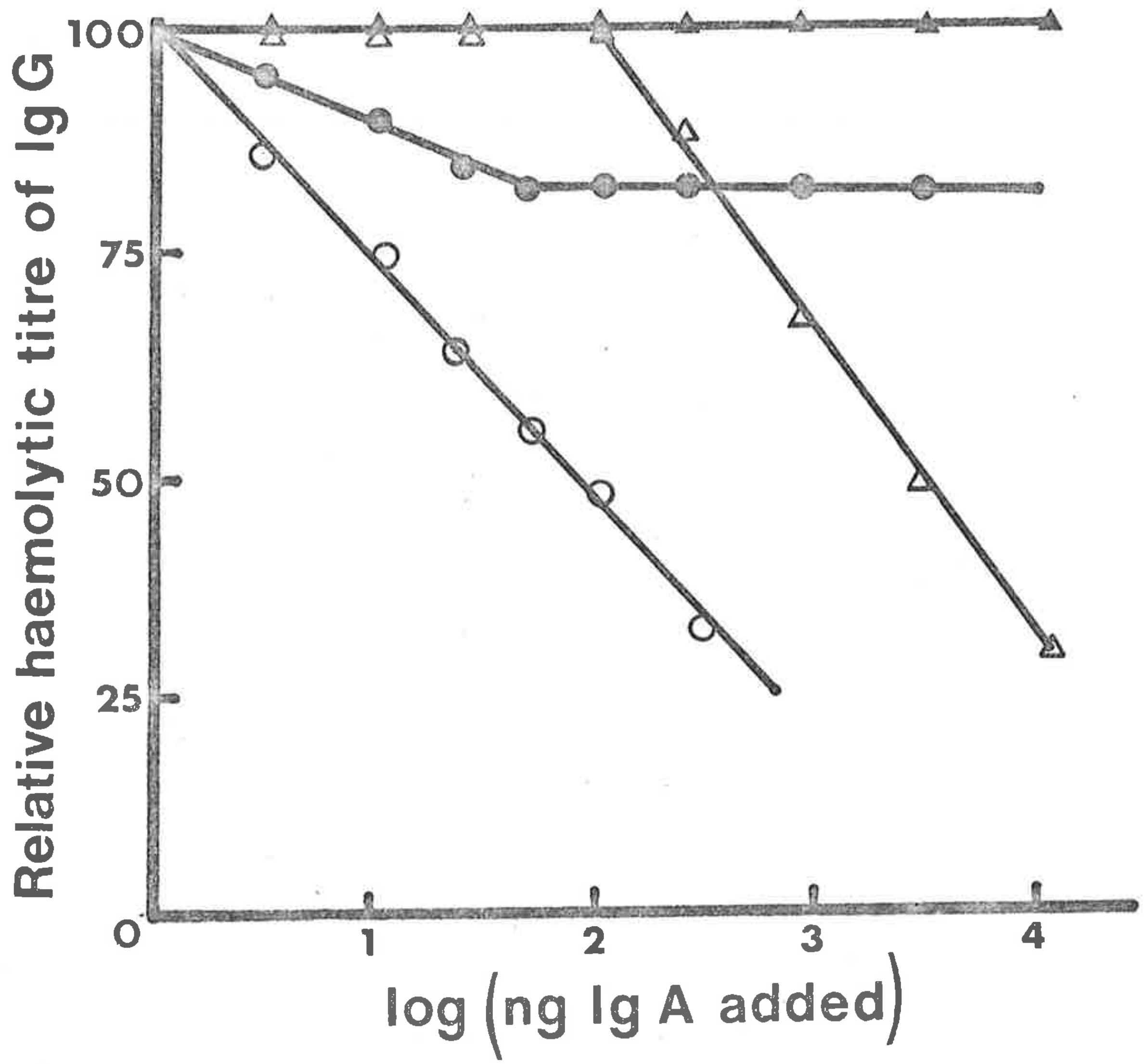
To gain some insight into the specificity of the blocking phenomenon and of the possible need for a (large) polymeric blocking molecule, trimeric (13S) and monomeric (7S) MOPC-315 IgA were each titrated for their capacity to interfere with the sensitisation of TNP-SRBC by TNP-specific or SRBC-specific mouse IgG_{2a} antibodies for lysis at a low level of C. To do this, the dilution of IgG which effected exactly 50% haemolysis was determined for each of a number of different concentrations of IgA, as well as in the absence of IgA (control). The relative amount of IgG antibody which, (mixed with the IgA) was required to sensitise the cells for exactly 50% haemolysis, was plotted against the IgA concentration (Fig. 3.4).

The trimeric (13S) IgA strongly inhibited sensitisation of

FIGURE 3.4

Lysis of TNP-SRBC by TNP-specific or SRBC-specific mouse IgG_{2a} antibody: inhibition by polymeric and monomeric IgA.

Inhibition was measured by performing replicate titrations of IgG antibody (0.2 ml; 2-fold serial dilutions). To each tube within a given set of dilutions was added 0.2 ml of VB^{2+}-BSA (control) or 0.2 ml of the same containing a constant amount of IgA. Each tube then contained 0.4 ml of antibody to which was added 0.2 ml of 0.14% TNP-SRBC with rapid mixing. After incubation for 30 min (37°), 0.2 ml of C (diluted 1/150) was added, the tubes were mixed and incubated at 37° for 60 min. They were then immediately centrifuged and the OD_{415} of each supernatant was measured. The dilution of IgG which effected exactly 50% haemolysis was thus determined for each level of IgA as well as in the absence of IgA (control). Each point represents the haemolytic titre of the IgG antibody relative to the uninhibited titre (100%) at the indicated level of IgA. The mean number of IgG_{2a} antibody molecules available to each target cell at the uninhibited endpoint dilution (relative titre = 100%) was 3,000 (TNP-specific IgG_{2a}) or 5,300 (SRBC-specific IgG_{2a}) (see Ey *et al.*, 1980). Polymeric IgA, O, ●; monomeric IgA,△,▲. TNP-specific IgG_{2a} O, △; SRBC specific IgG_{2a} ●,▲.



Relative haemolytic titre of IgG
100
75
50
25
0
1
2
3
4
log (ng Ig A added)

the cells with the TNP-specific IgG, only 13,000 mol/cell (mean number of molecules available per cell) of IgA being required to double (from 3,000 to 6,000 mol/cell) the amount of IgG antibody necessary for 50% lysis. The inhibitory effect of the IgA could be counteracted by increasing the amount of IgG in the sensitising mixture. This is to be expected however, because more C-fixing IgG duplets would be formed using greater quantities of IgG, necessitating more IgA to inhibit lysis (Perelson & Wiegel, 1979). A similar effect was observed using 7S IgA (Fig. 3.4), although this was a considerably less effective inhibitor since 1.2×10^6 mol/cell were necessary to halve the 50% lytic titre of the IgG. Thus, on a molar basis, the efficiency of the 13S molecules to inhibit sensitisation of the TNP-SRBC with TNP-specific IgG_{2a} was 100 times that of the 7S molecule. The greater bulk of the 13S molecule probably enables it to interfere by steric hindrance, and its greater binding avidity (relative to the 7S molecule) may also contribute to make it a more effective blocking antibody.

In contrast to the preceding results, 7S IgA was unable to affect the ability of SRBC-specific IgG molecules to sensitise TNP-SRBC for lysis. This observation, that 7S IgA blocks lysis of TNP-SRBC sensitised with TNP-specific but not with SRBC specific IgG, indicates that the blocking effect of 7S IgA probably involves competition with IgG molecules for binding sites on the cells. On the other hand, the lytic titre of the SRBC-specific IgG was progressively reduced by increasing

amounts of 13S IgA until a plateau phase (80% of original titre) was reached using levels equivalent to >10,000 mol/cell. The inhibition of lysis of TNP-SRBC sensitised with SRBC-specific IgG by the 13S molecule cannot be explained by competition for binding sites (as 7S IgA at much higher concentrations was not able to block this lysis) and it must therefore have been acting by some other mechanism, such as the steric interference of C1 binding to IgG duplets*.

3.3 Discussion

The results presented in this chapter demonstrate that the MOPC-315 IgA myeloma protein does not fix haemolytic C, and that it can act as a specific 'blocking antibody' in a model haemolytic system. These findings are consistent with the reported inability of IgA antibodies to fix C (Eddie *et al.*, 1971; Colten & Bienenstock, 1974; Heddle & Rowley, 1975) and with earlier studies which showed that IgA antibodies could interfere with *in vitro* C-mediated bacteriolysis (Zinneman *et al.*, 1964; Eddie *et al.*, 1971; Griffis, 1975). Unfortunately these workers did not measure the number of specific antibody molecules binding to the cells.

In the present work, the numbers of sensitising (IgG) antibody and 'blocking' (IgA) molecules bound to target cells at various

---

* Since the C1 complex (IC1q:2C1s:2C1r) is very large (742,000 daltons) with 6 elongated collagen-like arms (Porter & Reid, 1978), the binding of 13S IgA (510,000 daltons) in close proximity to an IgG duplet could feasibly prevent the binding of C1 to the duplet.

levels of inhibition have been determined. The inhibitory efficiencies of polymeric (13S) and monomeric (7S) MOPC-315 IgA have also been compared, since individuals from a number of vertebrate species apparently carry a high proportion of polymeric IgA in their lymphatic system (Orlans *et al.*, 1978; Vaerman *et al.*, 1973; Lemaitre-Coelho *et al.*, 1978; Kaartinen *et al.*, 1978).

Trimeric (13S) MOPC-315 IgA was capable of strongly inhibiting the lysis of TNP-SRBC sensitised with DNP-specific rabbit IgG or TNP-specific mouse IgG_{2a} antibodies. When cells were sensitised with mixtures of IgA and IgG, the efficiency of 'blockage' by IgA was found to depend on the absolute amount of IgG available (Figs. 3.2 & 3.4). For instance, the lysis of cells sensitised with 1.2 μg of DNP-specific rabbit IgG (32,000 molecules available per cell), which was sufficient to cause total lysis, was reduced to 50% in the presence of 1 μg of 13S IgA (8,100 mol/cell), *i.e.* at a molar (IgG:IgA) ratio of 4:1. Using 9.3 μg of IgG antibody, 70 μg of IgA (equivalent to a molar ratio of 0.45:1) was required to similarly reduce lysis to 50%. The extent of inhibition effected by a given amount of 13S IgA depended on the order in which the cells were exposed to the two antibodies, being least pronounced when IgA was added to cells presensitised with IgG (Fig. 3.3). Since the data of Table 3.1 indicated that no loss of bound IgG from IgG-presensitised cells occurred upon subsequent exposure to levels of IgA sufficient to inhibit C-dependent haemolysis, it was concluded that 13S IgA molecules

may interfere with the activation of C by IgG duplets (Humphrey and Dourmashkin, 1969; Perelson & Wiegel, 1979) present on the cells.

When 13S and 7S IgA molecules were compared for their capacity to inhibit the sensitisation of cells with the more avid TNP-specific mouse IgG_{2a} antibodies (Fig. 3.4), the 13S molecules were found to be considerably more efficient (100-fold, on a molar basis), than the 7S molecules. This difference did not seem to be totally due to differences in the avidity of the IgA molecules. For example Ey *et al.*, (1980), using the same C and IgG_{2a} antibody, found that more than 100,000 mol/cell of TNP-specific mouse IgG_1 were required to halve the lytic titre of the IgG_{2a}. This figure can be compared with the 13,000 mol/cell of 13S IgA and $1x10^6$ mol/cell of 7S IgA observed here (Fig. 3.4). The concentration of N-ε-DNP-L-lysine required to halve the TNP-SRBC agglutinating titre, which is a measure of relative binding site affinity (Steward, 1977) was 0.5 μM for both IgG_1 and IgG_{2a} (Ey *et al.*, 1980) and 60 μM for the 13S IgA (see Section 2.B.6). Thus, even though the 13S IgA exhibited an affinity for TNP much lower than that of the IgG_1 antibody, it was nevertheless considerably more efficient in inhibiting sensitisation of cells by the IgG_{2a} antibodies. The size of the 13S molecules therefore seems to be a dominant factor in mediating the observed 'blockage' of lysis.

In contrast, the lower 'blocking' efficiency of the 7S IgA

compared with the IgG_1 (above) can probably be attributed entirely to the difference in binding-site affinity of the two molecules. The mechanism of 'blockage' in this instance probably involves saturation of antigenic sites by the 7S blocking antibodies, which decreases the incidence of IgG duplets on the cells.

The sensitisation of TNP-SRBC with SRBC-specific mouse IgG_{2a} was also reduced, although only partially, by 13S IgA (Fig. 3.4). The 7S IgA had no effect. Because the 13S molecule exhibited no specificity for unmodified SRBC (*i.e.* it recognised only the TNP moiety on TNP-SRBC), it seems that the partial inhibition of SRBC-specific lysis must occur by a non-specific mechanism. The IgA may perhaps bind to a TNP site adjacent to an IgG duplet (considered essential for C fixation and lysis, Humphrey & Dourmashkin, 1969), and sterically interfere with the binding of CI.

On the other hand, if the IgA was situated next to a bound IgG molecule and sterically prevented the binding of a second IgG molecule (to form a duplet), then the second IgG would be available to bind to a distant site where the probability of forming a duplet would be the same as that at the 'blocked' site (Perelson & Wiegel, 1979). No inhibition of lysis would be expected in this case.

In summary, the results demonstrate that polymeric IgA antibody is an extremely effective inhibitor of C mediated

lysis of cells sensitised with IgG antibodies. The inhibition is largely antigen-specific, although some degree of non-specific inhibition has been observed. The inhibition of lysis by monomeric (7S) IgA molecules is substantially less efficient and is entirely antigen-specific. Two mechanisms of inhibition are apparent. The first, requiring saturating amounts of IgA, appears to involve competition with IgG molecules for antigenic sites on the target cells, thereby reducing the incidence of C-fixing IgG duplets. The second, requiring polymeric IgA, seems to involve interference with the activation of C by IgG duplets present on sensitized cells.

The ability of IgA antibodies to inhibit C-mediated phenomena *in vivo* probably depends on the absolute as well as relative levels of IgA and C-fixing IgG antibodies. The concentration of C may also be important, since the efficiency of IgG antibodies for sensitising cells for lysis is reduced at low C concentrations (Ey *et al.*, 1980). Levels of IgA antibodies sufficient to exert 'blocking' effects can occur *in vivo* however, as evidenced by the earlier work with human sera (Hall & Manion, 1953; Zinneman *et al.*, 1959). The lymph and sites of local IgA synthesis are regions where C-mediated phenomena would be most susceptible to inhibition by IgA, since in these areas the concentration of polymeric IgA is usually several-fold higher than that of serum (Orlans *et al.*, 1978; Vaerman & Heremans, 1970; Vaerman *et al.*, 1973, Birbeck *et al.*, 1979). Because the lymphatic IgG concentration is usually equal to or less than that of serum, the IgA concentration in the lymphatics may often equal or exceed that of IgG.

It is therefore conceivable that IgA antibodies may inhibit some C-mediated reactions at localised tissue sites or within the lymph. These aspects are considered further in Chapter 5.

3.4 Summary

The polymeric and monomeric forms of the DNP/TNP-specific MOPC-315 IgA myeloma protein were examined for their ability to inhibit the C-dependent lysis of TNP-SRBC sensitised with IgG antibodies. On a molar basis, polymeric IgA was 100 times more effective as an inhibitor than monomeric IgA. The inhibition could be counteracted by increasing the level of sensitising IgG and was antigen-specific, although an element of non-specific inhibition was observed using polymeric, but monomeric, IgA. It is concluded that polymeric IgA may be able to inhibit lysis of sensitised cells by sterically interfering with fixation of complement to IgG dimers.

CHAPTER 4

Inhibition of complement consumption by IgA

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4.5 Summary

CHAPTER 4

Inhibition of complement consumption by IgA

4.1 Preamble

The experiments described in Chapter 3 revealed that 7S or 13S IgA antibodies can effectively inhibit the lysis of IgG-sensitized target cells by complement (C). The inhibition of lysis was postulated to occur by one of two mechanisms, either by direct competition between molecules of IgA (7S or 13S) and C-fixing IgG for antigenic sites on the TNP-SRBC membrane, or by preventing the fixation/ activation of C1 by complement-fixing IgG duplets.

To determine whether IgA antibodies also block C-activation in reactions other than those involving target cell surfaces, the effect of IgA on C-activation in two cell-free systems was examined. The first utilised the soluble, haptenated protein, DNP-BSA, which, like dinitrophenylated human serum albumin, does not require the presence of specific antibody for activation of C (Boackle *et al.*, 1974; Iida *et al.*, 1976; Lint *et al.*, 1976; Loos & Konig, 1977; Loos & Thesen, 1978). This protein was chosen in order to examine the influence of IgA in the absence of competing antibodies. The second system, in which C was activated by complexes formed between soluble antigen (TNP-KLH) and IgG antibodies, was used to examine the effects of IgA on C-activation when the IgA, pre-mixed with IgG, was competing for antigenic determinants.

Trinitrophenylated and dinitrophenylated antigens were used interchangeably throughout this work, since it has been reported (Potter, 1972) that MOPC-315 IgA has similar affinities for both haptens.

4.2 Results

4.2.1 $DNP_{46}BSA$

4.2.1.a) Characterization

To check that the extensive dinitrophenylation of BSA had not caused the molecules to become highly aggregated, a sample of $DNP_{46}BSA$ (Section 2.A.4.a)) was chromatographed on Sephacryl S-200. The elution profile (Fig. 4.1) revealed that the $DNP_{46}BSA$ eluted as a single peak (as measured by OD_{360} and OD_{280}) concurrent with the ^{125}I-labelled IgG marker. Since gel filtration separates molecules on the basis of their Stoke's radii, rather than on molecular weights, the results suggested that either the molecular weight of the $DNP_{46}BSA$ was about 150,000 daltons (approximately twice the theoretical molecular weight), or that the molecule had unfolded appreciably as a result of its dinitrophenylation. It was clear from these results that the preparation was not significantly aggregated.

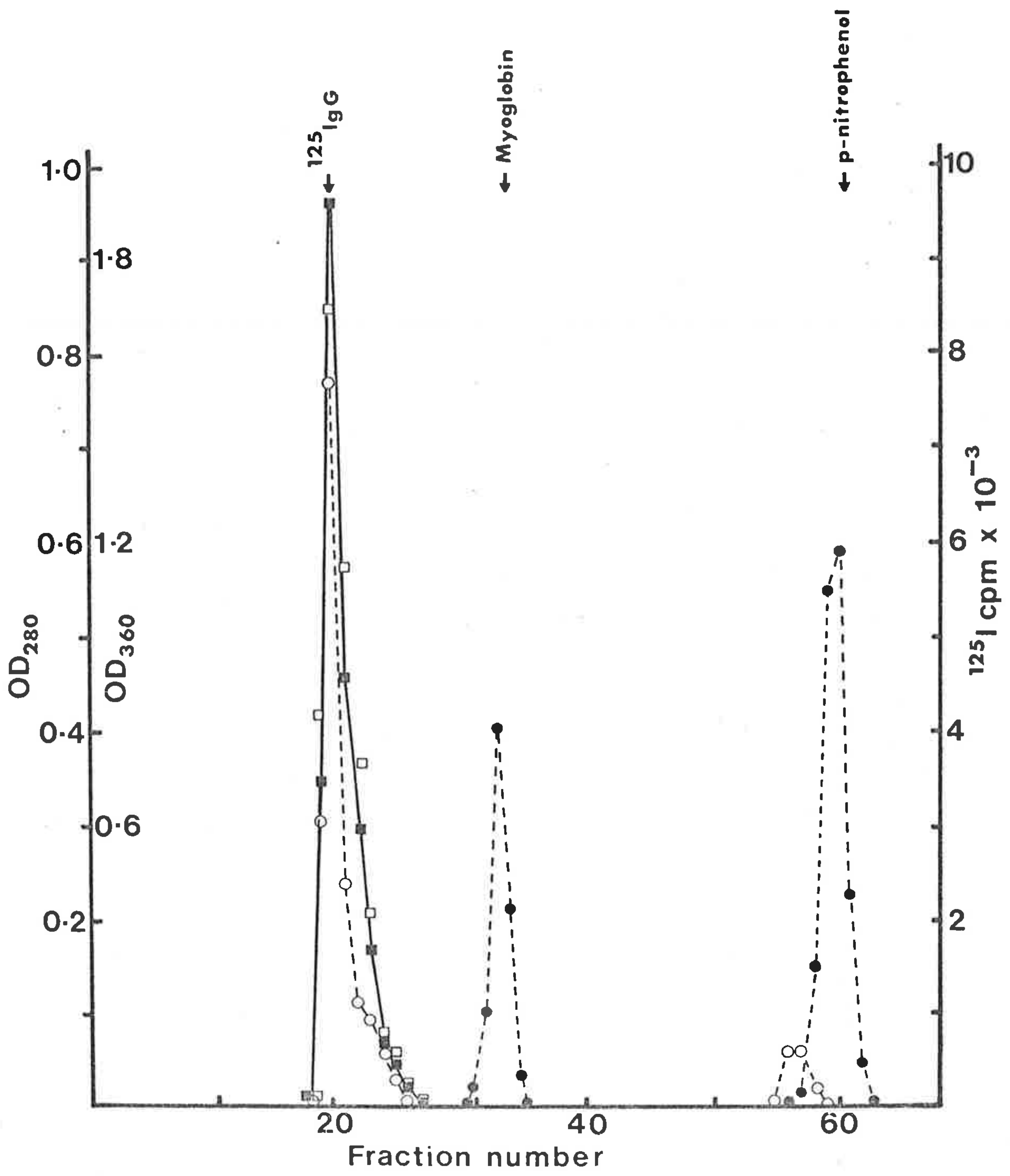
4.2.1.b) Complement consumption

Loos & Konig (1977) have reported that dinitrophenylated human serum albumin, which activates C by the alternate pathway (Konig *et al.*, 1974), can also interact with and precipitate purified Clq. It is at present unknown, however, whether this interaction leads to activation of the Cl complex. The extent

FIGURE 4.1

Chromatography of $DNP_{46}BSA$ on Sephacryl S-200

A 1.0 ml sample containing 2 mg of $DNP_{46}BSA$ plus ^{125}I-labelled IgG (1.6×10^6 cpm), myoglobin (0.5 mg) and p-nitrophenol (8 μ moles) was applied to the top of a 2.5 x 80 cm column of Sephacryl S-200 equilibrated at room temperature with TESA. The flow rate was 52 ml/hr and 2.6 ml fractions were collected. Fractions were assayed for radioactivity and for their optical density at 280 nm and 360 nm. Solid lines represent $DNP_{46}BSA$, dotted lines the intrinsic markers. ■, OD_{360}; □, OD_{280} (of DNP-BSA); o, radioactivity; ●, OD_{280} of myoglobin and p-nitrophenol.



^{125}IgG
Myoglobin
p-nitrophenol
1·0
1·8
0·8
0·6 1·2
0·4
0·6
0·2
OD_{280}
OD_{360}
10
8
6
4
2
^{125}I cpm x 10^{-3}
20
40
60
Fraction number

of C activation was found to be dependent on both the degree of substitution by DNP and on the concentration of DNP-HSA (König *et al.*, 1974).

To test whether C activation by the $DNP_{46}BSA$ preparation was also concentration-dependent, serial dilutions of $DNP_{46}BSA$ were incubated at 37° with C (14 CH_{50} units) and the activity remaining after one hour was determined. The results of this experiment are depicted in Fig. 4.2. The DNP-BSA was found to fix C in a concentration dependent manner, 8 μg being sufficient to effect 50% consumption (7 CH_{50} units) and 125 μg causing 100% consumption (14 CH_{50} units). These quantities may be compared with the amount of $DNP_{46}HSA$ (approx. 4 μg/ml) required to bind 50% of Cl (1.1 μg/ml; Loos & Konig, 1977) and with the quantity of aggregated IgG (230 μg) required to consume 58 CH_{50} units of C (Iida *et al.*, 1976). It must be noted, however, that in the former study the workers were measuring loss of Cl activity and in the latter study, the CH_{50} unit differs from that employed in the present experiments due to differences in the assay procedures.

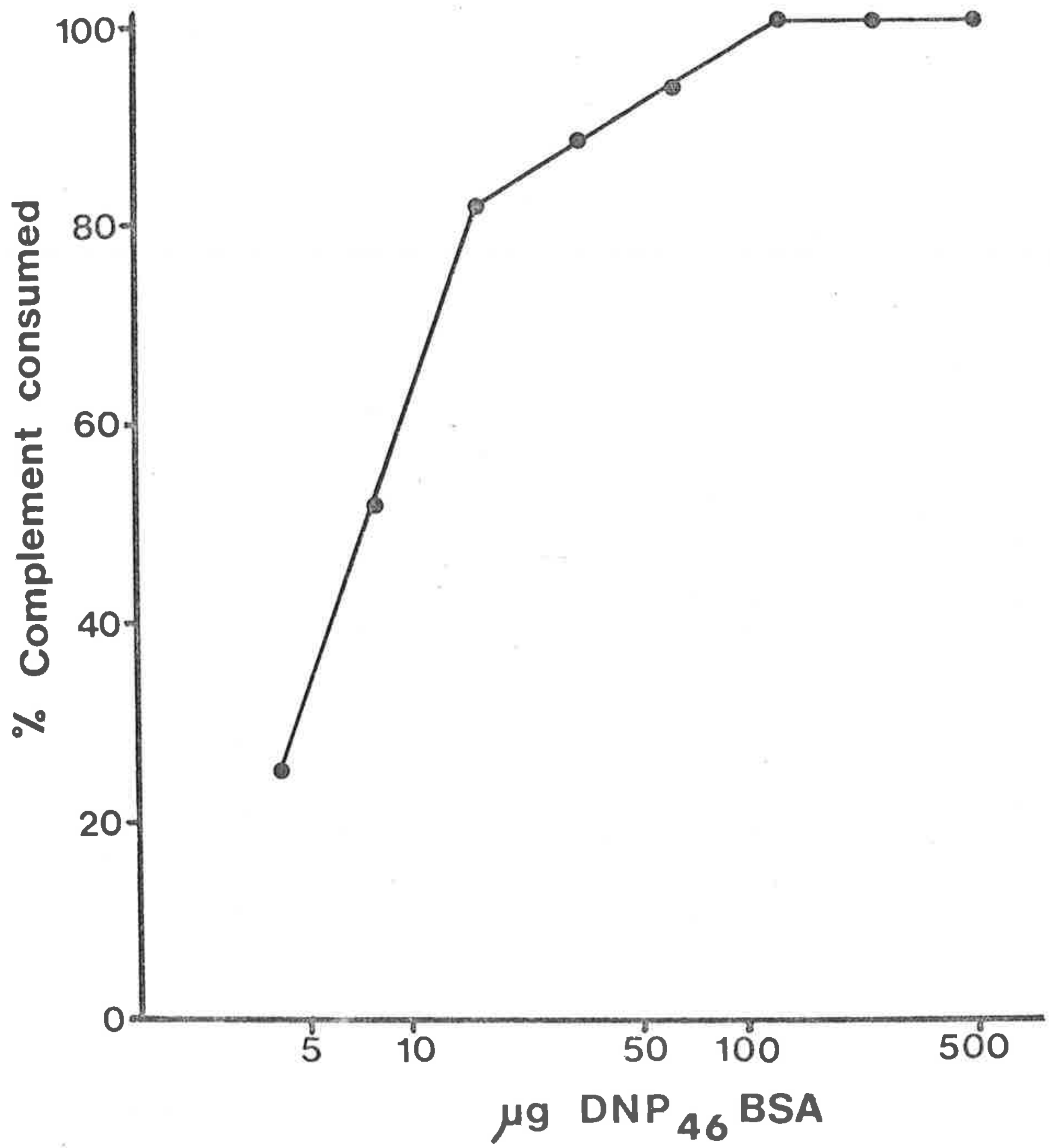
4.2.1.c) Inhibition of complement consumption by 7S and 13S IgA

In order to assess the capacity of IgA to interfere with the ability of DNP-BSA to fix C, 50 μg portions of DNP-BSA were pretreated with various dilutions of 7S or 13S MOPC-315 IgA (150 min at 37°; then O/N at 4°) prior to addition of C. A concentration dependent inhibition in C consumption was achieved (Fig. 4.3, top), similar amounts of 7S or 13S IgA

FIGURE 4.2

Complement consumption by $DNP_{46}BSA$

Serial twofold dilutions (0.2 ml) of $DNP_{46}BSA$ were mixed with 0.4 ml (14 CH_{50} units) of complement and incubated for 60 min at 37°. Each sample was then adjusted to 0.8 ml by the addition of saline (0.2 ml) before being serially diluted in twofold steps. To each dilution was added haemolysin-sensitized SRBC (0.2 ml of 2%). The contents of each tube were mixed and then incubated at 37° for 30 min, before being centrifuged. The OD_{541} of each supernatant was then measured to determine the residual CH_{50} titre of each sample (2.A.12.b).



100
80
60
40
20
0
% Complement consumed
5
10
50
100
500
μg DNP 46 BSA

producing equivalent degrees of inhibition. Complete inhibition was observed with the addition of 300 μg of either IgA preparation, while 50% inhibition was effected by 50 μg of 7S or 100 μg of 13S IgA. On a molar basis, 13S IgA was a slightly more efficient inhibitor than 7S IgA (Fig. 4.3, bottom). For example, 0.36 moles of 7S IgA or 0.17 moles of 13S IgA per mole of DNP-BSA reduced complement consumption by 50%. The finding that such a small relative number of IgA molecules could inhibit complement consumption by DNP-BSA indicated that blockage did not require all of the available DNP sites to be saturated by IgA. In order to prevent C consumption, the IgA molecules must have in some way prevented the access of C molecules to sites on the DNP-BSA molecules. As appreciable inhibition of C consumption was observed at IgA:DNP-BSA ratios of 1:1 to 10:1 (i.e. near precipitin equivalence) it seems most likely that an IgA/DNP-BSA lattice was formed. One would expect that sites required for C-activation would be concealed within such a lattice.

4.3 Antigen-antibody complexes

4.3.1 Complement consumption

The extent of complement consumption by antigen-antibody complexes has recently been shown to depend on the relative proportions of antigen and antibody comprising the complexes (Fig. 4.5; Rajnavölgyi *et al.*, 1978). For this reason, IgG antibodies were titrated against a fixed amount of antigen (TNP-KLH) and the mixtures were tested for their ability to

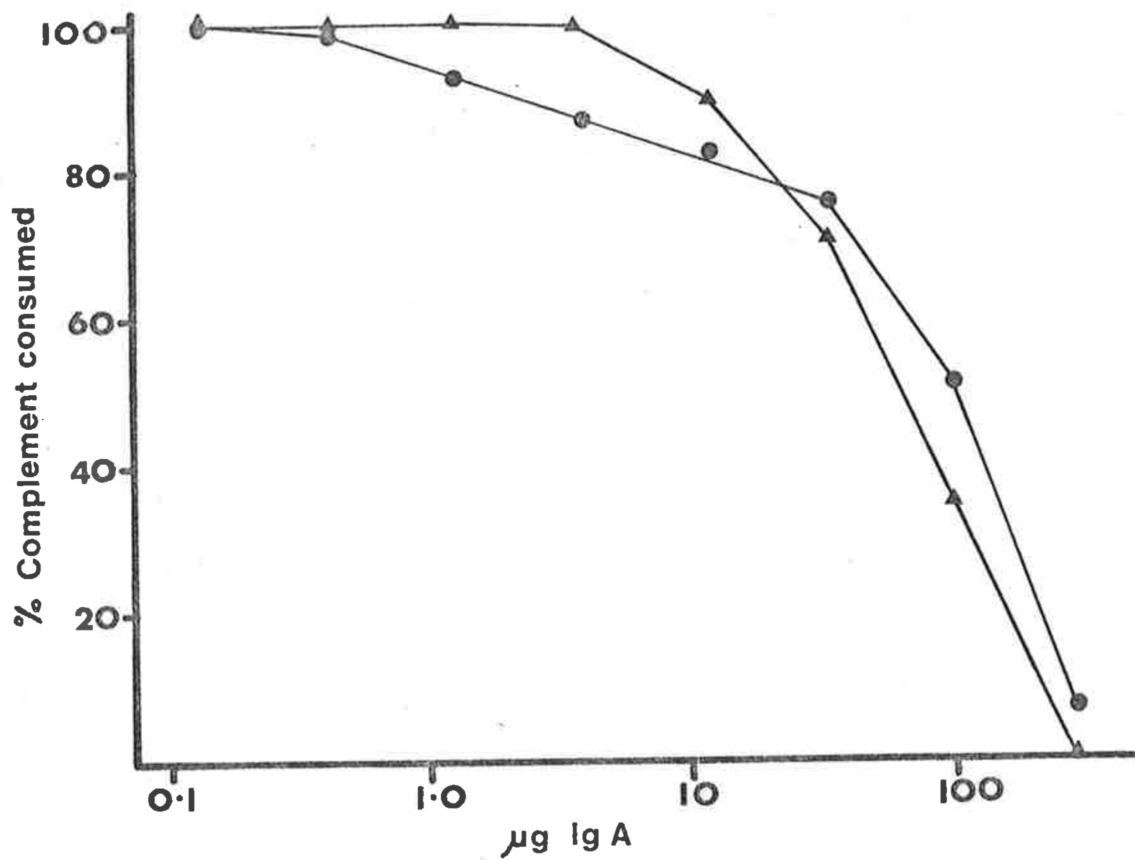
FIGURE 4.3

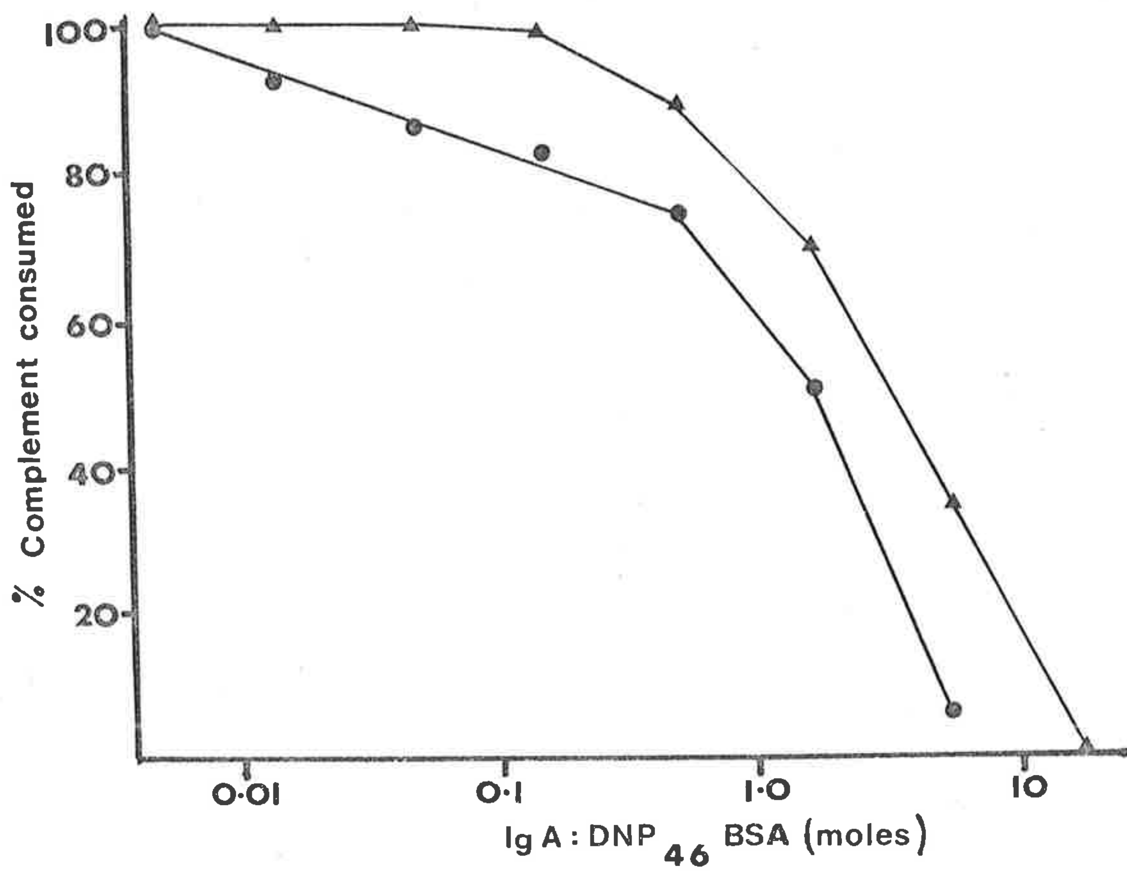
The ability of 7S and 13S IgA to prevent complement consumption by DNP-BSA

Serial twofold dilutions of 7S or 13S IgA (0.1 ml) were each mixed with a constant amount of $DNP_{46}BSA$ (50 μg in 0.1 ml). The mixtures were incubated for 150 min at 37° and then for 18 hours at 4°. Complement (0.4 ml) was then added and the samples were mixed and incubated for 60 min at 37°. Saline (0.2 ml) was then added to each tube, the samples were diluted serially in twofold steps, and these were then mixed with haemolysin-sensitized SRBC (0.2 ml of 2%). Lysis was determined as described for Fig. 4.2.

TOP: C consumption versus amount of IgA added.

BOTTOM: C consumption versus molar ratio, IgA : DNP-BSA ▲, 7S IgA; ●, 13S IgA.


100
80
60
40
20
% Complement consumed
0·1
1·0
10
100
μg Ig A


100
80
60
40
20
% Complement consumed
0·01
0·1
1·0
10
Ig A : DNP 46 BSA (moles)

fix C. A proportional increase in the amount of C consumed was observed with increasing amounts of DNP-specific rabbit IgG (Fig. 4.4). Consumption was maximal using 20 μg of antibody. Adding antibody in excess of this amount (up to 10x) did not result in further increases in complement consumption. These findings were consistent with the results of Rajnavölgyi *et al.*, (1978) who observed a similar relationship between increasing antibody levels and complement consumption (Fig. 4.5). For example, these authors found that the consumption of C by 1 μg of the antigen-antibody mixture was maximal at an antibody: antigen ratio of approximately 10:1 (w/w) and that further increases in this ratio caused no significant increase in consumption. Experiments using the same amount of antigen-antibody precipitates, revealed that little C was consumed by the insoluble complexes formed at the precipitin equivalence, while the precipitates formed in antibody excess were significantl more efficient in activating complement. The ratio of antibody: antigen present in the precipitate therefore determines the ability of the precipitate to fix C. The relevance of these observations in blocking is discussed in Section 4.3.2.a).

4.3.2 Inhibition of complement consumption by IgA

4.3.2.a) The effect of antibody concentration

To investigate the quantitative aspects of blockage, various amounts of 13S and 7S IgA, were mixed with two different levels of IgG antibody (54.25 μg and 13.56 μg). A constant amount of antigen was added, the mixtures were incubated and the capacity of the resultant complexes to consume C was determined. At the

FIGURE 4.4

Consumption of complement by antigen-antibody complexes

Serial dilutions (0.1 ml) of DNP-specific rabbit IgG were mixed with 5 μg of TNP-KLH (0.1 ml) and incubated for 150 min at 37° and then O/N at 4°. After adding complement (0.4 ml; 14 CH_{50}) and incubating for a further 60 min (37°), saline (0.2 ml) was added to each tube and the samples were assayed for complement consumption in the usual fashion (Fig. 4.2).

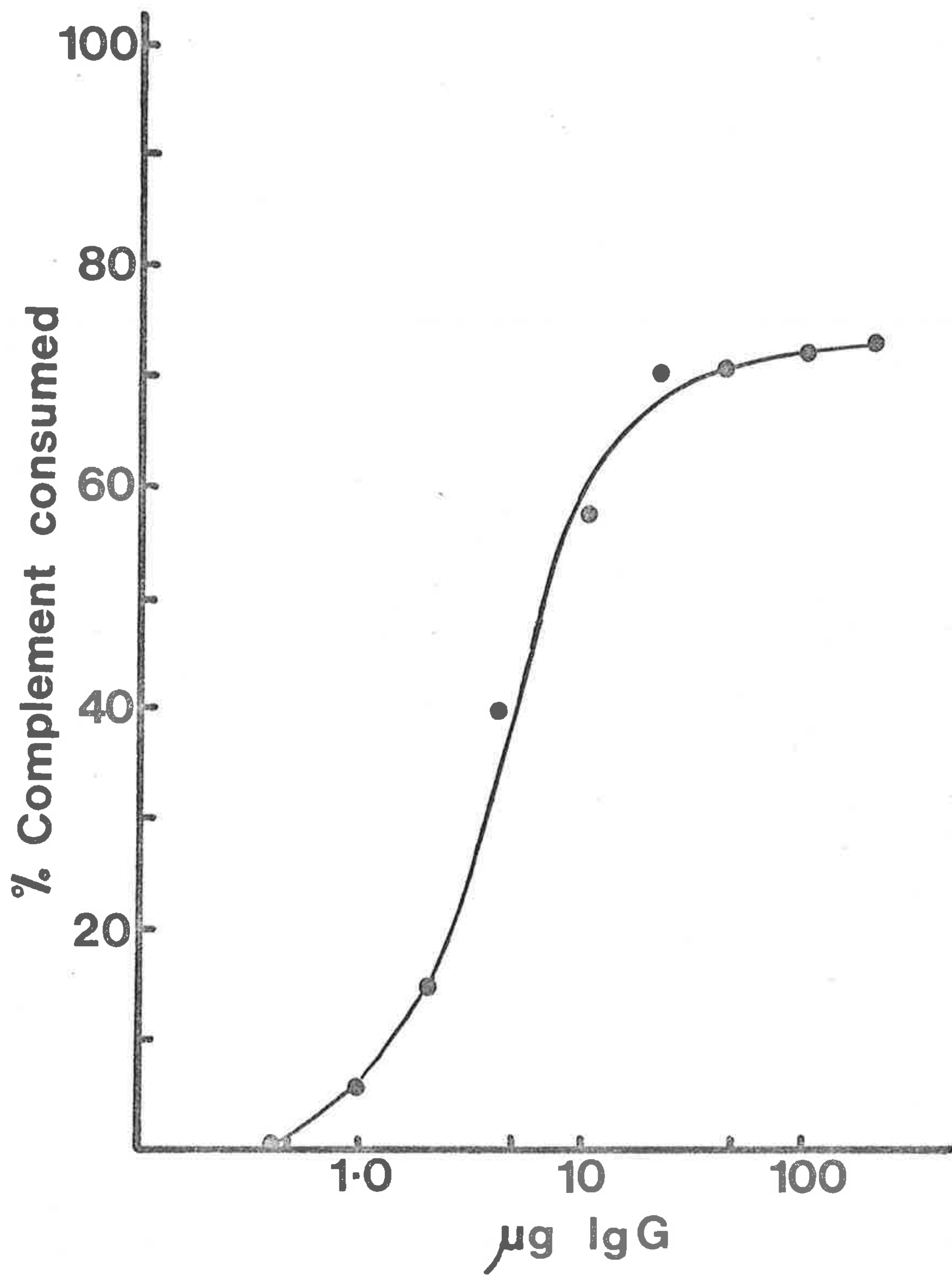
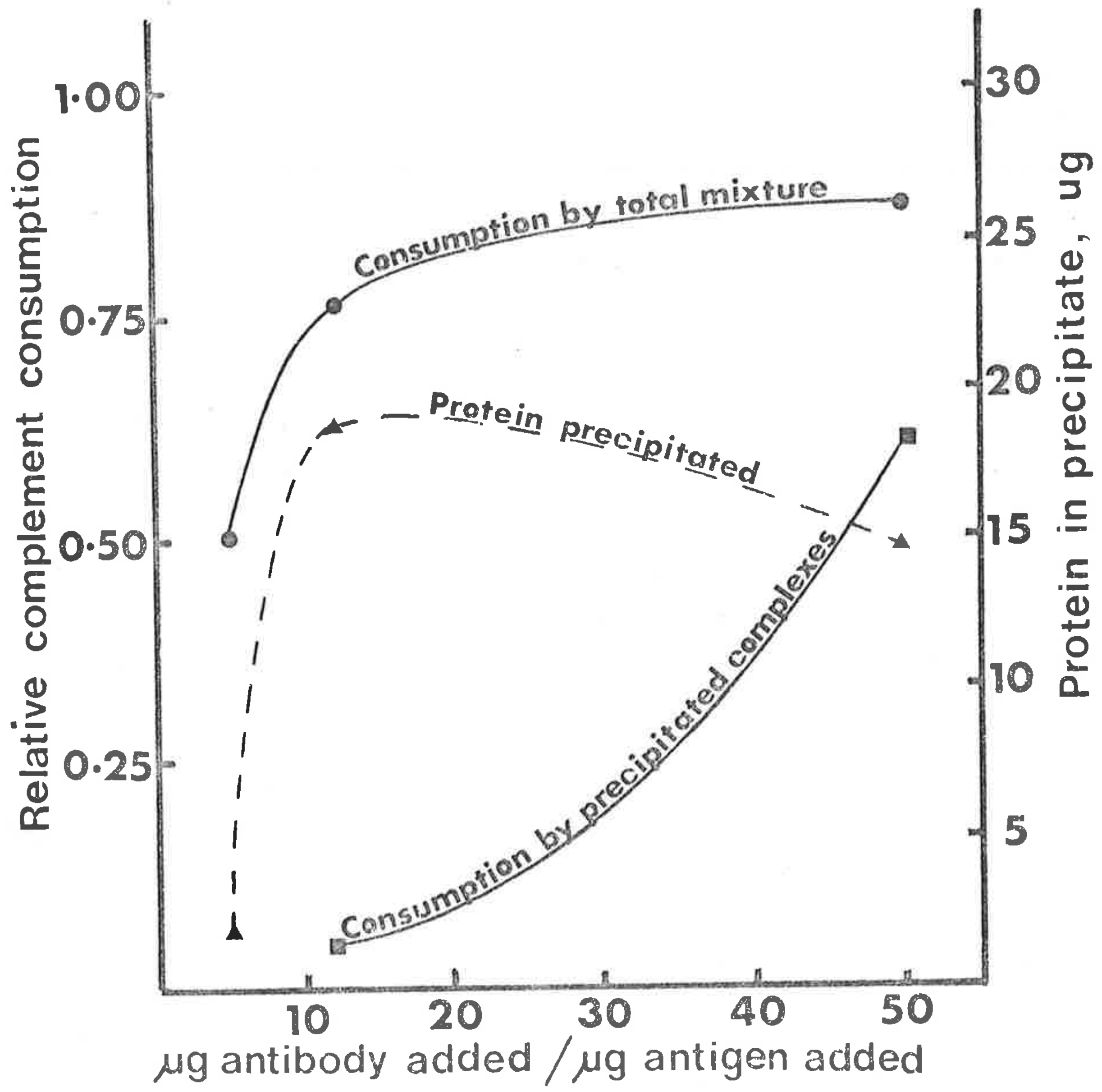

100
80
60
40
20
% Complement consumed
1·0
10
100
μg IgG

FIGURE 4.5

The activation of complement by soluble and insoluble BSA:anti-BSA complexes

Data adapted from Rajnavölgyi *et al.*, (1978:Fig.1.A)

The assay consisted of mixing BSA with different amounts of anti-BSA antibody. The consumption of complement by 1 μg of protein from the BSA:anti-BSA mixture, or from the antigen-antibody precipitate (isolated by centrifugation) was assessed.


1·00
0·75
0·50
0·25
Relative complement consumption
30
25
20
15
10
5
Protein in precipitate, ug
Consumption by total mixture
Protein precipitated
Consumption by precipitated complexes
10
20
30
40
50
µg antibody added / µg antigen added

higher level of IgG (54 μg), the addition of 7S or 13S IgA, in amounts up to 8-10 times that of the IgG, was ineffective in preventing C activation (Fig. 4.6, top). It is noteworthy however that small amounts of IgA were able to slightly enhance complement consumption (by about 10%). At this level of IgG (54 μg), the antigen (5 μg of TNP-KLH) must almost certainly have been saturated with antibody. Thus, the addition of the IgA could be expected to result in competition between the two types of antibody for the limited number of binding sites on the antigen, thereby reducing the number of antigen-bound IgG molecules available to fix C.

Since complexes formed in the presence of excess IgA (up to 10-fold with respect to IgG) showed little reduction (<20%) in their capacity to consume C, it seemed clear that a significan proportion of the available IgG molecules must be inhibited from binding before an appreciable reduction in C-fixing ability is observed. It is useful to consider these results with respect to Figure 4.4, where it can be seen that (in the absence of competing antibody), the number of IgG antibody molecules which must be "displaced" to effect a significant reduction in C consumption would depend on the total number of IgG molecules present. For instance, a 50% reduction in the amount of C consumed in the presence of 50 μg of IgG would require the "displacement" of at least 45 μg of IgG.

At the lower level of IgA (13.56 μg), the presence of relatively small amounts of IgA severely affected the ability of the complexes to consume complement (Fig. 4.6, bottom). For example, the amount of C consumed by complexes formed in the

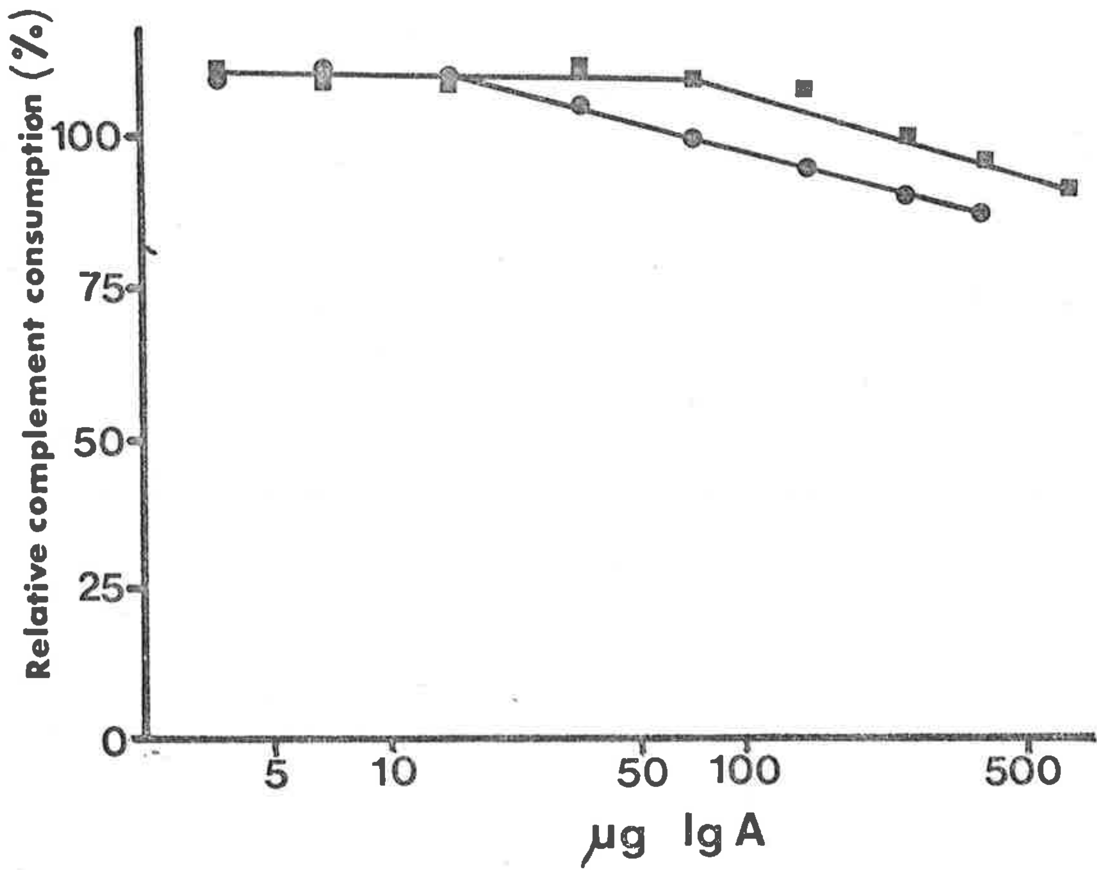
FIGURE 4.6

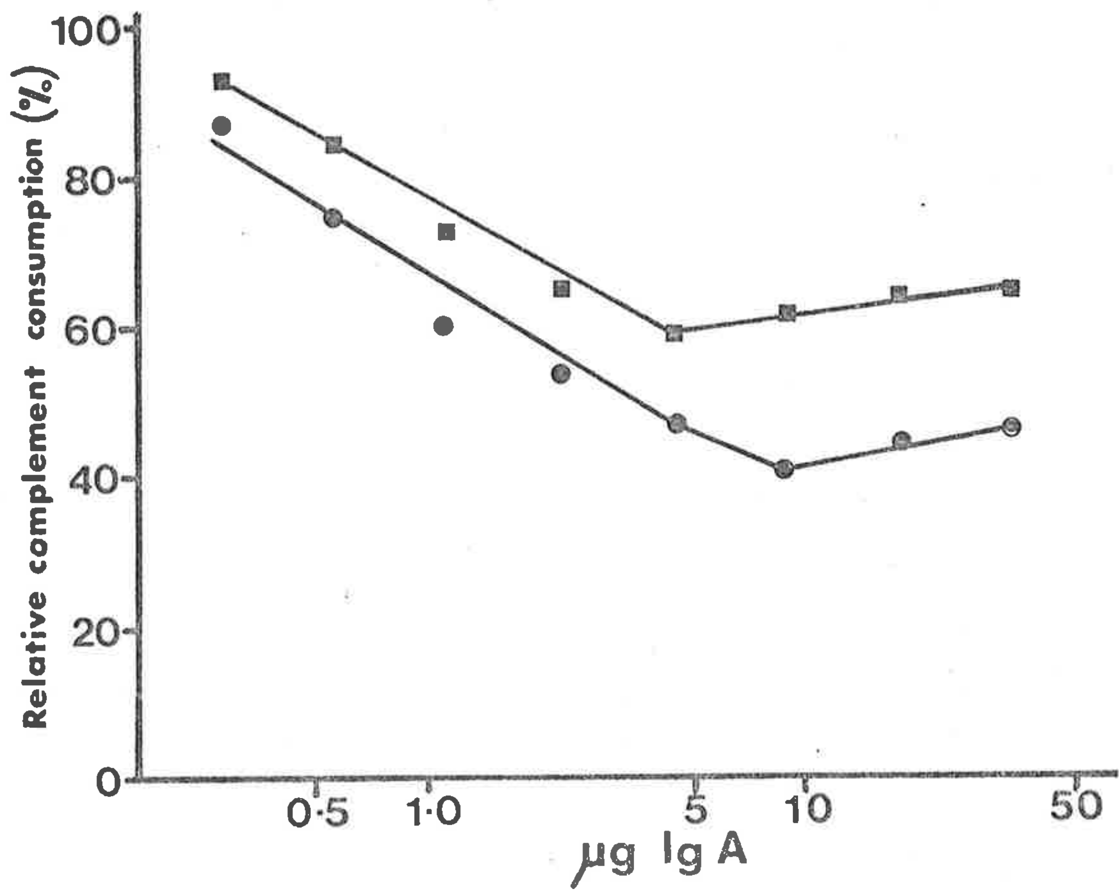
Complement consumption by antigen-antibody mixtures containing variable quantities of IgA

TNP-KLH (5 µg in 0.1 ml) was added to tubes containing a constant amount of IgG (50 µl) premixed with variable amounts of IgA (0.2 µg to 600 µg in 50 µl). The samples were incubated for 150 min at 37° and O/N at 4°, complement was added (0.4 ml, 14 CH_{50} units) and after further incubation (60 min; 37°), saline (0.2 ml) was added to each tube. The samples were titrated to determine their residual CH_{50} titres (see Fig. 4.2). Relative complement consumption is expressed as a percentage of the consumption observed with the IgG-antigen complex formed in the absence of IgA. Two fixed amounts of IgG antibody were used:

TOP: 54.25 µg; BOTTOM: 13.6 µg.

■, 7S IgA: ●, 13S IgA.


Relative complement consumption (%)
100
75
50
25
0
5
10
50
100
500
µg IgA


Relative complement consumption (%)
100
80
60
40
20
0
0·5
1·0
5
10
50
µg IgA

presence of 1 µg of 13S or 7S IgA was only 60% and 75%, respectively, of that consumed by complexes formed in the absence of IgA. Throughout the range of IgA concentrations tested, complexes containing 13S IgA fixed 10-20% less C than those formed in the presence of 7S IgA (Fig. 4.6, bottom). It is unlikely that the reduced C consumption observed with complexes formed in the presence of 13 µg of IgG and low levels of IgA (<5 µg) was due to competition between these molecules, as the antigenic sites on the TNP-KLH would presumably not be saturated. Furthermore, it seems improbable that 0.5 - 2.0 µg of IgA could compete effectively against 13 µg of IgG and prevent the latter from binding to 5 µg of antigen. It is possible that the IgA molecules may bind between potential C-fixing IgG duplets on the TNP-KLH and sterically prevent the attachment of C1 molecules. The larger bulk of the 13S IgA molecule could be expected to render it more efficient in blocking than 7S IgA, as was in fact observed (Fig. 4.6, bottom).

4.3.2.b) <u>The influence of complement concentration</u>

The concentration of C at secretory surfaces such as the lung is almost certainly much lower than the concentrations used in the blocking experiments described so far (Reynolds & Thompson, 1973; Newhouse *et al.*, 1976; Robertson *et al.*, 1976; Gross *et al.* 1978). Because low C concentrations have been shown by Ey *et al.* (1980) to enhance the ability of IgG_1 antibodies to block the sensitization of TNP-SRBC (for C mediated lysis), by IgG_2 antibody, it was decided to examine the blocking activity of IgA at two C concentrations (Fig. 4.7). The relative blocking

FIGURE 4.7

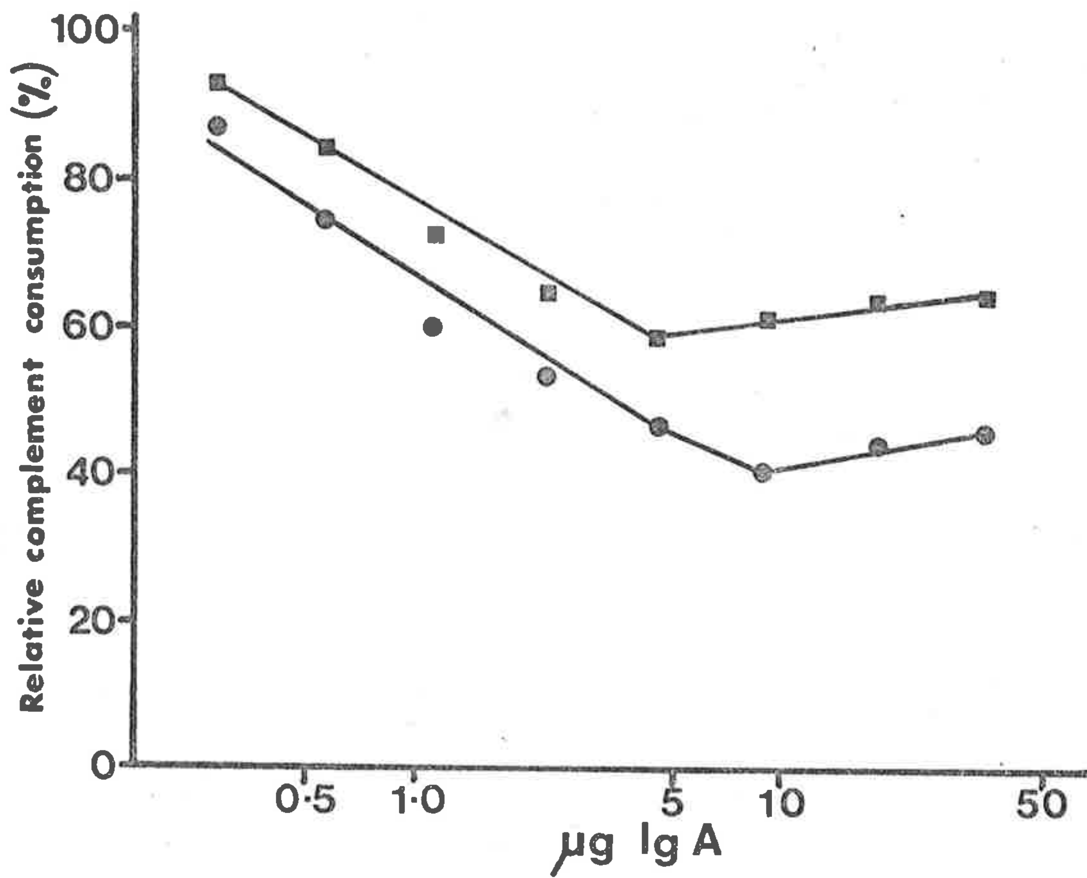
Effect of complement concentration on the ability of IgA to inhibit complement consumption by antigen-antibody complexes

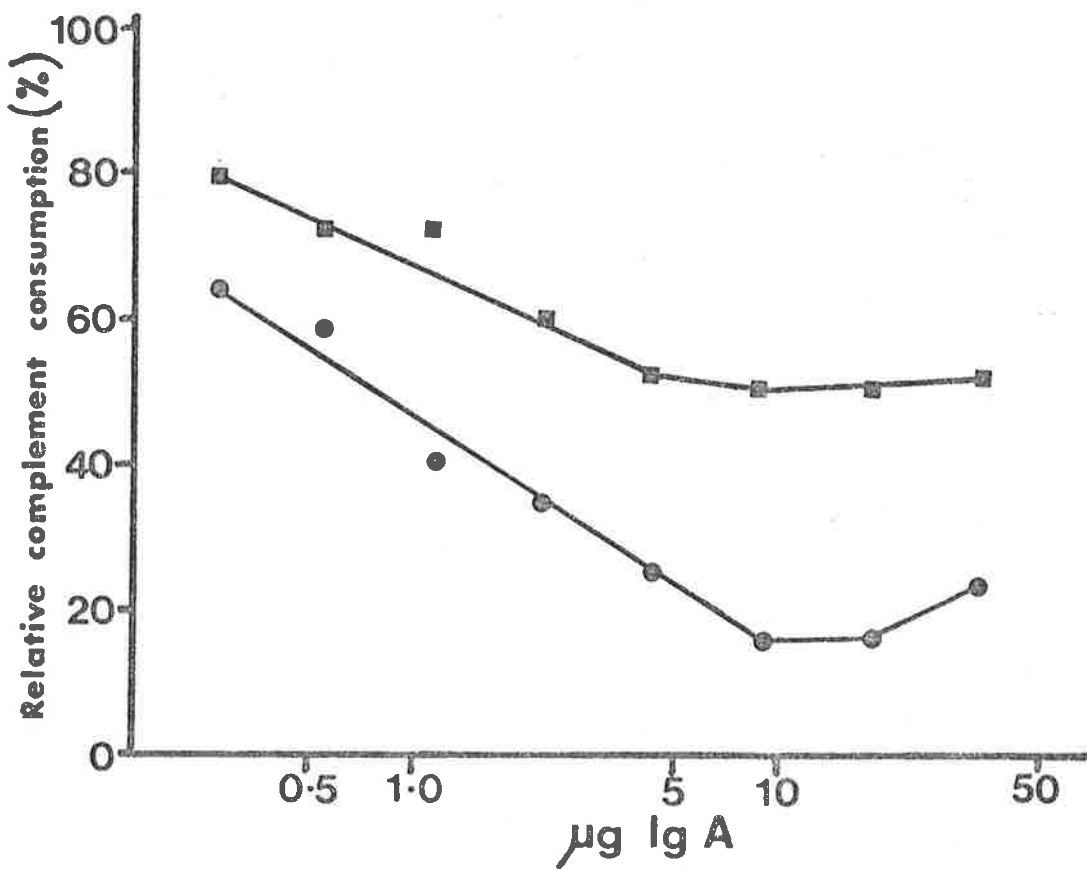
Duplicate dilutions of IgA were each mixed with a constant amount of IgG (13.6 μg in 50 μl) prior to the addition of TNP-KLH (5 μg; 100 μl). The tubes were incubated for 150 min at 37° and then O/N at 4°. After addition of complement (0.4 ml), the tubes were incubated for 60 min at 37°, saline (0.2 ml) was added to each tube and the samples were titrated for residual C activity (Fig. 4.2). Relative complement consumption is expressed as a percentage of the consumption observed with the IgG-antigen complex formed in the absence of IgA.

TOP: Consumption in the presence of 14 CH_{50} units of C (100% = 9 CH_{50}'s)

BOTTOM: Consumption in the presence of 7 CH_{50} units of C (100% = 4.48 CH_{50}'s)

Fig.4.7 (top) is the same as Fig.4.6 (bottom) and is reproduced here for ease of comparison.


Relative complement consumption (%)
100
80
60
40
20
0
0·5
1·0
5
10
50
μg Ig A


Relative complement consumption (%)
100
80
60
40
20
0
0·5
1·0
5
10
50
μg Ig A

efficiency of 7S IgA was marginally increased upon halving the C concentration used in the assay (e.g. by 5% at 5 μg and by 10% at 0.2 μg). In contrast, the inhibitory effect of 13S IgA on the ability of the complexes to consume C was much greater at the lower complement concentration (e.g. reduced by 25% over the range of 13S IgA used). When the inhibitory effect of the two IgA preparations were compared in terms of the molar ratio of IgA to IgG (Fig. 4.8), it was apparent that for any given IgA:IgG ratio at a particular C concentration, 7S IgA produced less than one half the inhibition observed with 13S IgA. Thus in the presence of 14 CH_{50} units of complement (in a reaction volume of 0.6 ml) and at a 1:10 ratio of IgA to IgG, the 7S and 13S IgA inhibited consumption by 25% and 50% respectively. At half this C concentration, relative C consumption was reduced by 35% and 75% respectively.

4.4 Discussion

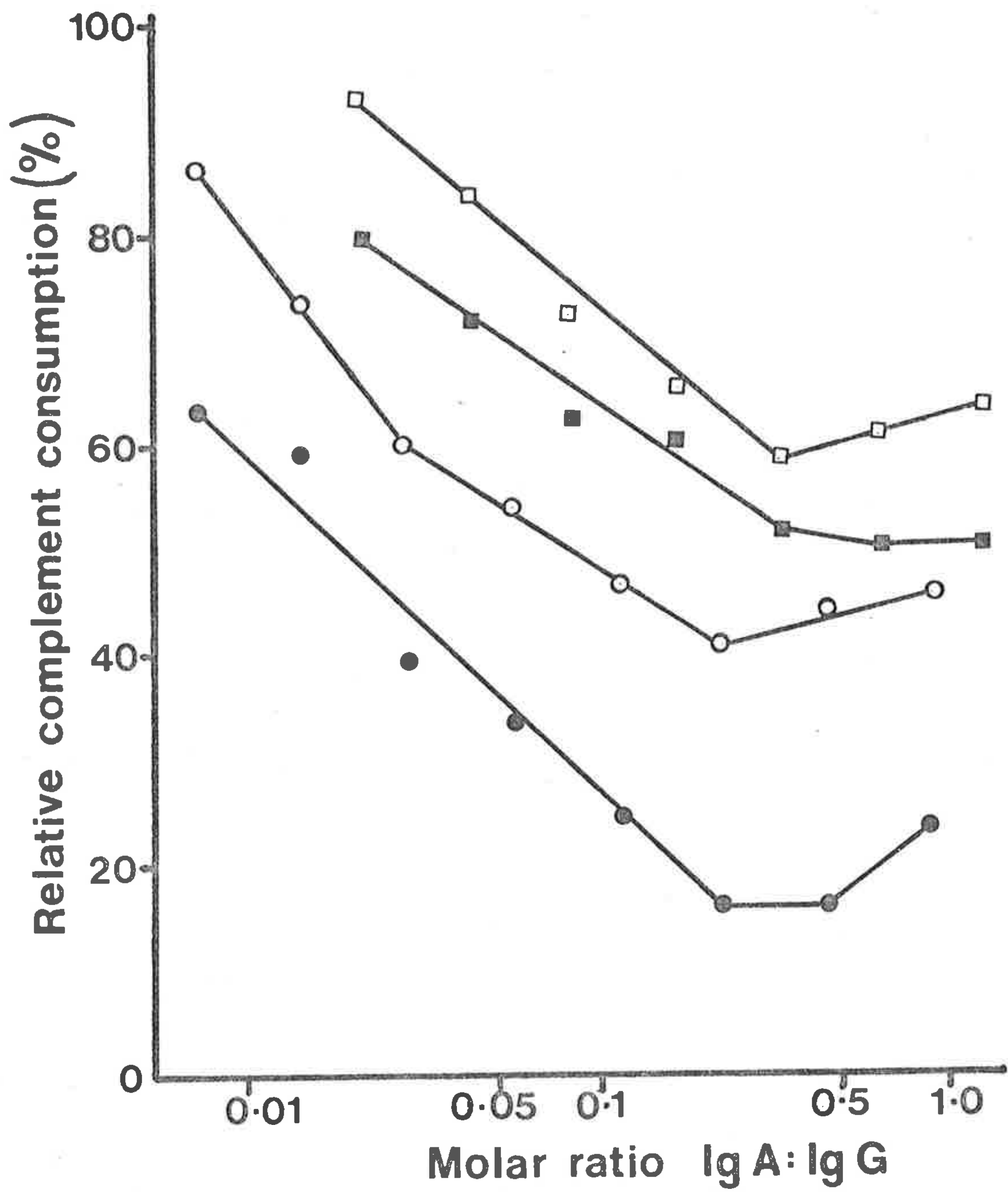
Complement may be activated within the body by a variety of substances including polyanions, such as lipopolysaccharide and antigens complexed to C-fixing antibodies. C-fixing antibodies such as IgM and some IgG subclasses are normally produced as part of the humoral immune responses to many antigens. Non-C-fixing antibodies, such as certain subclasses of IgG and in particular IgA are also elicited. The ratios of C-fixing to non-C-fixing antibodies elicited may well be important in the subsequent immune responses to antigen. For example the high titres of specific antibody found in patients

FIGURE 4.8

Change in complement consumption with changing ratios of IgA:IgG

Data from Fig. 4.7 is expressed in terms of the molar ratio of IgA:IgG added to TNP-KLH. A constant amount (13.6 µg) of IgA was added to dilutions of IgA, which were then mixed with 5 µg of TNP-KLH and incubated (150 min at 37°; O/N at 4°) prior to addition of 0.4 ml of C.

Complement: 14 CH_{50} units, □, o; 7 CH_{50} units, ■, ●. 7S IgA, □, ■; 13S IgA, o, ●.



100
80
60
40
20
0
Relative complement consumption (%)
0·01
0·05
0·1
0·5
1·0
Molar ratio IgA : IgG

with a number of chronic infections, such as meningitis and brucellosis (Griffiss, 1975; Hall & Manion, 1953; Zinneman *et al.*, 1959) have been shown to inhibit the bactericidal activity of normal serum *in vitro*. For these reasons it was considered important to investigate complement fixation by C-fixing antigens complexed with IgA and by antigen-antibody complexes formed in the presence of IgA.

The results showed that both monomeric (7S) and trimeric (13S) MOPC-315 IgA could significantly reduce the consumption of complement by the complement-activating $DNP_{46}BSA$. The activation of C was efficiently inhibited at low molar ratios of IgA:DNP-BSA, 50% inhibition being observed at molar ratios of 0.36:1 (7S) or 0.17:1 (13S) IgA.

Antigen-antibody complexes containing IgA were also found to fix less C than those containing antigen and IgG alone. The effect of IgA on the ability of the antigen-antibody complexes to fix C depended on the ratio of antigen-IgG present and upon the concentration of C with which the complexes were incubated. For example, the presence of even large amounts of IgA (up to 500 μg) had virtually no effect (<10% inhibition) on the ability of antigen-antibody complexes, formed in IgG antibody excess (54 μg of IgG : 5 μg antigen), to consume C. At lower concentrations of IgG (13 μg), however, IgA had a pronounced effect, 5 μg of 13S IgA reducing C consumption by approximately 50%. The relative inhibitory activity of IgA was further enhanced at lower C concentrations. For example, halving the level from 14 to 7 CH_{50} units (per 600 μl) increased the inhibition observed with 5 μg of 13S IgA from 47% to 76%.

The ability of IgA antibodies to inhibit C consumption by DNP-BSA or antigen-antibody complexes may result from either of the following mechanisms. Firstly, when the IgG antibody concentration greatly exceeds that required for precipitin equivalence and the antigen molecules become saturated with antibody, the IgA and IgG molecules would compete for antigenic sites. The extent of inhibition should in this case be proportional to the IgA:IgG ratio, since fewer C-fixing IgG duplets would be formed as the ratio is increased. Secondly, IgA molecules attached to the antigen may sterically block the fixation of C by the antigen (DNP-BSA) or by antigen-bound IgG duplets. In the case of $DNP_{46}BSA$, the molecules may be cross-linked by IgA to form a lattice, in which potential C-fixing sites would be concealed. Under conditions of vast IgA excess, when soluble complexes comprising single DNP-BSA molecules saturated with IgA would be favoured over a lattice structure, the IgA molecules themselves could be expected to sterically inhibit the activation of C. In the case of antigen-antibody complexes formed at low concentrations of IgG, the IgA may bind between (or in close proximity to) IgG duplets on the TNP-KLH molecule, thereby inhibiting the binding of C1 to these duplets.

In accordance with the findings of Ey *et al.*, (1980), the C-activation by antigen-antibody complexes containing IgA was blocked to a greater extent at low C concentrations. This is to be expected since the efficiency of the C1 → C9 activation sequence depends on the overall C concentrations. Consequently, any process which reduces the activation of C (e.g. the presence of IgA) will be amplified at lower C concentrations.

The ability of IgA to block or inhibit C-activation may have particular relevance *in vivo*. For instance, the extensive fixation of C which may occur during periods of high or chronic antigenic challenge can sometimes result in a number of deleterious conditions, such as the tissue damage which occurs after the production of auto-antibodies. The initiation of such reactions may normally be blocked, at least partially, by IgA antibodies. The lack of IgA, such as occurs in IgA-deficient patients, may lead to an increased incidence of pathological conditions associated with C-mediated phenomena, such as rheumatoid arthritis, systemic lupus erythematosus, thyroiditis and some forms of pernicious anaemia (Tomasi & Grey, 1972; Cooper *et al.*, 1971).

4.5 Summary

The polymeric and monomeric forms of IgA were examined for their capacity to inhibit complement consumption by the C-activating protein, DNP-BSA and by antigen-antibody complexes. On a molar basis, 13S IgA was slightly more efficient in inhibiting C consumption by DNP-BSA. When present during the formation of antigen-antibody complexes, 13S IgA was considerably more effective than 7S IgA in reducing the ability of the complexes to fix C. The degree of inhibition observed was related to the concentration of C used in the assay, being greater as the concentration was decreased.

CHAPTER 5

Inhibition of hypersensitivity reactions by IgA

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CHAPTER 5

Inhibition of hypersensitivity reactions by IgA

5.1 Preamble

During desensitization of allergic individuals, such as hay fever sufferers, the serum levels of IgG and IgA antibodies have been found to increase (Stokes *et al.*, 1974; Deuschl *et al.*, 1977; Wilkie *et al.*, 1978). The appearance of these antibodies in serum has been postulated to give rise to raised titres of blocking antibodies in the mucous of the respiratory tract. These antibodies would presumably neutralize allergens and prevent them from binding to cell-bound reaginic antibody in the underlying mucosa (Giessen *et al.*, 1976; Schumacher & Jeffery, 1979). It is as yet unclear which antibody class is most effective in neutralization, or whether such neutralization does occur at all. For this reason, MOPC-315 IgA was examined for its ability to inhibit two "classic" hypersensitivity reactions, active cutaneous anaphylaxis and the Arthus reaction.

5.2 Results

5.2.1 Active cutaneous anaphylaxis (A.C.A.)

5.2.1.a) The reaction

Cutaneous anaphylaxis is an immediate-type hypersensitivity reaction which can be induced by the subcutaneous injection of antigen into sensitized animals (Lehrer, 1977; Watanabe &

Ovary, 1977; Coulson, 1976). Antigen crosslinks homocytotrophic antibodies bound to mast cells or basophils, causing the release of histamine and other vasoactive amines, which leads to an increase in the permeability of the local capillaries of the skin. The reaction is easily visualized, after the intravenous injection of a suitable dye (such as Evans blue), as a localized area of intense blueing which appears within 5-10 minutes of antigen injection. The reaction intensifies over 30 minutes, after which it subsides to be undetectable by 60 minutes. In mice, both the IgE and IgG_1 antibody classes are effective at eliciting this reaction.

5.2.2 The effect of antigen dose

In this study (BALB/C x C57 Bl) F_1 mice which had been immunized with $DNP_{20}BSA$ (Section 2.A.13.(d)) were tested for their capacity to elicit an A.C.A. reaction when challenged intradermally with varying doses of TNP-KLH. Injection of increasing amounts (2-16 μg) of TNP-KLH led to a proportional increase in the size of the reaction (Photo 5.1). A positive reaction to BSA (16 μg) was also observed (Photo 5.1#6). The reactions observed in these mice disappeared within 60 min, as expected for an A.C.A. reaction (Lehrer, 1977; Watanabe & Ovary, 1977). A 10 μg dose of antigen was chosen for all subsequent experiments and the reaction elicited by this dose was assigned a reaction intensity of 3. The reaction to TNP-KLH was completely inhibited when 20 μg of 13S MOPC-315 IgA was

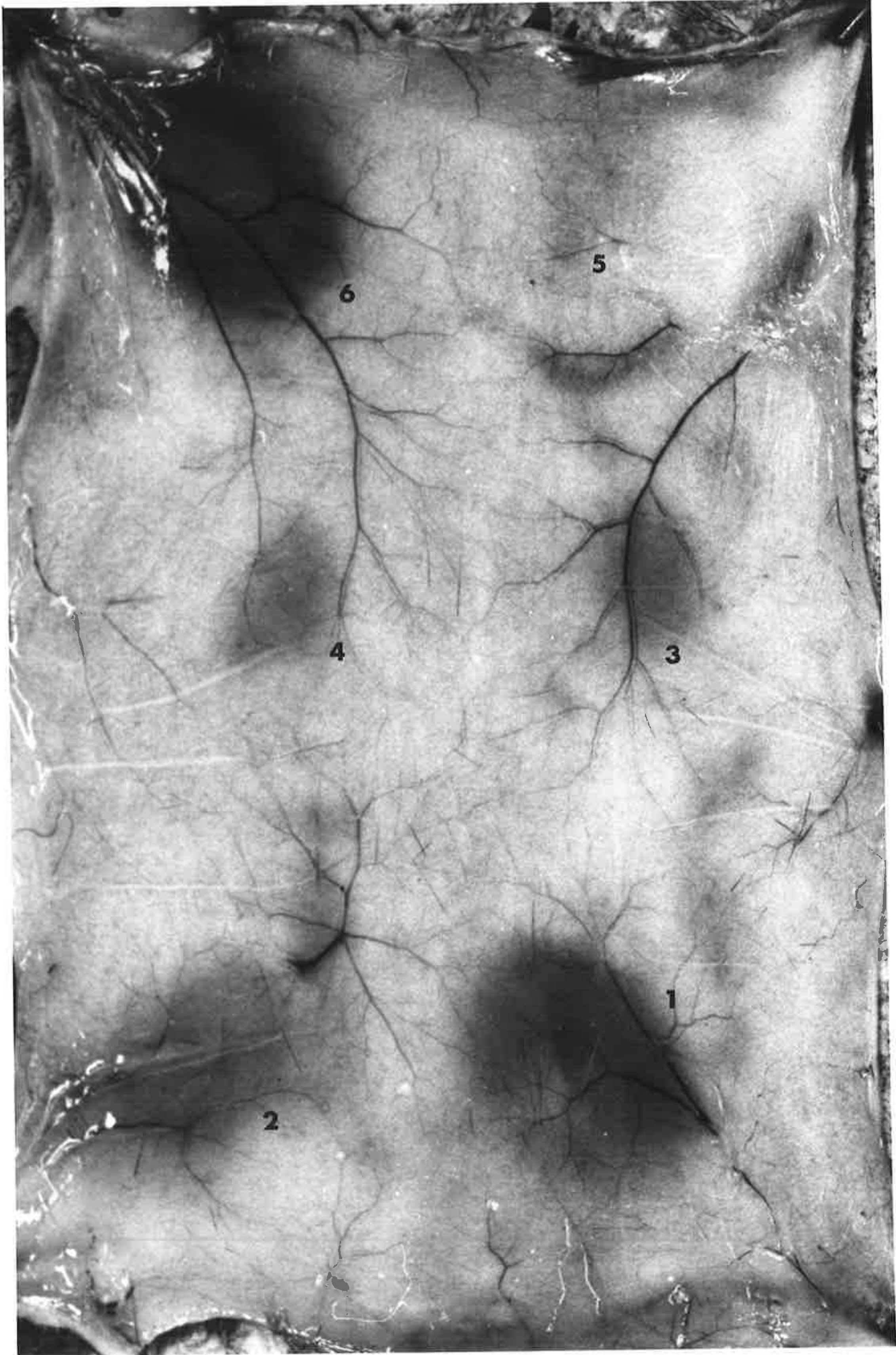
PHOTO 5.1

Change in A.C.A. reaction with increasing doses of antigen

Various amounts of antigen in 50 µl volumes of saline were injected into the shaved backs of DNP-BSA-immunized F_1 mice, several minutes after an intravenous injection of Evans blue (0.2 ml, 1%). The mice were killed half an hour after the antigen injection and the reflexed skin of each animal was examined for the presence of a reaction.

The reactions were elicited by injections of:-

1. 16 µg TNP-KLH
2. 8 µg TNP-KLH
3. 4 µg TNP-KLH
4. 2 µg TNP-KLH
5. 10 µg TNP-KLH + 20 µg 13S IgA
6. 16 µg BSA


6
5
4
3
1
2

premixed with the antigen prior to intradermal injection (Photo 5.1#5). For this reason, various doses of 7S and 13S IgA were tested for their capacity to block the A.C.A. reaction.

5.2.3 Reactions elicited by antigen-IgA mixtures

Aliquots of TNP-KLH were incubated with twofold dilutions of 7S or 13S IgA for 1 hour at 37° prior to injection into mice. The A.C.A. reaction was completely inhibited by the addition of 3 µg of IgA (Fig. 5.1 and Table 5.1) and as little as 0.19 µg caused a 50% reduction in the reaction. No difference in the blocking efficiency of 7S or 13S IgA could be discerned. Examples of different reaction intensities are shown in Photo 5.2. Areas of darkly pigmented skin (see Photo 5.2.#6) were often observed on the skin of the F_1 mice but were readily distinguishable from the blue A.C.A. reactions.

5.2.4 Specificity of the blocking phenomenon

To exclude the possibility that the IgA may be blocking the A.C.A. reactions elicited by TNP-KLH by non-specific mechanisms, the effect of IgA on the reaction elicited with BSA was examined. When high levels of IgA (180 µg) were incubated with 10 µg samples of BSA no discernable reduction in the intensity of the reactions occurred. As much lower levels of IgA (3 µg) completely blocked the A.C.A. reaction to TNP-KLH, the blocking activity observed with IgA clearly was an antigen-specific phenomenon, presumably involving

TABLE 5.1

Active cutaneous anaphylactic reactions induced by mixtures of TNP-KLH and MOPC-315 IgA

μg IgA	Reaction intensity*	
	7S IgA	13S IgA
12	-	-
6	-	1
3	-	-
1.5	2	-
0.75	0.5	0.5
0.38	2	2
0.19	2.5	2.5
0.094	4.5	4.5
0.047	6	6
-	6	6

Mice immunized with DNP-BSA were injected with Evans blue, followed by 10 μg aliquots of TNP-KLH which had been preincubated with different amounts of 7S or 13S IgA. After 30 min the mice were killed and the intensity of blueing on the reflexed skin determined. Each mouse was injected with a TNP-KLH control. Results are expressed as the additive reactions from two mice. These data are plotted in Fig. 5.1.

* Representative reaction intensities are shown in Photo 5.2.

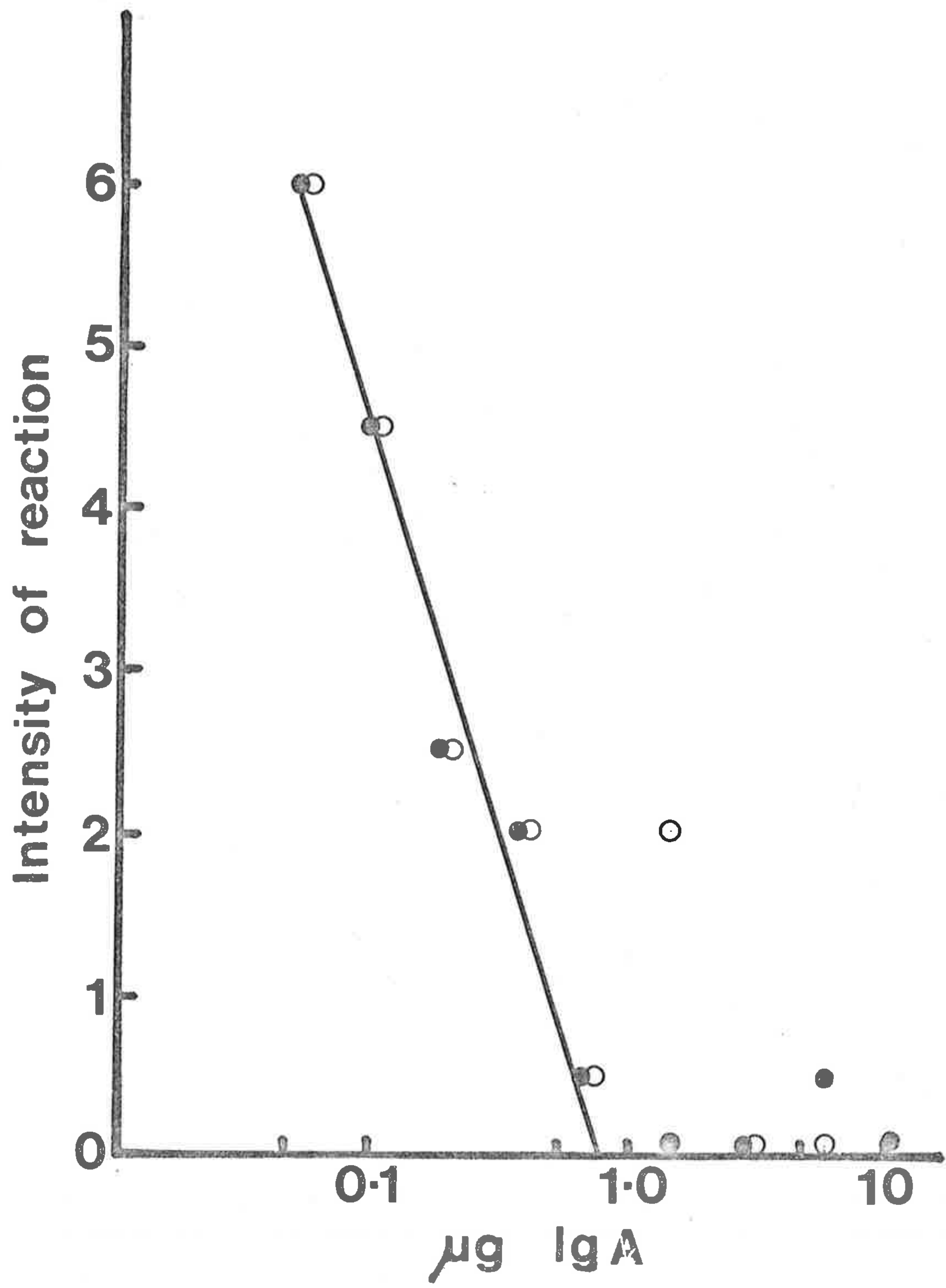
FIGURE 5.1

Active cutaneous anaphylactic reactions induced by mixtures of TNP-KLH and IgA

Plot of the data shown in Table 5.1. Mice immunized with DNP-BSA were injected with Evans blue, followed by 10 μg aliquots of TNP-KLH which had been preincubated with different amounts of 7S or 13S IgA. After 30 min the mice were killed and the intensity of blueing on the reflexed skin determined. Each mouse was injected with a TNP-KLH control. Results are expressed as the additive reactions from two mice.

Representative reaction intensities are shown in Photo 5.2.

o , 7S IgA ; ● , 13S IgA.



Intensity of reaction
6
5
4
3
2
1
0
0·1
1·0
10
μg IgA

neutralization of the antigen. A non-specific interaction of IgA with the cells responsible for the A.C.A. reaction can therefore be excluded.

5.2.5 Blockage by serum IgA

Although premixing IgA with TNP-KLH inhibited the A.C.A. reaction to this antigen, it was not known if IgA could compete with other classes of antibody present in serum and inhibit the initiation of A.C.A. reactions. For this reason, (BALB/C x C57 Bl) F_1 mice immunized with DNP-BSA, were injected intraperitoneally with 2×10^6 MOPC-315 tumour cells and tested at various stages during tumour growth for their A.C.A. responses to TNP-KLH. Immediately prior to killing, each mouse was bled from the retro-orbital plexus. Individual serum samples (0.2 ml) obtained in this fashion were passed down 0.2 ml columns of DNP-Sepharose (Section 2.A.5.(a)) and the DNP-specific antibodies were eluted with DNP-glycine (0.2 ml, 0.1 M). The immunoglobulin content of the eluted antibody samples was determined by radial immunodiffusion. The level of antibodies in the different immunoglobulin classes in individual mice and the intensity of the A.C.A. reactions observed in these mice were compared (Table 5.2; Fig. 5.2).

Increases in the level of serum IgA were correlated with decreases in the intensity of the A.C.A. reactions elicited in these mice (Correlation coefficient, (r) = -0.9524; $P < 0.001$). While the intensity of the A.C.A. reactions

TABLE 5.2

Comparison of serum antibody levels with the intensity of the active cutaneous anaphylaxis reaction elicited in individual tumour-bearing mice

Mouse	Antibody (mg/ml) IgG_1	IgA	IgG_1:IgA ratio	Intensity of the* A.C.A. reaction
1	2.35	<0.05	-	4
2	2.30	<0.05	-	6
3	1.63	<0.05	-	9
4	1.53	<0.05	-	9
5	0.95	<0.05	-	8
6	0.40	<0.05	-	8
7	0.37	0.132	2.83	6
8	0.60	0.140	4.28	6
9	0.38	0.200	1.90	4
10	0.31	0.256	1.21	2.5
11	0.72	0.280	2.59	3
12	0.25	0.410	0.61	0
13	0.40	0.466	0.86	1.5
14	1.70	0.660	2.58	1.5
15	0.27	0.990	0.27	1.5

Mice immunized with DNP-BSA and injected with MOPC-315 tumour cells were each challenged intradermally with 10 μg of TNP-KLH (3 sites) and 10 μg of KLH (3 sites). After 30 min the mice were bled, killed, skinned and the intensity of the reactions measured. Individual sera were assayed for the immunoglobulin content of specific anti-DNP antibodies. Reaction intensities are expressed as the sum of the 3 reactions. No reactions were observed when KLH, rather than TNP-KLH, was used as the eliciting antigen. Antibody concentrations refer to the original concentration in serum. These results have been plotted in Fig. 5.2.

* Representative reaction intensities are shown in Photo 5.2.

FIGURE 5.2

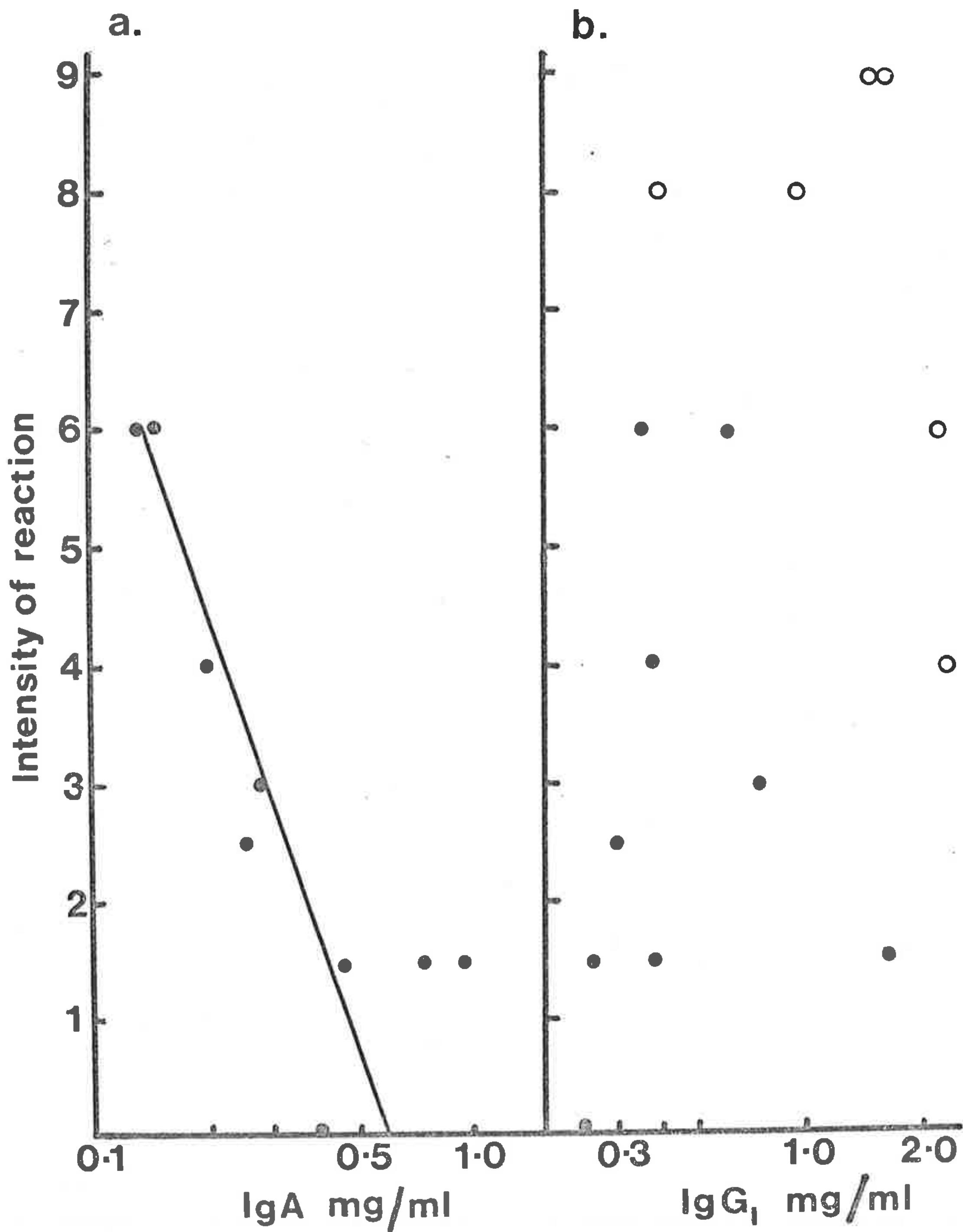
Comparison of serum antibody levels with the intensity of the active cutaneous anaphylaxis reaction elicited in individual tumour-bearing mice

Mice immunized with DNP-BSA and injected with MOPC-315 tumour cells were each challenged with 10 μg of TNP-KLH (3 sites) and 10 μg of KLH (3 sites). After 30 min the mice were bled, killed, skinned and the intensity of the reactions measured. Individual sera were assayed for the immunoglobulin content of specific anti-DNP antibodies. Reaction intensities are expressed as the sum of 3 reactions. Data from Table 5.2. Representative reaction intensities are shown in Photo 5.2.

a) Comparison of serum IgA anti-DNP antibodies with the intensity of the A.C.A. reaction. Correlation coefficient = 0.9524 ($P < 0.001$)

b) Comparison of serum IgG_1 anti-DNP antibodies with the intensity of the A.C.A. reaction. Correlation coefficient = 0.4351 ($P > 0.10$)

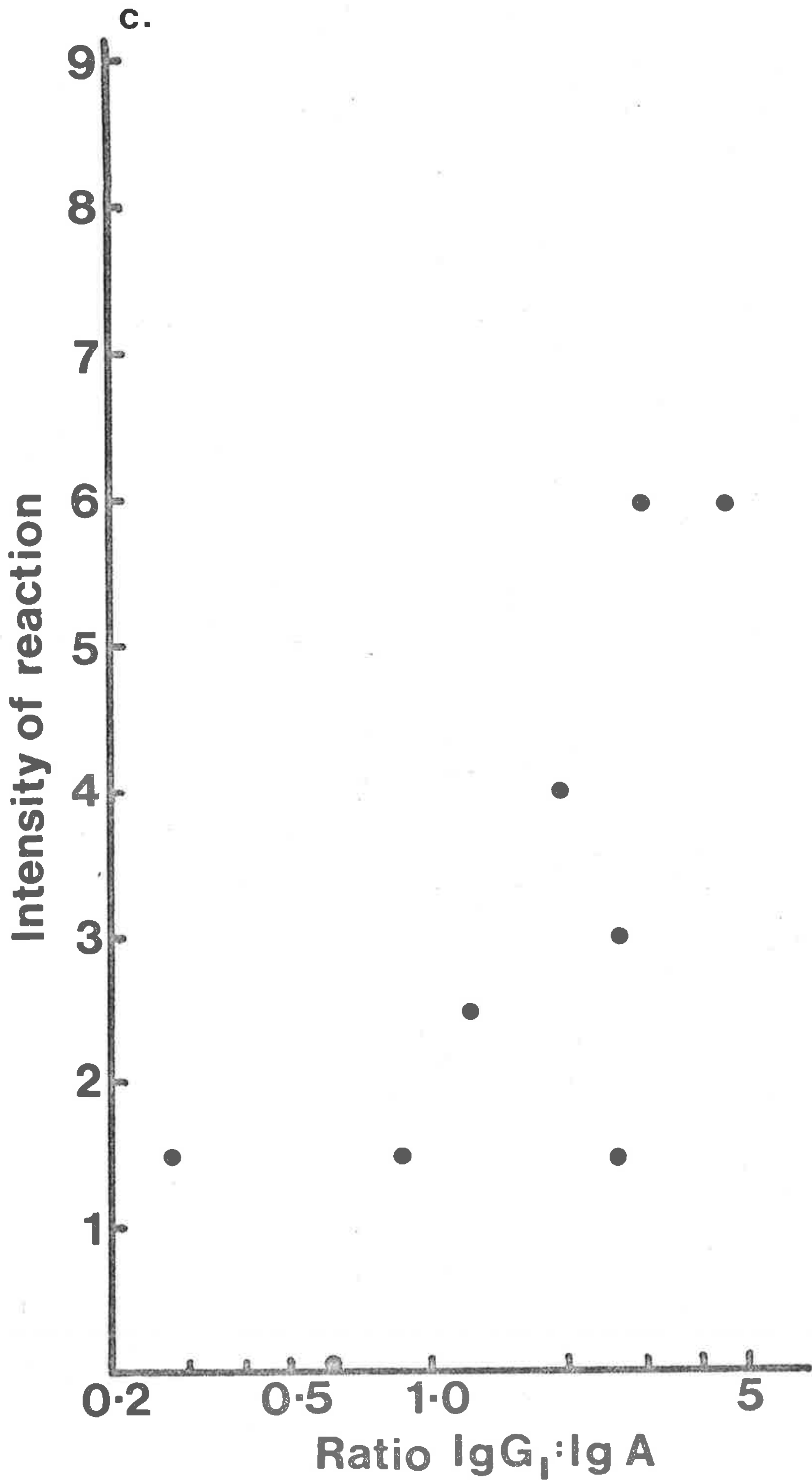
● Serum IgA $\geqslant$50 μg/ml; o Serum IgA <50 μg/ml.



a.
b.
Intensity of reaction
9
8
7
6
5
4
3
2
1
0·1
0·5
1·0
IgA mg/ml
0·3
1·0
2·0
IgG1 mg/ml

FIGURE 5.2 continued

c) Comparison of the ratio of IgG_1:IgA anti-DNP antibodies with the intensity of the A.C.A. reaction. Correlation coefficient = 0.7204 ($P > 0.02$). (Data from Table 5.2).


c.
Intensity of reaction
9
8
7
6
5
4
3
2
1
0·2
0·5
1·0
5
Ratio IgG_1:Ig A

bore a negative correlation with serum IgA antibody levels, there was no significant correlation with either the level of serum IgG_1 antibodies ($r = 0.4351$; $P > 0.10$) or the ratio of serum IgG_1:IgA ($r = 0.7204$; $P > 0.02$) (Fig. 5.2, a-c). It is noteworthy that the levels of serum IgG_1 antibodies tended to decrease as IgA levels rose, possibly due to a decrease in antigenic stimulation of IgG_1 antibody producing cells.

5.3 The Arthus reaction

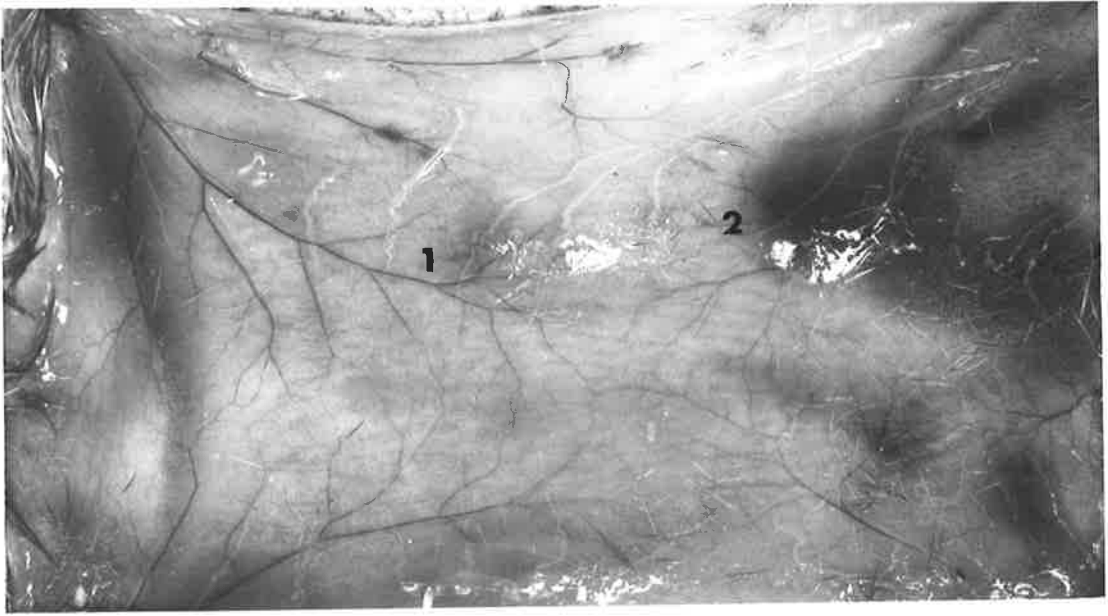
The results of Chapter 4 showed that IgA antibodies could block complement activation by antigen-antibody complexes. It was decided therefore to determine whether IgA could block the C-mediated Arthus reactions. This reaction occurs when antigen is injected intradermally into an immunized animal which has detectable levels of C-fixing, antigen-precipitating antibody (Cochrane, 1967; 1976; Yoshinga *et al.*, 1971). Antigen, complexed with antibody, is believed to precipitate extravascularly, whereupon complement is activated. During this process, polymorphonuclear leukocyte (PMN) chemoattractants are generated which attract these cells to the site of antigen deposition (see Chapter 6). Vasoactive amines, released from the infiltrating PMN cells, rupture the basement membrane causing oedema and haemorrhage, which can be visualized macroscopically by the prior injection of Evans blue dye. In mice, this reaction is not evident before 2 hours (see below). By 8 hours, mononuclear cells begin to enter the

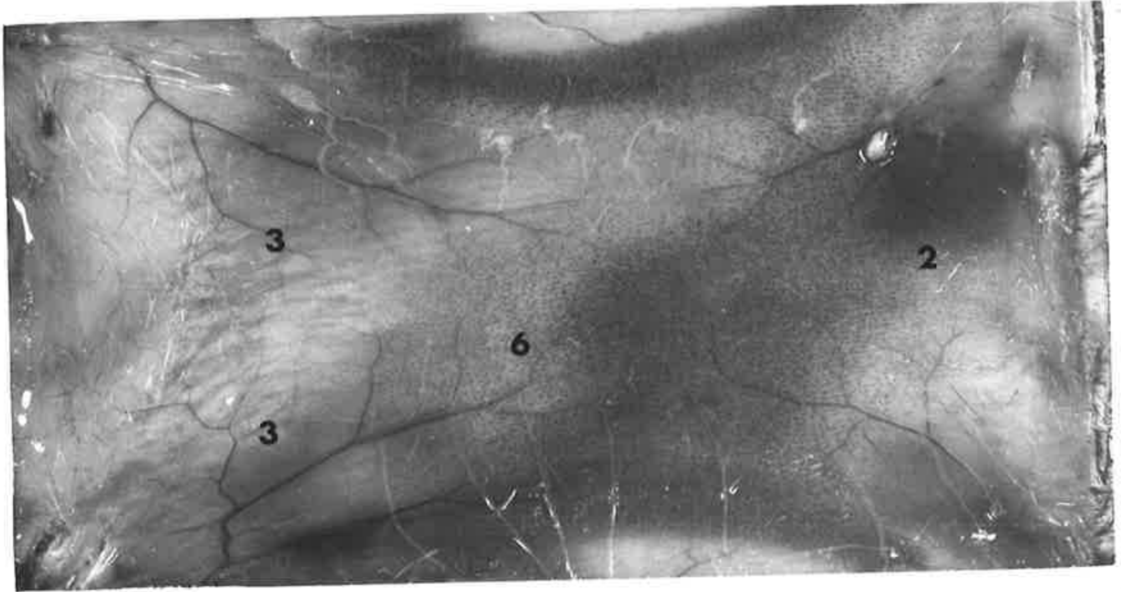
PHOTO 5.2

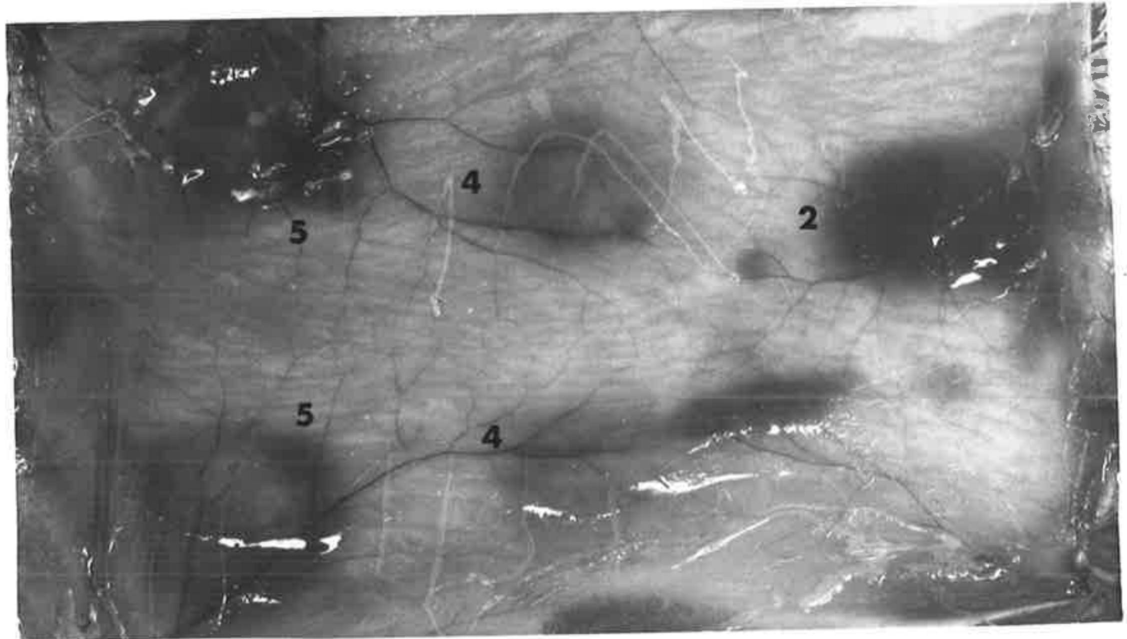
Representative active cutaneous anaphylaxis reactions intensities

Mice immunized with DNP-BSA were injected with Evans blue, followed by 10 μg aliquots of TNP-KLH which had been preincubated with different amounts of 7S or 13S IgA. Representative intensities of the reactions observed are shown.

1. Reaction intensity ; 0.5
2. Reaction intensity ; 3.0
3. Reaction intensity ; 1.0
4. Reaction intensity ; 2.0
5. Reaction intensity ; 2.5
6. Dark area of skin often seen in F_1 mice. This was easily distinguished from the blueing of an A.C.A. or Arthus reaction.


1
2


3
2
6
3


4
2
5
5
4

reaction site and healing commences, within 24 hours, macroscopic evidence of the reaction has disappeared.

5.3.1 Elicitation of the Arthus reaction in mice

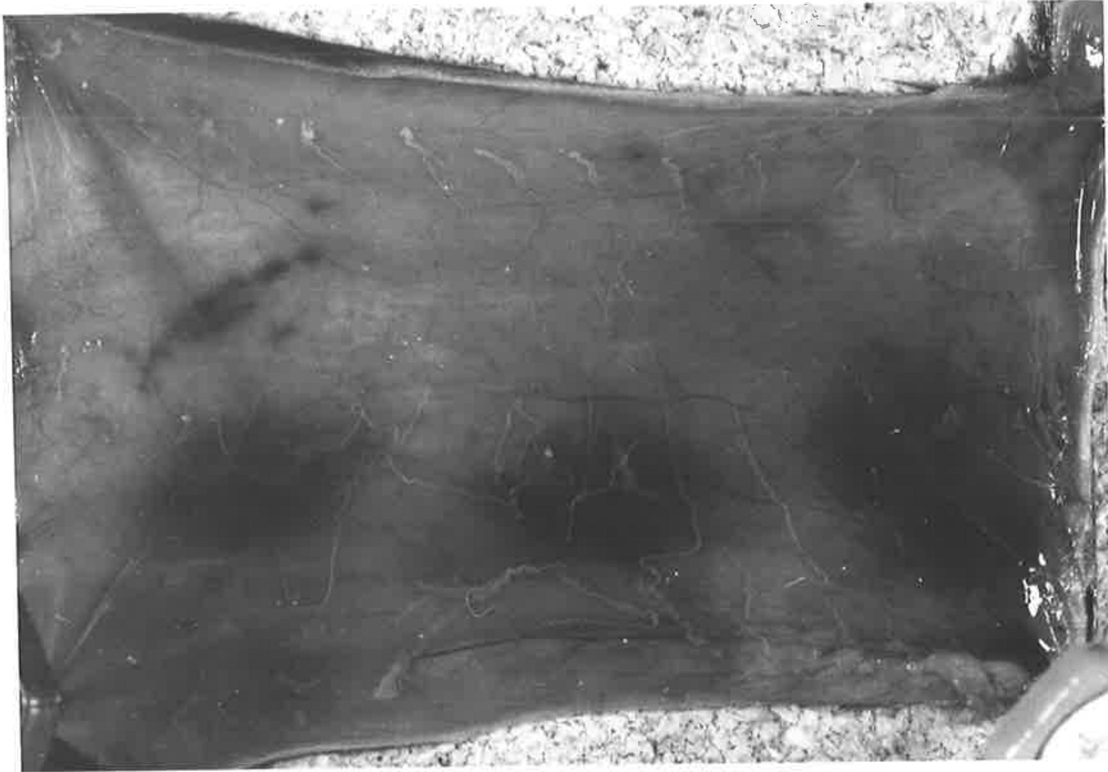
Hybrid (BALB/C x C57/Bl) F_1 mice immunized with DNP-*E. coli* (Section 2.A.13.(d)) produced an Arthus hypersensitivity reaction when challenged intradermally with TNP-KLH, following prior injection of Evans blue. It should be noted that the Arthus reaction was preceded by an A.C.A. reaction (Photo 5.3). However, this disappeared completely within 60 min of antigen challenge. The Arthus reaction was then demonstrable by the gradual onset of a blueing reaction 150 min after antigen challenge. The blueing reaction peaked by 5 hours and subsequently subsided until it was inapparent at 18 hours. Serum taken from these DNP-*E. coli* immunized mice, was able to elicit passive cutaneous anaphylactic reactions (see Section 2.A.13.(b)) in normal mice but did not produce reverse Arthus reactions. Furthermore, mice immunized with DNP-BSA gave strong A.C.A. reactions upon challenge with TNP-KLH but did not exhibit any Arthus reactivity. These results confirm that the later (Arthus) reaction was independent of the occurrence of an earlier A.C.A. reaction.

Injection of increasing amounts of TNP-KLH (2-20 μg) resulted in an increase in the area of the Arthus reaction which was elicited (Fig. 5.3). A challenge dose of 10 μg of TNP-KLH was used in all subsequent Arthus reactions.

PHOTO 5.3

A.C.A. and Arthus reactions elicited in DNP-*E.coli* immunized mice

DNP-*E.coli* immunized mice were challenged intradermally with 10 μg of TNP-KLH (3 sites) and 10 μg of KLH (3 sites). Mice were killed after either 30 min (top; A.C.A. reaction) or 5 hours (bottom; Arthus reaction). No reaction was elicited against KLH.



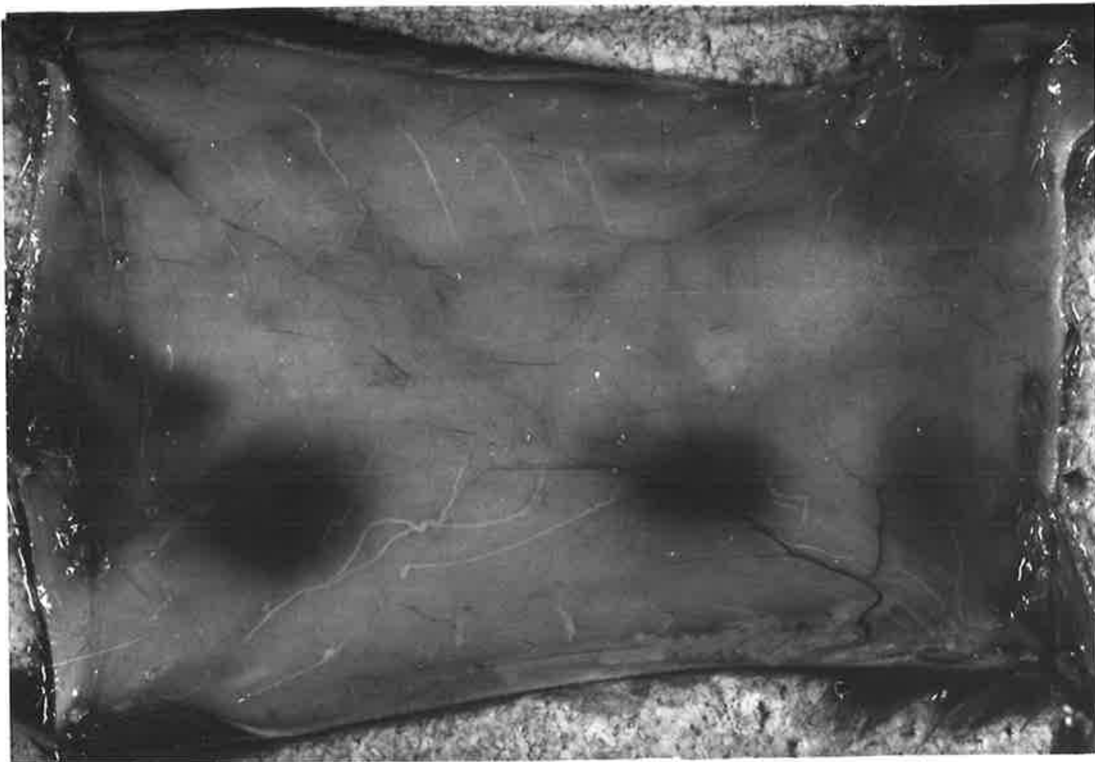
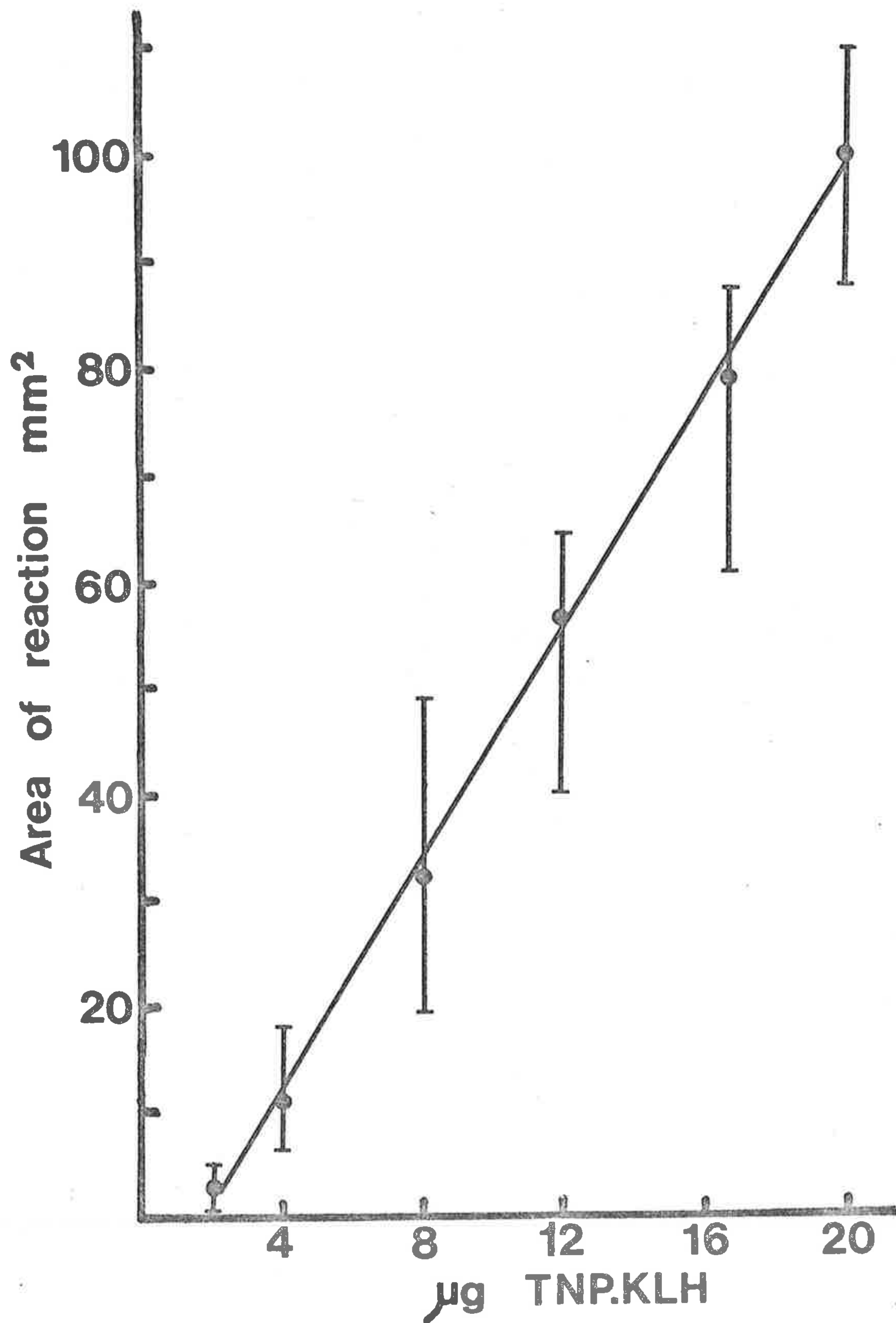


FIGURE 5.3

Increase in Arthus reaction
with increased levels of TNP-KLH

Mice immunized with DNP-*E.coli* were injected intravenously with 0.2 ml of 1% Evans blue and then challenged by intradermal injections of varying amounts of TNP-KLH (2,4,8,12,16,20 µg). After 5 hours the mice were killed and the size of the blueing reactions determined. The area of the reaction was calculated from two diameter measurements taken at 90°. Each point represents the average of 12 reactions. Vertical bars represent the range of readings obtained.


Area of reaction mm²
100
80
60
40
20
4
8
12
16
20
µg TNP.KLH

5.3.2 Reactions elicited by antigen-IgA mixtures

IgA was tested for its capacity to block the Arthus reaction by preincubating TNP-KLH with increasing amounts of 7S or 13S IgA (90 min; 37°) prior to injection into mice. Upon examination of the mice five hours after challenge, a decrease in the intensity of the Arthus reaction was observed only when $\geq$ 90 μg of IgA was used (Photo 5.4). There appeared to be no difference in the blocking ability of 7S or 13S IgA. The addition of as much as 180 μg of IgA caused only a 50% reduction in the intensity of the Arthus reaction (summed reaction intensity from 3 mice decreasing from 9 to 4.5).

5.3.3 Blockage with serum IgA

To determine whether IgA antibodies present in serum were able to inhibit an Arthus reaction, DNP-*E. coli* immunized mice (as used in Section 5.3 and 5.3.1) were injected intraperitoneally with 2x10^{6} MOPC-315 tumour cells and tested, at various stages during tumour growth, for their sensitivity to TNP-KLH and KLH. Five hours after challenge, the mice were bled, killed and their reactions evaluated. The class distribution of DNP/TNP specific antibody in the serum of each mouse was determined from radial-immunodiffusion tests. The reaction intensities observed at each of the three test sites were summed and these values are presented, together with the levels of specific antibody found in each mouse, in Table 5.3 and Figs. 5.4 and 5.5. Reactions were not observed when KLH, rather than TNP-KLH, was used as the

PHOTO 5.4

Arthus reactions elicited by mixtures of of TNP-KLH and MOPC-315 IgA

Mice immunized with DNP-*E.coli* were injected intravenously with Evans blue and then TNP-KLH which had been preincubated with various amounts of 7S or 13S IgA (90 min, 37°). The reaction was observed after 5 hours.

1. 10 μg of TNP-KLH
2. 10 μg of TNP-KLH + 180 μg of 13S IgA
3. 10 μg of TNP-KLH + 180 μg of 7S IgA
4. 10 μg of TNP-KLH + 90 μg of 13S IgA
5. 10 μg of TNP-KLH + 90 μg of 7S IgA

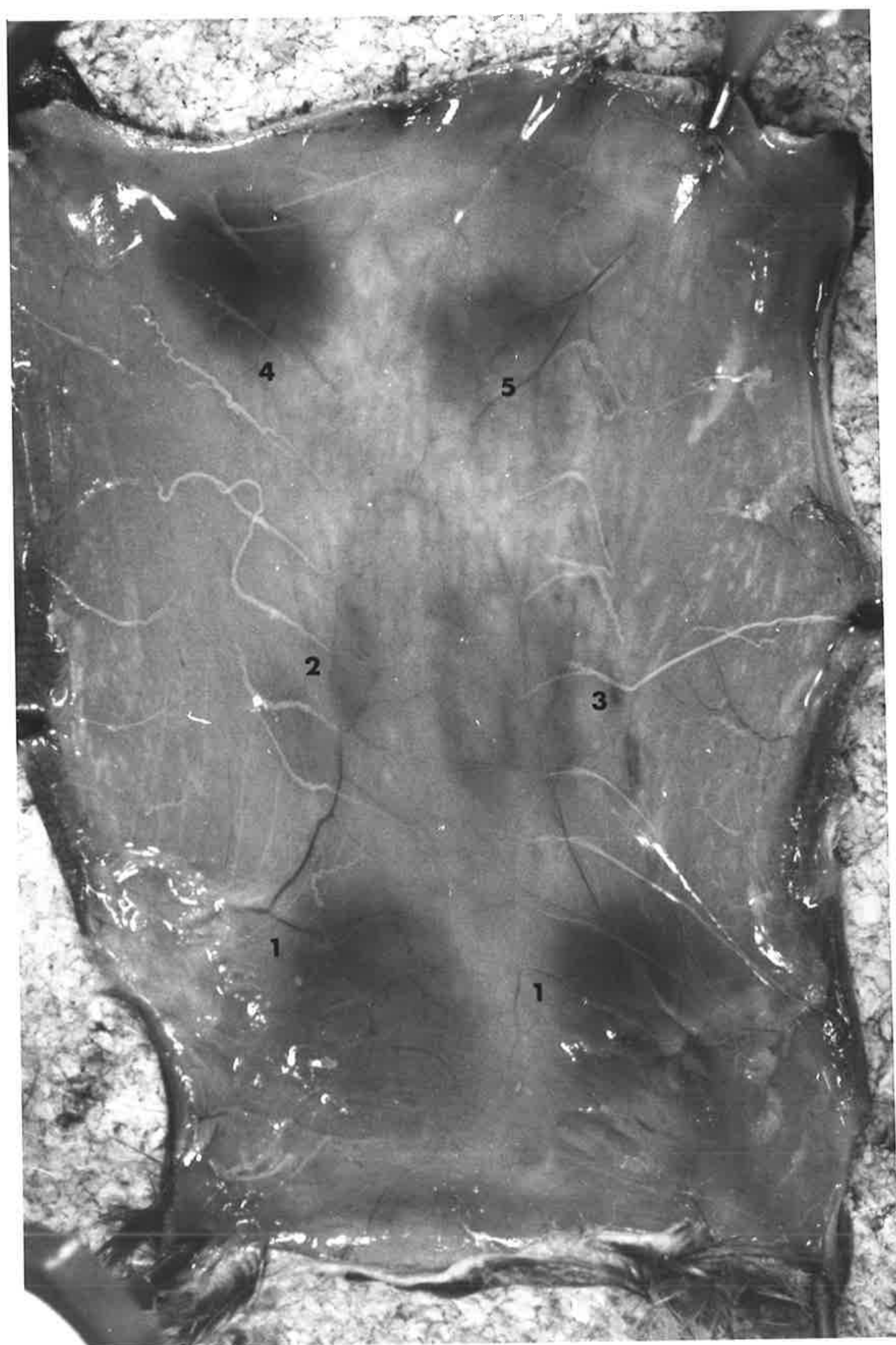

4
5
2
3
1
1

TABLE 5.3

Comparison of serum antibody levels with the intensity of the Arthus reaction elicited in individual tumour-bearing mice

	Antibody (mg/ml)				Ratio		Intensity of
Mouse	IgG_1	IgG_2	IgG_{1+2}	IgA	IgG_2:IgA	IgG_{1+2}:IgA	Arthus*
1	2.16	3.61	5.77	<0.05	-	-	9
2	1.69	3.11	4.80	<0.05	-	-	8
3	1.65	3.05	4.70	<0.05	-	-	7
4	1.29	2.95	4.24	<0.05	-	-	9
5	1.68	2.81	4.49	<0.05	-	-	8
6	1.84	2.58	4.42	<0.05	-	-	5
7	1.99	2.18	4.17	<0.05	-	-	9
8	1.38	2.05	3.43	<0.05	-	-	9
9	1.80	2.02	3.82	<0.05	-	-	5
10	0.95	1.92	2.87	<0.05	-	-	8
11	0.49	1.68	2.17	<0.05	-	-	7
12	1.66	1.64	3.30	<0.05	-	-	8
13	1.04	1.38	2.42	<0.05	-	-	7
14	0.51	0.79	1.30	0.22	3.59	5.9	5
15	0.24	1.03	1.27	0.52	1.90	2.4	1.5
16	0.91	1.61	2.52	0.53	3.05	4.75	3
17	0.24	1.72	1.96	0.60	2.78	3.26	3
18	0.26	1.11	1.37	0.79	1.28	1.73	2
19	0.97	1.23	2.20	0.93	1.37	2.45	2
20	0.97	1.63	2.60	1.00	1.63	2.6	2
21	1.05	1.22	2.27	1.33	0.93	1.53	1.5
22	0.35	1.36	1.71	2.48	0.55	0.69	1.5

Mice immunized with DNP-*E.coli* and injected with MOPC-315 were challenged intradermally with 10 μg of TNP-KLH (3 sites) and 10 μg of KLH (3 sites). After 5 hours the mice were bled, killed and the skin of the back was reflexed. The intensities of the reactions observed were summed. The serum obtained from each mouse was assayed for the immunoglobulin content of specific anti-DNP antibodies. The levels of IgG_{2a} and IgG_{2b}, determined separately, were summed and are shown as IgG_2. At no stage was a reaction observed against KLH. These results have been plotted in Figs. 5.4 and 5.5

* Representative reaction intensities are shown in Photo 5.2.

FIGURE 5.4

Comparison of serum antibody levels with the intensity of the Arthus reaction elicited in individual tumour-bearing mice

Mice immunized with DNP-*E.coli* and injected with MOPC-315 were challenged intradermally with 10 µg of TNP-KLH (3 sites) and 10 µg of KLH (3 sites). After 5 hours the mice were bled, killed and the skin of the back reflexed. The intensities of the reactions observed were summed. The serum obtained from each mouse was assayed for the immunoglobulin content of specific anti-DNP antibodies. Data from Table 5.3. Representative reaction intensities are shown in Photo 5.2.

a) Comparison of DNP-specific serum IgA and the reaction intensity. Correlation coefficient = -0.8521 ($P < 0.005$).

b) Comparison of DNP-specific serum IgG_1 and the reaction intensity. Correlation coefficient = 0.6521 ($P = 0.01$).

●, IgA $\geq$ 50 µg/ml o , IgA 50 µg/ml.

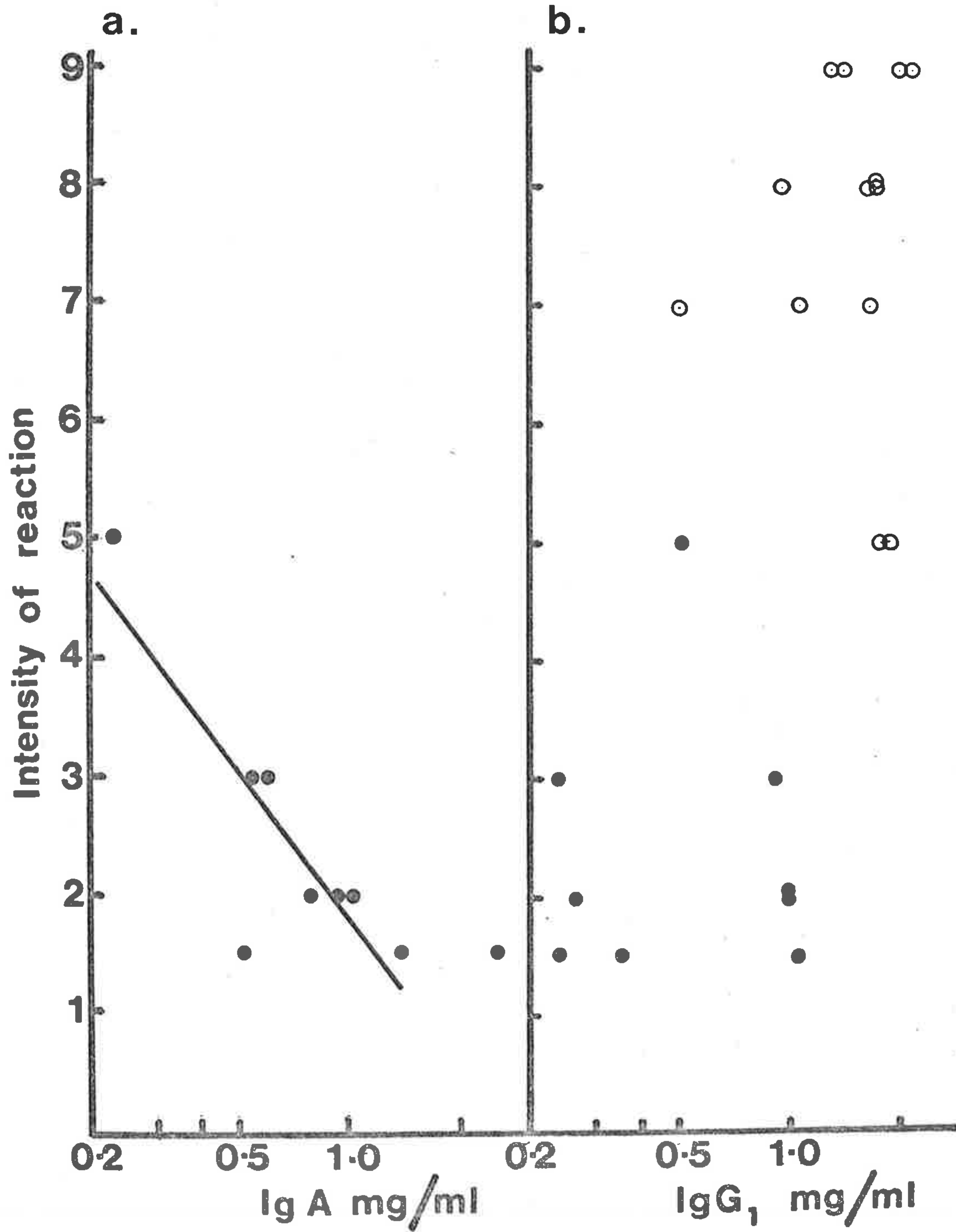
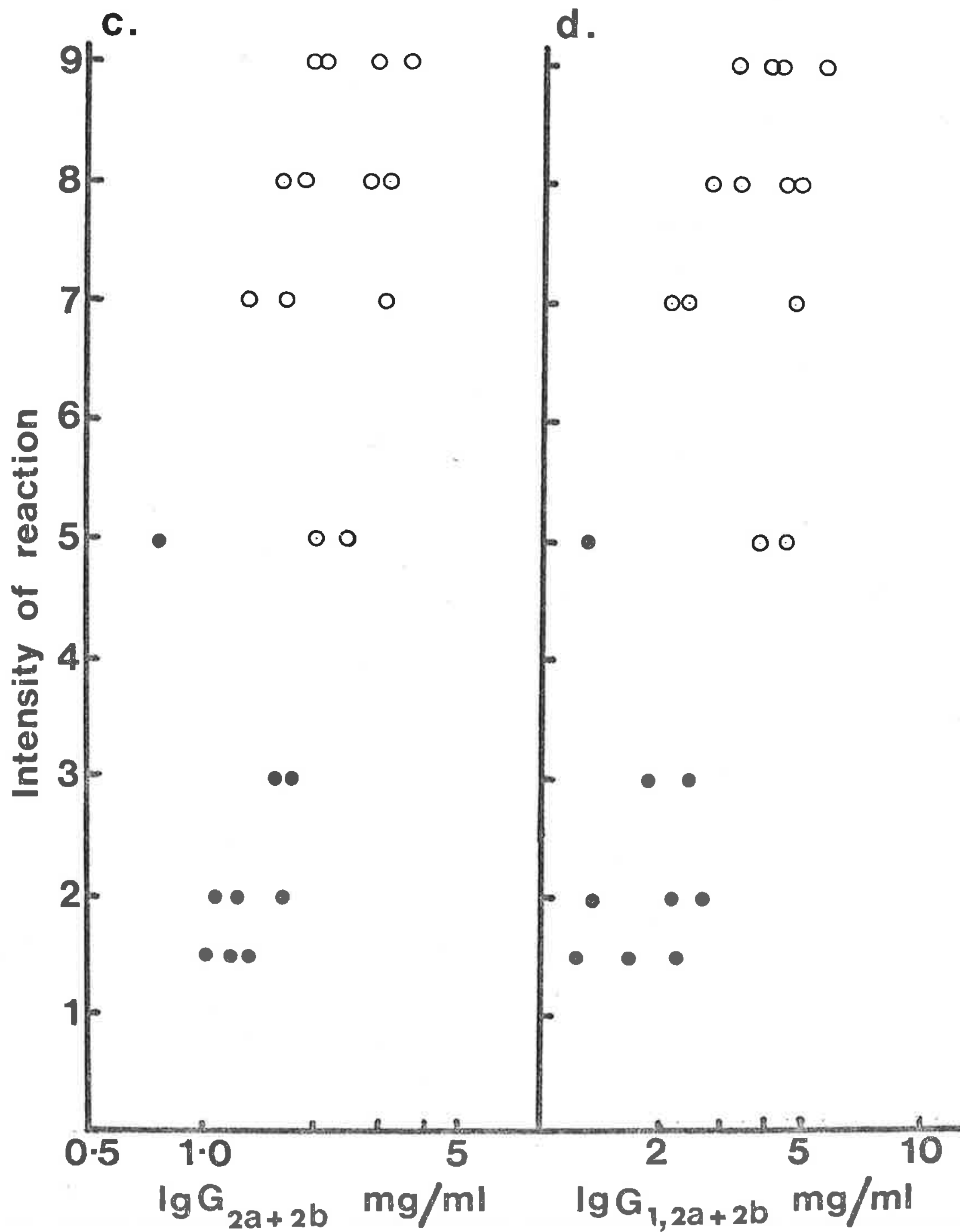

a.
b.
Intensity of reaction
9
8
7
6
5
4
3
2
1
0·2
0·5
1·0
Ig A mg/ml
0·2
0·5
1·0
IgG1 mg/ml

FIGURE 5.4 (continued)

c) Comparison of DNP-specific serum IgG_{2a+2b} and the reaction intensity. Correlation coefficient = 0.6170 ($P < 0.01$).

d) Comparison of DNP-specific serum $IgG_{1+2a+2b}$ and the reaction intensity. Correlation coefficient = 0.7059 ($P < 0.001$).

●, IgA $\geq$50 μg/ml o, IgA <50 μg/ml.



c.
d.
Intensity of reaction
9
8
7
6
5
4
3
2
1
0·5
1·0
5
2
5
10
IgG2a+2b mg/ml
IgG1,2a+2b mg/ml

eliciting antigen, showing that the reactions involved DNP/TNP-specific antibodies.

The intensity of the Arthus reactions elicited in tumour-bearing mice by TNP-KLH declined as the serum IgA antibody levels increased (Fig. 5.4a: $r = -0.856$; $P < 0.005$). The intensity of reaction showed a partial but significant correlation with the IgG_1, IgG_2 and IgG_{1+2} antibody levels ($r = 0.6521$, 0.6170, and 0.7059; $P = 0.01$, <0.01, <0.001 respectively)*. The overall level of IgG antibody in the serum decreased as the IgA antibody levels increased,

---

* As the size of the Arthus reaction is governed by the level of C-fixing IgG antibody, there should, in the absence of IgA antibodies, be a high correlation between the level of IgG antibody and the intensity of this reaction. Similarly, if IgA inhibits this reaction, a strong negative correlation should exist between the level of IgA antibody and the reaction intensity. The degree of correlation between reaction intensity and the level of one antibody will be affected, however, by the absolute level of the other. For these reasons, the effect of IgA upon the reaction intensity would be best determined from the IgG: IgA antibody ratios (e.g. Fig. 5.5). When this was done, a good correlation between this ratio and the reaction intensity was observed (IgG_2:IgA, $r = 0.7784$, $P < 0.02$; IgG_{1+2}:IgA, $r = 0.7849$; $P < 0.02$).

FIGURE 5.5

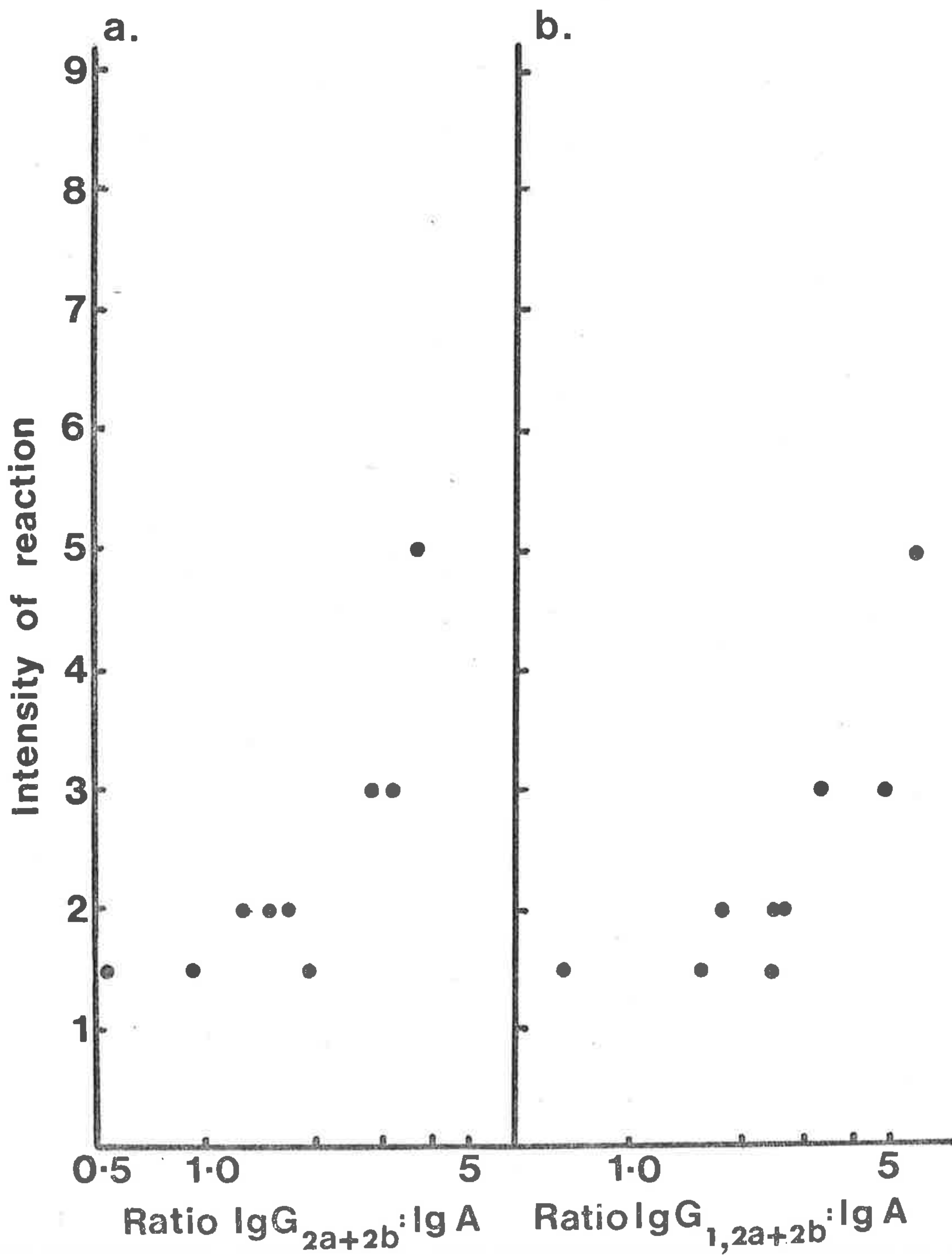
Comparison of DNP-specific serum IgG:IgA ratio and the intensity of the Arthus reaction

The weight ratio of serum IgG:IgA antibody in DNP-*E. coli* immunized mice (Table 5.3) plotted against the intensity* of the Arthus reactions observed in these mice 5 hours after challenge with three 10 μg doses of TNP-KLH.

a) Serum IgG_{2a+2b}:IgA
Correlation coefficient = 0.7784
($P < 0.02$).

b) Serum $IgG_{1+2a+2b}$:IgA
Correlation coefficient = 0.7849
($P < 0.02$).

* Refer to Photo 5.2.


a.
b.
Intensity of reaction
9
8
7
6
5
4
3
2
1
0·5
1·0
5
Ratio IgG_{2a+2b}:Ig A
1·0
5
Ratio $IgG_{1,2a+2b}$:Ig A

presumably because the IgA secreted by the tumour neutralized residual antigen and prevented further antigenic stimulation of the animals.

Significant reductions ($\geq$ 75%) in the intensity of the Arthus reaction was observed in tumour-bearing mice exhibiting levels of serum IgA antibody greater than one quarter of the IgG antibody levels.

5.4 Discussion

The data presented in this chapter demonstrate that serum IgA is highly efficient at blocking both the A.C.A. reaction and the C-mediated Arthus reaction. For example, mice possessing levels of serum (MOPC-315) IgA antibodies which were only one half that of IgG antibodies, showed significantly reduced (>50%) hypersensitivity reactions (A.C.A. and Arthus) upon antigen challenge. The blocking efficiencies of 7S and 13S IgA antibodies (when premixed with antigen) appeared to be similar.

The ability of IgA to block cutaneous anaphylaxis suggests that IgA antibodies, shown to increase in serum during the desensitization of allergic individuals (Stokes *et al.*, 1974; Deuschl *et al.*, 1977; Wilkie *et al.*, 1978), may be responsible for the observed loss of symptoms in these patients. This contrasts with the suggestion by these authors, that it is the IgG antibodies, which also increase during desensitization, which were predominantly responsible

for the loss of symptoms. The results presented above suggest that IgA antibodies may in fact be more important in desensitization than has been previously thought. Stimulation of IgA antibodies for desensitization would seem to be preferable to the stimulation of IgG antibodies for the following reasons:- Firstly, preferential secretion of IgA immunoglobulin (in the form of secretory IgA) normally occurs in the area in which these allergic reactions are initiated. Secondly, the synthesis of C-fixing IgG antibodies could result in the formation of C-fixing antigen-antibody complexes which would initiate Arthus reactions at the site of complex deposition. Stimulation of IgG reaginic antibodies (an IgG subclass as yet to be defined in man; IgG_1, in the mouse; Spiegelberg, 1974), on the other hand, would result in local A.C.A. reactions at the site of exposure to antigen.

While the data presented in this chapter do not provide a definitive explanation as to how IgA blocks the A.C.A. reaction, the opinions expressed in current literature are that blocking antibodies, induced during desensitization, function by neutralizing antigen before it can bind to cell-bound reaginic antibody. The subsequent release of histamine and various other mediators of inflammation would thereby be prevented (Turk *et al.*, 1970; Stanworth & Smith, 1973; Deuschl *et al.*, 1977; Giessen *et al.*, 1976).

The possibility that serum IgA might effectively block the Arthus reaction was infered from the results of experiments

described in Chapter 4. In the latter study, IgA was found to interfere with the ability of IgG antibodies to form C-fixing complexes with antigen. It appears likely that the blockage of the Arthus reaction by IgA is brought about by a similar mechanism. Thus, IgA antibodies present in the circulation would inhibit the formation of C-fixing complexes between IgG antibodies and antigen. In areas such as the secretory surfaces of the upper respiratory tract, where the level of complement is much lower than in serum (Newhouse *et al.*, 1976; Gross *et al.*, 1978; Robertson *et al.*, 1976; Kaltreider, 1976), the potential for IgA to inhibit Arthus reactions would be greatly enhanced (see Chapter 4).

5.5 Summary

MOPC-315 IgA antibodies present in the circulation or complexed with antigen, were examined for their capacity to inhibit active cutaneous anaphylaxis (A.C.A.) and Arthus reactions, normally elicited when the antigen was injected into sensitized mice. Low levels of 7S or 13S IgA mixed with antigen were able to prevent the expression of A.C.A. to the antigen TNP-KLH. Much higher levels (50-100x) of IgA (mixed with antigen) were required to inhibit the Arthus reaction to this antigen. Both the A.C.A. and Arthus reactions were markedly reduced in sensitized mice possessing detectable (>0.05 mg/ml) levels of circulating IgA antibody.

CHAPTER 6

Inhibition of polymorphonuclear leukocyte chemotaxis by IgA

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6.4 Summary

CHAPTER 6

Inhibition of polymorphonuclear leukocyte chemotaxis by IgA

6.1 Preamble

Chemotaxis, the directed movement of cells induced by a number of substances (chemoattractants or chemotaxins), is an important constituent of the inflammatory reaction, as it is the mechanism whereby phagocytic cells, such as polymorphonuclear (PMN) leukocytes and monocytes are attracted to areas of tissue damage or microbial invasion. Chemotaxis of PMN cells can be stimulated by a variety of factors including, the complement components C3a, C5a and the complex $C\overline{567}$, casein, plasminogen activator, substances present in bacterial culture filtrates and by factors released from activated leukocytes (Jensen & Esquenazi, 1975; Kaplan *et al.*, 1973; Keller & Sorkin, 1967; Ward *et al.*, 1968; Tack *et al.*, 1974; Becker, 1977). Stimulation of PMN chemotaxis by these chemotaxins apparently involves the binding of the chemoattractant to specific receptors on the cell surface (Showell *et al.*, 1976).

A number of clinical conditions exist which are associated with defective leukocyte chemotaxis (Quie & Kates, 1977). The defect seems to lie either in some cellular dysfunction, which inhibits cell movement, or from the presence of a factor in the serum of such patients which inhibits the movement of normal leukocytes. A number of individuals,

whose sera contain inhibitors of chemotaxis have been shown to possess markedly elevated levels of serum IgA, often associated with IgA-secreting myelomas (Van Epps *et al.*, 1974; Van Epps & Williams, 1976; Davis *et al.*, 1977; Quie & Kates, 1977). The observation by Lawrence *et al.*, (1975), that PMN cells have surface receptors for polymeric IgA, led Van Epps & Williams (1976) (on the basis of their own chemotaxis experiments) to postulate that IgA was able to block leukocyte chemotaxis by binding to the cells and sterically inhibiting the attachment of chemotaxins to their receptors. The latter authors examined the effect of human myeloma or colostral IgA on the chemotaxis of PMN cells to C-derived chemotactic factors and other chemoattractants, such as bacterial filtrate and casein. They did not examine whether the presence of IgA in antigen-antibody complexes would affect the capacity of complexes to stimulate chemotaxis.

In the preceding chapter, it was found that MOPC-315 IgA could inhibit the development of Arthus reactions which are believed to follow cellular chemotaxis to sites of antigen-antibody complex deposition. The results of Chapter 4 suggested, furthermore, that this phenomenon might result from the inhibitory effect (of IgA) on C-activation by antigen-antibody complexes. For these reasons, the ability of IgA to inhibit the generation of PMN leukocyte chemoattractants by antigen-antibody complexes was assessed. IgA was also incubated with PMN cells in an attempt to inhibit their chemotaxis to bacterial culture fluids and C-derived chemoattractants.

6.2 Results

6.2.1 Effect of complement depletion on chemotaxis

The chemotaxis of PMN cells initiated by antigen-antibody complexes and bacterial culture fluid has been reported to require the presence of serum (Stecher & Sorkin, 1969; Ward *et al.*, 1965; Boyden, 1961). Antigen-antibody complexes generate heat-stable chemotaxins from heat-labile complement components. Bacterial culture fluids, on the other hand, promote chemotaxis even in the presence of heat inactivated serum. In order to show that the chemotaxis of PMN leukocytes, observed in the following experiments, was dependent upon similar factors, chemotaxis was assessed in the presence of normal rabbit serum (NRS), heat inactivated serum (HI NRS) and rabbit serum depleted of Cl_q (NRS-Cl_q) (Table 6.1; Photo 6.I). The generation of chemotactic factors by TNP-KLH or complexes formed between TNP-KLH and antibody depended upon the presence of a functionally intact C pathway, since heat inactivation or Cl_q depletion of the serum markedly reduced the amount of chemotaxis observed. When the samples were heat inactivated after the generation of chemotaxins, however, chemotaxis appeared to be unaffected. Removal of Cl_q from the serum had little effect on chemotaxis to TNP-KLH alone, suggesting that this antigen activated C by the alternate pathway. The chemotaxins elicited by *E. coli* appeared to be heat stable, as no loss of chemotactic activity occurred when heated rabbit serum was used. In fact, enhanced chemotaxis (1½-2x) was seen upon heating, due perhaps to the presence of thermolabile inhibitors of this chemotaxin in serum.

TABLE 6.1

The importance of complement for chemotaxis

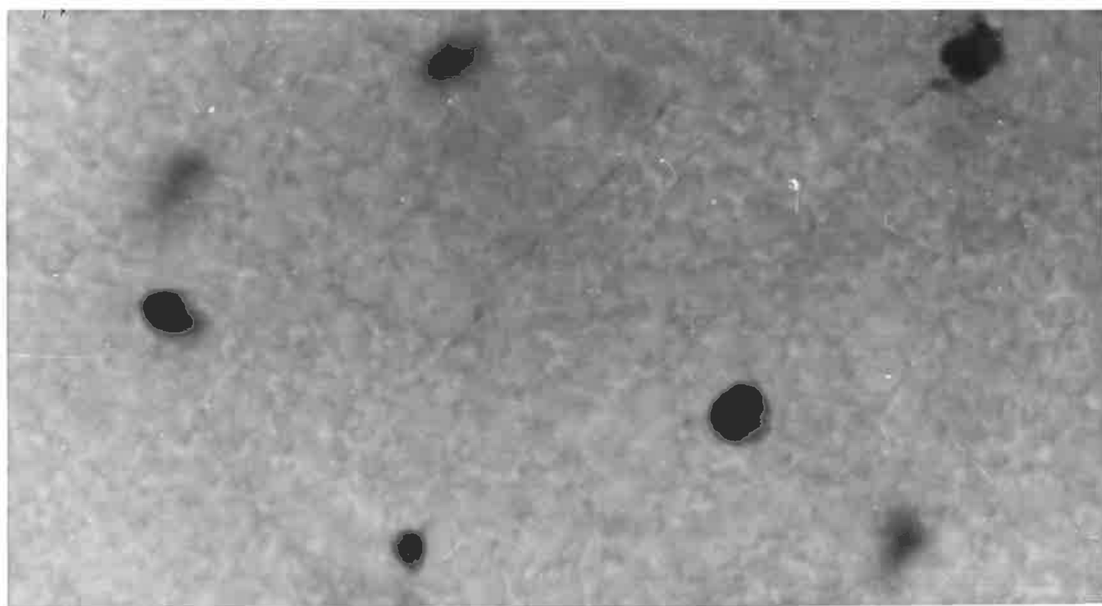
Activating agent	Number of cells/filter (10 fields)			
	NRS	HI-NRS	NRS-HI	Cl_q¯NRS
IgG.TNP-KLH	1,033	112	983	380
IgA.TNP-KLH	480	66	520	410
IgG.IgA.TNP-KLH	424	84	480	385
TNP-KLH	472	70	537	360
E. coli	2,450	4,215	3,520	N/D
saline	40	22	18	N/D

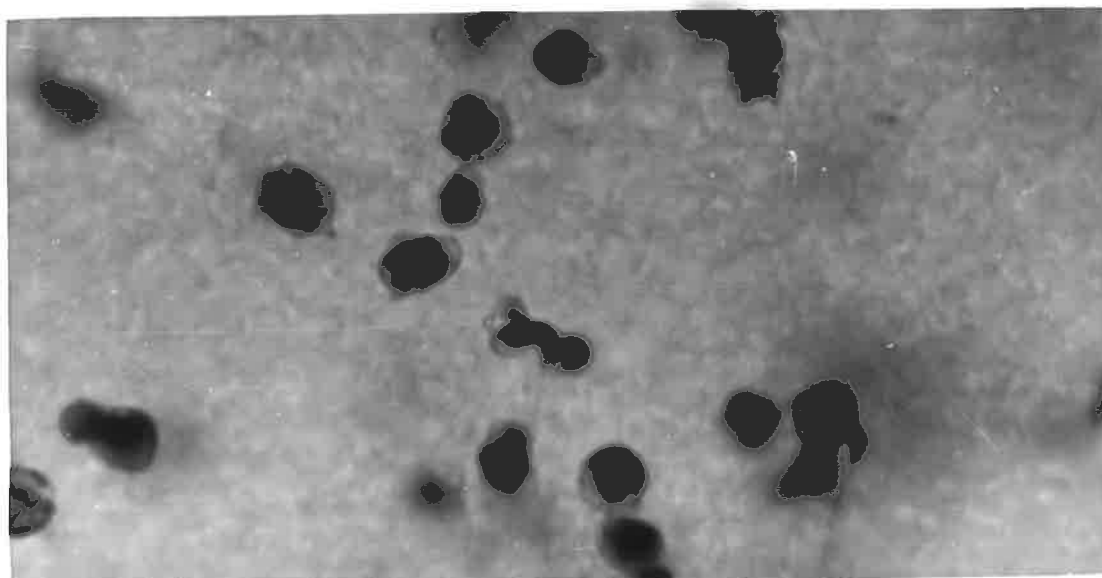
Movement of rabbit PMN leukocytes ($1x10^6$) towards chemotaxins generated by different substances in the presence of normal rabbit serum (NRS), heat inactivated serum (HI-NRS), Cl_q-depleted serum (Cl_q¯NRS) during a 1 hr incubation at 37°. The effect of heat-inactivating samples (30 min, 56°) after this 1 hr incubation was also examined (NRS-HI). Serum was depleted of Cl_q by passage down a rabbit IgG-Sepharose column (10 mM EDTA, 22°; KOLB *et al.*, 1979). The effluent serum was dialysed against saline overnight before use. The activating agents were used in the following amounts: Rabbit anti-DNP IgG, 214 µg; 13S MOPC-315 IgA, 925 µg; TNP-KLH, 100 µg; *E. coli*, 200 µl O/N culture. Serum, treated as indicated, was included in the medium on each side of the filter. The total number of PMN cells which had migrated completely through the filter was determined from 10 high-power fields (400x). Each value represents the average for 3 filters.

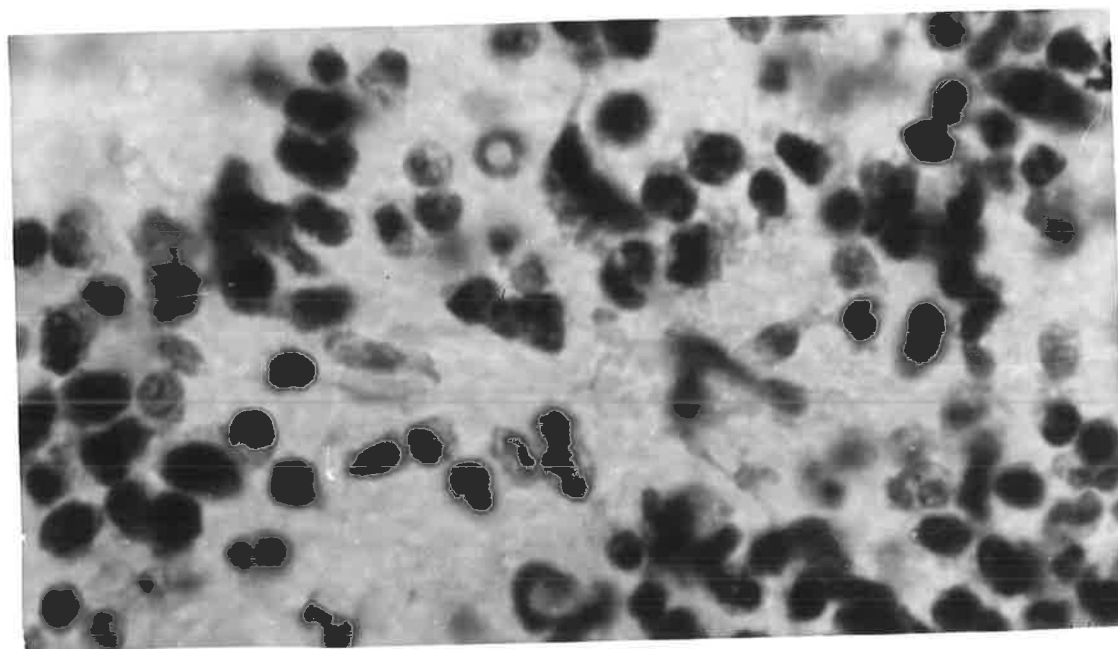
PHOTO 6.1

Chemotaxis of polymorphonuclear leukocytes to different substances

Movement of rabbit PMN leukocytes towards the following substances:- top: saline (200 µl); middle: TNP-KLH.IgG complexes (100 µg of TNP-KLH; 214 µg of anti-DNP IgG); bottom: *E. coli* (200 µl of O/N culture). Each substance had previously been incubated with normal rabbit serum (60 min, 37°; see Table 6.1). Chemotaxis was assessed after 2 hr of incubation (37°). Photo represents 1 high power field (400x).







6.2.2 The effect of MOPC-315 IgA on chemotaxis

a) Chemotaxis to antigen-antibody complexes containing IgA

Antigen-antibody complexes, formed between TNP-KLH, anti-DNP IgG antibody and various amounts of 13S MOPC-315 IgA, were tested for their ability to stimulate chemotaxis in the presence of normal rabbit serum. The results, presented in Table 6.2, indicated that chemotaxis to complexes formed with a 1.5 molar excess of IgA over IgG was markedly reduced compared to that observed using complexes containing no IgA. A proportional decrease in chemotaxis was observed as the amount of IgA present during the formation of the complexes was increased. A similar decrease in complement consumption was found using antigen-antibody complexes formed in the presence of IgA (see Section 4.3.2(a)). In contrast, chemotaxis to *E. coli* did not appear to be affected even at very high levels of IgA (3,700 μg).

It appeared therefore that the reduction of chemotaxis to TNP-KLH.IgG complexes formed in the presence of IgA was most probably due to a reduction in the capacity of the complexes to fix C. However, in view of the findings of Van Epps and Williams (1976; mentioned earlier), it was decided to examine whether the inhibition of chemotaxis might be the result of a direct interaction of IgA with the cells.

TABLE 6.2

Chemotaxis to antigen-antibody complexes containing IgA

Antibody added to antigen to form complex (μg)		Number of cells/filter (10 fields) migrating towards		
IgG	IgA	Antigen-antibody complexes		*E. coli*
		Expt. 1	Expt. 2	
-	-	28	132	554
214	-	159	512	-
-	3,700	-	-	501
-	925	87	147	-
214	925	80	149	-
214	463	91	211	-
214	231	102	342	-
214	116	136	354	-
214	58	-	476	-

Chemotaxis of PMN cells to TNP-KLH or complexes formed between TNP-KLH and antibody (rabbit anti-DNP IgG, 13S MOPC-315 IgA or mixtures of the two; O/N, 37°). As a control, IgA was also incubated with *E. coli* (60 min, 37°) prior to addition to chemotaxis chambers. Chemotaxis was allowed to proceed for 2 hr at 37° before the filters were removed, fixed and stained. The total number of PMN cells which had migrated completely through the filter were determined from 10 high power fields (400x). Each value represents the average of 3 filters. The number of cells added to the upper chamber was 5×10^5 (Expt. 1) and 1.2×10^6 (Expt. 2).

b) Chemotaxis of polymorphonuclear leukocytes preincubated with IgA

Preincubation of PMN cells with 13S IgA caused a marked reduction in the chemotaxis stimulated by TNP-KLH.IgG complexes (Table 6.3). The inhibitory effect was proportional to the amount of IgA added. For example, the number of cells which had migrated through to the lower side of the filter was reduced by 60% using 325 μg of IgA, and by 90% using 3.7 mg. The inhibitory effect was shown to be non-specific, as the chemotaxis to an unrelated chemoattractant (*E. coli*), was also reduced (by 54% using cells preincubated with 3.7 mg of MOPC-315 IgA).

Van Epps & Williams (1976) reported that the ability of cells to respond to chemotactic stimuli was partially regained (>40% recovery within 2.5 hours) when, after incubation with IgA, they were washed and incubated in medium. In view of these findings, it was considered that in the experiment of Table 6.3, some of the inhibitory activity of the IgA may have been lost (through the effects of dilution) during the period of assay (2.5 hr). To investigate this possibility, cells incubated with either 13S or 7S MOPC-315 IgA were compared for their ability to migrate towards a chemoattractant during a shorter period of incubation (60 min). As very few cells would have migrated through the filter within this time, cell movement was evaluated by measuring the distance into the filters which the leading cells had moved (Wilkinson, 1977; Kemp *et al.*, 1979). Both 7S and 13S IgA were found to inhibit

TABLE 6.3

Chemotaxis of polymorphonuclear leukocytes preincubated with IgA

Amount of IgA incubated with cells (μg)	Number of cells/filter (10 fields) migrating towards		
	Antigen	Complex	*E. coli*
-	1,980	4,042	4,117
3,700*	-	360	-
3,700	-	468	1,928
1,850	-	986	-
930	-	1,196	-
465	-	1,495	-
235	-	1,544	-

Cells ($1x10^6$) were preincubated with the indicated amounts of 13S MOPC-315 IgA (60 min, 22°) before being mixed with medium and added (with the IgA) to the upper chambers. Antigen (100 μg of TNP-KLH), antigen-antibody complexes (100 μg of TNP-KLH + 214 μg anti-DNP IgG, incubated O/N, 37°) or *E. coli* (200 μl of O/N culture) was added to the lower compartment. Chemotaxis was allowed to proceed for 2.5 hr at 37°. The total number of PMN cells which had migrated completely through the filter were determined from 10 high power fields (400x). Each value represents the average of 3 filters.

* After incubation of the cells with IgA, they were washed three times before addition to the chemotaxis chambers.

cell movement towards the chemoattractants generated by the antigen-antibody complexes (Fig. 6.1). For instance, movement was reduced by 90% when 250 μg (1250 μg/ml) and 60 μg (300 μg/ml) of 7S and 13S IgA respectively, were incubated with the cells, prior to their addition to the chemotaxis chamber. Polymeric (13S) was significantly more efficient in inhibiting chemotaxis (4 x w/w or 12 x mole/mole) compared to 7S IgA. As little as 11 μg of 13S or 44 μg of 7S IgA was capable of reducing chemotaxis by 50%. These results are similar to those of Kemp *et al.,* (1979) who found that approximately 30 μg of aggregated IgG and 600 μg of 7S IgG was required to achieve a 50% reduction in chemotaxis towards 10% normal serum. Van Epps & Williams (1976), however, observed little correlation between the level of serum IgA macroglobulin and the degree of inhibition of chemotaxis.

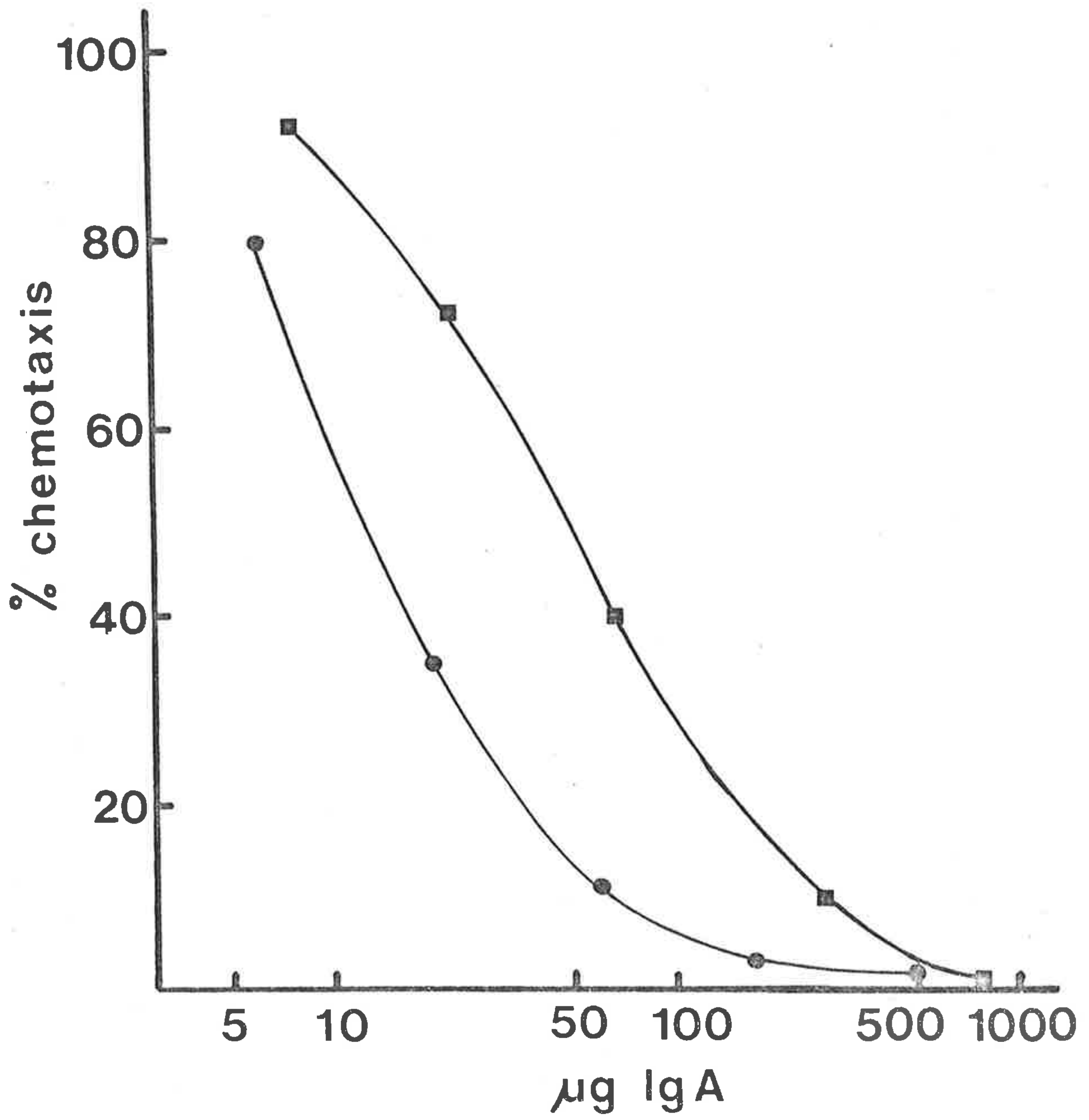
The observation that IgA could partially inhibit chemotaxis to bacterial culture fluid (Table 6.3) suggested that cell-associated IgA could sterically inhibit the binding of the relevant chemoattractants to the cell surface. In this respect it could be expected that 13S IgA (due to its larger bulk) would be a more effective inhibitor of chemotaxis than 7S IgA. This was indeed the case, as shown by the data (previously discussed) of Fig. 6.1.

When the results of the experiments described in Table 6.3 and Figure 6.1 were compared, it appeared that IgA may be slowly dissociating from the PMN cells. For instance, with

FIGURE 6.1

Inhibition of PMN leukocyte chemotaxis by cell-associated IgA

Dilutions of IgA (7S or 13S; 200 µl) were mixed with 1×10^6 packed PMN cells and incubated (60 min, 22°) prior to introduction into chemotaxis chambers. Chemotaxis to TNP-KLH IgG complexes (100 µg of TNP-KLH, 214 µg of anti-DNP IgG) was allowed to proceed for 60 min (37°) after which the filters were removed and stained. The distance which the cells had moved into the filter was then determined. The results (an average for 3 filters) are expressed as a percentage of the movement elicited in the absence of IgA. 13S IgA, ●——●; 7S IgA, ■——■ .



100
80
60
40
20
% chemotaxis
5
10
50
100
500
1000
μg IgA

the shorter chemotaxis assay (60 min; Fig. 6.1), 130 μg of 13S IgA reduced chemotaxis to antigen-antibody complexes by 95%, whereas in the longer assay (Table 6.3; 2.5 hrs), 3,200 μg was required to reduce chemotaxis by 90%.

The results obtained by premixing cells with IgA (Section 6.2.2(b)) suggested that at least some of the inhibition of chemotaxis (to antigen-antibody complexes) observed in Section 6.2.2(a) may have been due to the direct interaction of IgA with the polymorphs.

6.3 Discussion

There are three possible mechanisms by which chemotaxis may be inhibited. These involve the regulation of chemotaxin formation, the inactivation of these factors, or effects on the cells which cause them to become refractive to chemotactic stimuli. IgA could conceivably inhibit chemotaxis by two of these mechanisms. For example, the ability of IgA antibodies to reduce the activation of C by antigen-antibody complexes (Chapter 4) may enable it to inhibit the generation of C-derived chemotactic factors. It has also been suggested by Van Epps & Williams (1976) that cell-bound polymeric IgA may inhibit the binding of chemotaxins to receptors on the cell surface. In this study, mouse MOPC-315 IgA was examined for its ability to inhibit the chemotaxis of rabbit PMN leukocytes to antigen-antibody complexes, either when present in the complexes or when preincubated with the cells.

The results presented in this chapter are consistent with the findings of Chapters 4 and 5, which showed that IgA could inhibit complement consumption by antigen-antibody complexes, and that serum IgA was capable of reducing the intensity of the Arthus reaction elicited in sensitized mice bearing the MOPC-315 plasmacytoma. The inhibition observed when IgA was incubated with the chemoattractants was antigen-specific, as the addition of high levels of IgA to *E. coli* culture fluid did not inhibit the chemotaxis elicited by this chemoattractant.

When the cells were preincubated with IgA in the absence of antigen, however, chemotaxis was non-specifically inhibited. Chemotaxis towards antigen-antibody complexes was more effectively reduced than that stimulated by *E. coli* chemotaxins. These findings are similar to those of a number of workers who have reported chemotaxis to be reduced in the presence of serum from patients with high levels of polymeric IgA (Van Epps & Williams, 1976; Davis *et al.*, 1977; Van Epps *et al.*, 1974). In contrast to the findings of Van Epps & Williams (1976), however, 7S (monomeric) IgA was also found to be effective in inhibiting chemotaxis. Van Epps & Williams (1976) postulated that inhibition was due to steric blockage of binding of the chemoattractant to the PMN elukocytes by cell-bound IgA. This would explain why the 13S IgA was a better inhibitor of chemotaxis than 7S IgA (12:1 mole/mole). The effect of IgA on rabbit PMN leukocytes appeared to be reversible, as was also shown by Van Epps & Williams (1976), using human leukocytes, since

a significant drop in its inhibitory action was noted during longer periods of incubation.

It is not clear whether blockage of chemotaxis by IgA has any functional significance. It is conceivable, however, that high concentrations of IgA antibody may reduce the severity of inflammatory reactions involving PMN cells in areas in which these reactions would not be beneficial. Such areas would include the subepithelial tissues associated with the gut and respiratory tract which are involved in absorption and secretion. Inflammatory reactions which could decrease the functional efficiency of these tissues, are potentially undesirable. This may account for the increased incidence of various pathological conditions (such as malabsorption and coeliac disease) frequently observed in IgA-deficient patients (Crabbé & Heremans, 1967; Bull & Tomasi, 1968; Ammann & Hong, 1971, Beale *et al.*, 1971; Scott *et al.*, 1977).

6.4 Summary

MOPC-315 IgA was examined for its ability to inhibit the generation of PMN leukocyte chemotaxins by antigen-antibody complexes which contained IgA, and also for its capacity to inhibit the chemotaxis of these cells towards *E. coli* and C-derived chemotaxins. In agreement with the results of chapter 4, IgA, when present during the formation of TNP-antibody complexes, was effective in specifically inhibiting the generation of C-derived chemotaxins by these complexes. Preincubation of PMN cells with 7S or 13S IgA resulted in a proportional decrease in chemotaxis of these cells to *E. coli* or to antigen-antibody complexes.

CHAPTER 7

DISCUSSION

Since the initial discovery of the IgA class of immunoglobulin in serum (βχ-globulin, Grabar & Williams, 1953), a vast amount of data has been accumulated concerning the structure, synthesis, distribution and secretion of this immunoglobulin in a number of vertebrate species. The finding that IgA is the predominant immunoglobulin in the secretions of many species (Vaerman & Heremans, 1970; 1972; Cebra & Robbins, 1966; Porter, 1969; Crabbé *et al.*, 1970; Tomasi & Grey, 1972) has stimulated further work aimed at elucidating the function(s) of this immunoglobulin in these secretions. As a result, secretory IgA has been shown to have an important role in the protection of secretory surfaces against bacterial and viral infections, and a possible role in preventing the uptake of antigen across these surfaces (see Section 1.3(b); Keller & Dwyer, 1968; Ogra *et al.*, 1968; Ganguly *et al.*, 1973; Fubara & Freter, 1973; Gibbons, 1974; Rogers & Synge, 1978). It is at present unclear, however, whether the non-secreted IgA present in lymph and serum has any functional significance within the tissues. Little attention seems to have been given to this possibility.

Since IgA is unable to elicit many of the reactions demonstrable with other immunoglobulin classes (e.g. complement

activation, cell lysis, opsonization), any role which it may fulfil within the tissues must be distinct from those of IgG, IgM or IgE. A number of experiments (see Section 1.6) have shown, however, that IgA antibodies can, under certain circumstances, block reactions (such as bacteriolysis, phagocytosis and hypersensitivity) elicited by other antibody classes (Hall *et al.*, 1971; Stokes *et al.*, 1974; Griffiss, 1975; Van Epps *et al.*, 1978; Wilton, 1978; Schumacher & Jeffery, 1979). It is therefore possible that the presence of IgA antibodies in the tissues may serve to inhibit or control the reactions of other antibody classes, especially at sites of high IgA concentrations, although, few workers have given this concept any serious consideration (Van Epps & Williams, 1976; Wilton, 1978).

In this thesis, IgA myeloma proteins having known antigen-binding specificity were examined for their capacity to inhibit various antibody-dependent reactions, with a view to assessing the possibility that IgA may function as a blocking antibody. Myeloma proteins, rather than natural antibody, were chosen for this study because of the inherent difficulties in eliciting and purifying adequate amounts of specific antibodies of the IgA class. It was anticipated that IgA isolated from the three mouse plasmacytomas, J539, S107 and MOPC-315, which secrete IgA proteins specific for β-D-galactoside, phosphorylcholine and nitrophenyl analogues respectively (Potter, 1972), could be used. However, due to problems in maintaining

the J539 and S107 tumours and difficulties in obtaining reagents necessary for the purification of their secreted IgA, MOPC-315 IgA was used in all experiments. This protein, which has a marked affinity for DNP and TNP (Dourmashkin *et al.*, 1971; Eisen *et al.*,1968; Green *et al.*, 1971; Potter, 1972), was purified on DNP-Sepharose affinity columns (Porter, 1950; Farah & Awdeh, 1972; Goetzl & Metzger, 1970; Loos & König, 1977) followed by chromatography on Protein A-Sepharose, anti-μ-chain-Sepharose and Sepharose 6B. This resulted in preparations which were more than 99% pure with respect to IgA.

The use of these IgA preparations, represents the first time that pure, monospecific IgA has been examined on a quantitative basis for its capacity to interfere with reactions elicited by other classes of antibody (c.f. Hall *et al.*, 1971; Griffiss, 1975).

The reactions in which the inhibitory capacity of IgA was studied are outlined below. Initially, MOPC-315 IgA was used to block lysis of IgG antibody sensitized TNP-SRBC. It was found that 7S IgA could effectively compete with anti-DNP IgG for antigen binding sites on the cell surface and prevent lysis of the cells by C. Although 13S IgA presumably can, at high concentrations, also act in this fashion, at much lower concentrations it was found to sterically inhibit the attachment of C1 to IgG duplets bound to the cell surface. These observations led to further experiments in which "fluid phase" IgG/IgA/TNP-KLH

complexes and complexes formed between IgA and the C-fixing antigen $DNP_{46}BSA$ were examined, using various levels of IgA, for their capacity to consume C. Once again MOPC-315 IgA was found to successfully compete with IgG for binding sites on the TNP-KLH molecules, thereby reducing the C-fixing activity of the antigen-antibody complexes formed in antibody excess. Both 7S and 13S IgA sterically inhibited C-fixation by $DNP_{46}BSA$ and IgG/TNP-KLH complexes formed in antigen excess. The blocking activities of MOPC-315 IgA outlined above was found to be antigen-specific, since J539-IgA (specific for β-D-galactoside) could not block lysis of anti-DNP IgG sensitized TNP-SRBC, while S107-IgA (specific for phosphorylcholine) failed to block C-activation by $DNP_{46}BSA$.

As a result of these experiments in which IgA was observed to block C-activation, MOPC-315 IgA was examined for its capacity to inhibit the C-mediated Arthus hypersensitivity reaction. A series of experiments designed to investigate the ability of MOPC-315 to block the active cutaneous anaphylaxis reaction were also carried out. Both types of reaction were reduced when the IgA was preincubated with the antigen before challenge or present within the mice. Blockage was antigen specific.

Because a number of workers (Van Epps *et al.*, 1974; Van Epps & Williams, 1976; Davis *et al.*, 1977; Quie & Kates, 1977) had shown that high levels of IgA, bound to human

PMN cells, could reduce the chemotaxis of these cells, it was thought that the blockage of the Arthus reaction by IgA might have been due, at least in part, to a similar mechanism. For this reason, MOPC-315 IgA was tested for its ability to block chemotaxis of rabbit leukocytes, either when incubated with the cells, or when present during the formation of antigen-antibody complexes. Both mechanisms were found to operate. The inhibitory effect of IgA on the chemotaxin-generating ability of antigen-antibody complexes was antigen-specific. However, when IgA was added to the cells chemotaxis, to both antigen-antibody complex generated chemotaxins and the unrelated *E.coli*-generated chemoattractants, was reduced.

There is evidence in the literature to suggest that IgA is able to block bacteriolysis, phagocytosis and PMN chemotaxis, although little is known about the mechanism(s) through which this blockage occurs. From the present work, however, two mechanisms appeared likely. The first involved direct competition between IgA and IgG for antigenic sites. The second entailed steric interference by IgA of either C-fixation (an antigen-specific effect) or of the binding of chemotactic factors to IgA-tested PMN cells (a non-specific effect).

Competition between IgA and IgG for antigen resulted in effective blockage of lysis of TNP-SRBC sensitized with IgG/7S IgA mixtures and in inhibition of C-activation by antigen-antibody complexes formed in antibody excess. The

extent of inhibition was proportional to the ratio of IgG:IgA. Similarly, IgA competed favourably with IgG antibodies to block A.C.A. reactions in sensitized animals injected with TNP-KLH. These results are comparable to the findings of a number of other workers. Ey *et al.*, (1980) found that non-C-fixing IgG_1 molecules could effectively compete with IgG_{2a} antibodies and block the C-dependent lysis of TNP-SRBC sensitized with these two antibody classes. The efficiency with which the IgG_1 molecules inhibited lysis depended upon the ratio of IgG_1:IgG_{2a} antibody and on the level of C used in the lytic assay. It is highly probable that such competitive inhibition of lysis by IgA also occurred when mixtures of IgA and IgM or IgA and IgG serum fractions (containing antibody), when added to bacteria, were found to inhibit lysis of meningococcus, salmonellae and brucellae by complement (Zinneman, 1964; Eddie *et al.*, 1971; Hall *et al.*, 1971; Griffiss, 1975).

The observation that low levels of serum IgA antibody (one half that of serum IgG_1 antibody) could specifically reduce (by >50%) the A.C.A. reaction suggests that stimulation of antibodies of the IgA class would be highly beneficial in the desensitization of allergic individuals. Furthermore, the levels of IgA antibody found in patients might provide a better measure of desensitization than can be obtained from the IgG antibody levels (Stokes *et al.*, 1974; Deuschl *et al.*, 1977; Wilkie *et al.*, 1978). Stimulation of IgA rather than IgG antibody during desensitization

is likely to be advantageous for the following reasons. Firstly, the induction of C-fixing IgG may lead to Arthus reactions upon subsequent contact with antigen. On the other hand, stimulation of the non-C-fixing IgG_4 subclass (in man) or the IgG_1 subclass (in mice) would result in A.C.A. type reactions as these antibodies are, like IgE, homocytotrophic for mast cells and basophils (Stanworth & Smith, 1973; Giessen *et al.*, 1976). Secondly, desensitization of mucosal surfaces might be effected through the preferential stimulation of secretory IgA antibodies since other classes of antibody are not actively secreted.

IgA, in particular 13S IgA, was found to inhibit a number of C-dependent reactions by sterically interfering with the attachment or activation of C. The number of IgG molecules required to effect lysis of TNP-SRBC was markedly increased (from 3,000 to 6,000 molecules per cell) by the addition of only 13,000 molecules of 13S IgA, in conditions where the TNP antigenic sites were not saturated. Similarly, low molar ratios of IgA to $DNP_{46}BSA$ (5:1) or IgA to IgG (0.1:1), were sufficient to significantly reduce (by >50%) the activation of C by $DNP_{46}BSA$ or IgG-antigen complexes (formed at antigen excess). It was concluded that the degree of C-activation was reduced, either by the formation of an IgA/DNP-BSA lattice by which potential C-fixing sites were occluded, or through the binding of 7S or 13S IgA molecules in close proximity to IgG duplets present on the TNP-KLH molecules.

The observation that chemotaxis of PMN leukocytes to two different chemoattractants (antigen-antibody complexes and *E.coli* could be significantly reduced by prior incubation of the cells with high levels of IgA (3.7 mg/ $1.5x10^6$ cells) is consistent with the concept presented in current literature, that polymeric IgA, bound to the surface of PMN cells, can sterically inhibit the attachment of a number of chemotaxins present on the cells (Lawrence *et al.*, 1975; Van Epps & Williams, 1976; Wilton, 1978). In contrast to the results obtained by these workers, however, preincubation of PMN cells with 7S MOPC-315 IgA also inhibited their chemotaxis to antigen-antibody complex-derived chemotaxins, although polymeric (13S) IgA was (on a molar basis) some 12 times more efficient. This may be due to the larger bulk of the 13S molecule or to an higher affinity of the PMN cells for polymeric IgA (Van Epps & Williams, 1976; Lawrence *et al.*, 1975; Wilton, 1978; Davis *et al.*, 1977).

The Arthus hypersensitivity reactions were inhibited at relatively low serum concentrations of MOPC-315 IgA (greater than half that of IgG). This was attributed to an inhibition of C-activation. These levels of IgA (> 0.50 mg/ml) may also have been sufficient to affect the interactions of chemoattractants with PMN leukocytes. It is noteworthy, however, that much higher levels of IgA (12-40 mg/ml of serum) were required by Van Epps & Williams (1976) to inhibit the movement of human PMN cells. Such mechanisms could account for the increased

incidence of recurrent infections observed in many patients with high levels of serum IgA (Van Epps & Williams, 1976; Davis *et al.*, 1977; Quie & Cates, 1977).

In conclusion, the findings of this thesis confirm the initial postulate that IgA antibodies have the potential to block antibody-mediated reactions, both *in vitro* and *in vivo*. Whether a sufficient level of IgA antibody exists in the various tissues of normal individuals for such inhibitory effects to occur is at present unknown. It is clear, however, that major perturbations in IgA concentrations can be correlated with increases in the incidence (in the case of IgA deficient subjects) of certain inflammatory reactions or (in patients with high serum IgA levels) in the frequency of recurrent infections (Ammann & Hong, 1971; Stokes *et al.*, 1974; Quie & Kates, 1977; Van Epps *et al.*, 1974, 1978; Van Epps & Williams, 1976; Davis *et al.*, 1977). It would therefore seem useful to re-examine a number of IgA-associated pathological conditions (such as IgA glomerulonephritis) with the aim of determining whether the IgA is eliciting or controlling the reactions responsible for these diseases. Furthermore, in order to treat these conditions it would seem desirable to determine ways of stimulating or inhibiting the production of the relevant IgA antibodies.

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