THE EXPRESSION OF ALTERNATIVE PATHWAY-MEDIATED HAEMOLYSIS BY SERA FROM SELECTED ANIMALS.

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The kinetics of alternative-pathway mediated haemolysis were studied in sera from a number of vertebrate species. Rabbit erythrocytes in a veranol buffer supplemented with magnesium chloride and ethylene glycol bis tetraacetic acid (EGTA) were used as a target for these assays. Human sera showed significant variations in the kinetics of the lytic reaction at dilutions above 1:2. A number of factors appeared to contribute to these variations and no single determinant of reaction kinetics could be identified. Sera from domesticated and feral animals showed little variation within any species, but marked differences were seen between the species studied. Badger, ferret and hedgehog exhibited potent alternative pathway activity while mouse serum showed very little. The results of kinetic assays of alternative pathway haemolytic activity and total haemolytic complement activity were compared with those of conventional, endpoint, assays. No classical pathway activity could be elicited from mouse serum. Inbred strains of mice showed significant differences in the kinetics of alternative pathway mediated haemolysis between the males and females of each strain examined, and between some of the strains examined. These differences did affect the spread of mycobacteria after subcutaneous and intravenous injection. Human sera with a defective ability to opsonise yeasts showed normal lytic activity in the kinetic assay. In contrast, sera from children and adults with obstructive jaundice which exhibit enhanced opsonic activity also showed accelerated reaction kinetics in the lytic assay.
To the Memory of Dorothy Peters, Louis Pillemer
and Rodney Porter.

"...Very often a solution turns on devising some means of quantifying phenomena or states that have hitherto been assessed in terms of "rather more," rather less," or "a lot of," ......... To quantify is not to be a scientist, but goodness, it does help......

Sir Peter Medawar, 1979
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1.1 THE MAMMALIAN IMMUNE SYSTEM

The immune system is a diverse collection of cells and secreted (or humoral) products but so far studies of innate and acquired differences in immune responsiveness have tended to concentrate on particular aspects of the immune system, notably lymphocyte responses (Dausset and Contu, 1980). The work to be described here seeks to broaden these studies by examining the complement system, particularly the alternative pathway of complement activation, using a novel application of an assay of complement activity.

Attempts to study individual components of the immune system, such as the alternative pathway, must take account of the possible influence of other mechanisms at all stages because the immune system contains such a diversity of processes. The host defences of each individual are a complex mosaic resulting from the interplay of genetic and environmental influences as well as the intricate interactions of the components themselves (Table 1-1).

Although tissue-based reactions are probably of greatest importance in immune responses, the ready availability of blood samples has made this the medium of choice for the study of the cellular and humoral components of the immune system, particularly in man, where ethical considerations severely limit the scope for the study of tissue samples. While the results of investigations performed on blood samples have proved invaluable, there are certain disadvantages to their use which will emerge
The cells of the immune system come originally from the haemopoietic (blood-forming) tissues of the body, and arise from a population of relatively undifferentiated cells known as stem cells. The stem cell pool is maintained by regular cell division. Hormones produced by other cells in the haemopoietic tissues act on the stem cells causing them to differentiate along a variety of developmental pathways (Nicola and Vadas, 1984; Figure 1-1).

In most cases, the cells released from the haemopoietic tissues are functionally mature, or nearly so. Thymus-derived or T lymphocytes however, complete their maturation in the thymus gland (Haynes, 1984), under the influence of hormones secreted by the epithelial cells of the thymus (Stites, Caldwell and Pavia, 1980). Thymus-derived lymphocytes are close to functional maturity when released into the bloodstream.

Each of the cell types depicted in Figure 1-1 is recognised by means of certain distinctive features. Originally the classification was made on the basis of their morphology and staining reactions (Bessis, 1973). These are now supplemented by biochemical, serological and functional criteria (Cawley and Burns, 1980; Klinman, 1981; Reinhertz and Schlossman, 1981; Taussig, 1984a).

Erythrocytes, platelets, and neutrophil polymorphs remain in the bloodstream in normal circumstances and approximately 50% of the neutrophil polymorphs in the bloodstream circulate freely. The remainder are intimately associated with the endothelial cells which
It is thought that these are the cells which move into the tissues when inflammatory reactions cause changes in capillary permeability. In transit to the tissues are the eosinophil polymorphs, basophil polymorphs and monocytes. Tissue basophils were named mast cells by early histologists. Monocytes which have entered the tissue spaces are called macrophages or histiocytes. Tissue eosinophils have not been given a distinctive title, possibly because they were rarely observed by histologists. It is generally accepted that macrophages differ in some respects from blood monocytes. Important distinctions have been described in their surface chemistry and in their functional capabilities (Simon, 1979; van Furth and Willemze, 1979). Macrophages appear to display further changes if they take up long-term residence in certain tissues and organs. Lymphocytes display patterns of migration which are quite different from those described above. They leave the bloodstream in specialised blood vessels known as post-capillary venules or high endothelial venules (Weissman et.al., 1978) found in lymph nodes and other lymphoid tissues. The capillaries in other tissues and organs adopt this morphology during chronic infections (Ford, 1983). Such changes are said to be associated with the movement of lymphocytes into inflamed regions. Lymphoid tissues are those in which large populations of lymphocytes are found, and they often display a high
Primary lymphoid tissues are the sites of lymphocyte development (Taussig, 1984a), in adult humans the bone marrow and thymus gland. Secondary lymphoid tissues are the areas in which the mature lymphocytes reside during intervals in their travels around the body. With the exception of the spleen they consist of little more than lymphocytes, macrophages, and antigen processing cells in a connective tissue framework. A variety of secondary lymphoid tissues have been recognised. They show various degrees of complexity in their organisation, ranging from the sub-mucosal aggregates of lymphocytes described above, to large regional lymph nodes. Many of the cellular responses associated with lymphocyte-mediated immunity occur in secondary lymphoid tissue.

Bone marrow derived (B) lymphocytes tend to remain in the secondary lymphoid tissues for long periods, while thymus derived (T) lymphocytes recirculate more regularly. This recirculation takes them from the lymph nodes to the lymphatic vessels, which lead back to the bloodstream. Thus even the furthest reaches of the lymphatic system are subjected to regular visitation by T lymphocytes, and the greater part (ca 70%) of the blood lymphocytes are found to be T lymphocytes (Taussig, 1984a).

The blood plasma is an aqueous solution of electrolytes, small organic molecules and proteins. Many of these proteins have transport functions, while others are involved in other aspects of homeostasis (Allison,
are produced by the liver. Two groups of proteins which do not fall completely within this classification are of particular interest, antibodies (immunoglobulins; Steward, 1984) and complement. Antibodies are secreted as a result of B lymphocyte activation during an immune response. The specificities of the antibodies found in the blood plasma generally reflect the body's recent antigenic experience. The complement proteins stand out as a powerful adjunct to antibody activity, and as a valuable defence system in their own right.

When an inflammatory episode causes changes in capillary permeability, neutrophil polymorphs move into the tissues. Plasma proteins move with them, forming an inflammatory exudate (Simon, 1979). Until recently it was generally accepted that small amounts of these proteins cross the walls of capillaries under normal circumstances (the process of transudation), providing a supply of important proteins in the extravascular spaces. The concentrations achieved in the tissue fluids by means of transudation have not been widely studied, but are said to be significantly less than those seen in blood plasma (Hay, 1979). The ratio of small to large proteins in lymph, is said to be higher than that found in the plasma, suggesting some form of filtration.

Local antibody production in the tissues is well documented however (Bienenstock and Befus, 1980), and all the antibody classes may be found in the tissues, regardless of their molecular weight. In addition,
macrophages secrete the complement components needed to enhance their functions, creating regions of alternative pathway activity around themselves. In this model, the complement proteins found in the blood plasma are secreted by the hepatocytes, and only enter the tissues when inflammatory reactions alter the capillary permeability. Such events would accentuate the irregular distribution of the complement components in the tissue spaces. The secretion of complement proteins by macrophages has been demonstrated in a series of carefully designed experiments, but the model developed from these results has not received universal acceptance (Ezekowitz, 1983).

1.2 PHAGOCYTOSIS AND THE IMMUNE SYSTEM

Neutrophil polymorphs and macrophages possess highly developed capacities for phagocytosis. They are able to ingest "foreign" materials as well as denatured "self" components. The ingested materials are subjected to the actions of microbicidal substances and digestive enzymes (Stossel, 1974; Bellanti, 1971). In most instances these will degrade them to soluble fragments which can be excreted or reutilised, as appropriate. These systems are not totally effective and a number of microorganisms can survive within the phagocytic cells (Edelson, 1982; Iglesias, 1984). They provide significant challenges to the success of the immune system, since phagocytic cells are the focus of most of the diverse mechanisms found in the immune system.
which are subsequently ingested will be considered in
due course, when the mechanisms by which the complement
system interacts with its activators are discussed.
Neutrophil polymorphs are short lived cells (Wilkinson,
1983). When confronted with "foreign" material, they
are as likely to discharge the contents of their
cytoplasmic granules into their environment as to
indulge in phagocytosis (Figure 1-2).
There has been an extended debate on the extent to which
cells of the monocyte-macrophage lineage maintain their
numbers by cell division in the periphery (van der Meer,
1980). The difficulties of experimentation in this
field have hampered the resolution of this problem. The
microbicidal and digestive systems found in these cells
have not been studied to the same extent as those of the
neutrophil polymorphs in man, but work done in other
species indicates that they possess a similar range of
activities to the neutrophil polymorph, but that the
individual systems are generally less potent in the
monocyte-macrophage line (Stossel, 1974).
Phagocytic cells are a feature of the host defences of
most animals and their functions are undoubtedly crucial
to the control of infectious disease (van Furth and
Willemze, 1979). In man, numerous diseases have been
associated with failure of neutrophil polymorph
production (Horwitz, 1982). In every case the
deficiency has been associated with a marked
susceptibility to infection. Inborn errors of
metabolism which are manifested in neutrophil polymorphs
example a deficiency of the enzyme NADPH oxidase prevents the production of adequate amounts of hydrogen peroxide which is necessary for the oxidative killing of bacteria (Figure 1-2). Few innate deficiencies of the monocyte-macrophage system have been reported, but the vital role of these cells in host responses to infectious diseases is well documented (Rosenthal, 1980).

Innate deficiencies of lymphocytes, such as the Di George syndrome and the Severe Combined Immuno-deficiencies (Hayward, 1977) are associated with recurrent, life-threatening infections which begin soon after birth. These observations suggest that phagocytes alone cannot provide adequate protection against infection in warm-blooded animals. The evolution of lymphocytes has contributed a variety of mechanisms which help to contain the threat from pathogens and their toxic products, as well as others which attract phagocytes to the areas of lymphocyte activation, and also enhance the metabolic activity of the recruited cells (Table 1-1).

The activation of B and T lymphocyte clones requires the concomitant activation of helper (or inducer) T lymphocyte populations. An additional requirement is that antigenic determinants must be presented to the lymphocytes in an organised array, associated with certain of the cell surface proteins coded by genes in the major histocompatibility complex (Owen and Crumpton, 1980), on the surface of so-called accessory cells.
Macrophages can perform this function in-vitro, and were once thought to do so in-vivo (Rosenthal, 1980). Recent work has attributed in-vivo accessory cell function to a number of specialised cells known as antigen processing cells (Chestnut and Grey, 1984).

The cellular and humoral defence mechanisms described so far, are those which have clearly defined roles in immunological reactions in-vitro and in-vivo. Current research literature contains numerous reports of other mechanisms as well as novel interactions of the systems already described. All have been demonstrated with relative ease in-vitro. For some their relevance to host defences is emerging as suitable technology becomes available, for others this exercise has proved more difficult and it is not realistic to make assumptions about their physiological significance at present. Table 1-1 includes two important examples of such mechanisms, the K cell phenomenon (Adams, Lewis and Johnson, 1983) and the natural killer cell (Koren and Herberman, 1983; Klein, 1983).

The serious consequences of lymphocyte deficiencies described earlier confirm that these benefits are regularly employed in normal individuals. Nevertheless, they cannot be used effectively in the absence of phagocytes, which remove much of the debris produced during lymphocyte responses. Furthermore, the products of these responses cannot be disseminated through the body fluids and tissues in effective amounts in the first few days of an infection which has not been previously encountered. It is therefore important to
available to deal with an infection before the products of lymphocyte activation are deployed effectively. The alternative pathway of complement activation is one such mechanism, arguably one of the most important (Fearon and Austen, 1980).

The overt consequences of complement activation by either pathway include the recruitment of neutrophil polymorphs (and possibly macrophages) to the site of activation (vascular permeability and chemotaxis) and enhanced interaction between the complement-coated activator and these phagocytes (opsonisation). Finally the disruption of the plasma membrane of some cellular targets (lysis, one form of cytotoxicity, Table 1-1) is also seen.

Activation of the alternative pathway can therefore provide a readily available substitute for some of the effector mechanisms of lymphocyte-mediated immunity (Table 1-1). The prominent feature of this substitution is the recruitment of phagocytic cells, and enhancement of their activity through opsonisation of the material which caused activation. Furthermore, the alternative pathway is inevitably activated in the wake of classical pathway activation, so that it is also involved in the expression of antibody-mediated immunity when lymphocyte activation has occurred. The complement system will now be considered in greater detail.
The term complement was coined by Ehrlich at the turn of the century to describe a heat labile component of animal sera which was needed to complement the activity of antibodies in order to produce cell lysis. As with immunoglobulins, the detailed structure of the complement system was not open to investigation until the technology of protein chemistry developed in the 1950's.

The complement system is a collection of proteins (Lachmann, 1975; Brown and Frank, 1981; Reid and Porter, 1981) found in the blood plasma and other body fluids (Table 1-2). A variety of inflammatory stimuli can activate the system, causing the proteins to interact in certain defined sequences. The products of these reactions are responsible for a number of effects on the surrounding tissues which can make a significant contribution to the body's attempts to eliminate the agent causing the inflammation.

The observation of serum-mediated cell lysis led to the discovery of the complement system, and today it is one of the well-known consequence of complement activation. It occurs because a protein complex formed by the association of several complement components is inserted into the plasma membrane or cell wall of the target. When eukaryotic cells such as the mammalian erythrocyte are used as targets, the effects of lysis are dramatically obvious. The significance of lysis in the control of microbial infections is less certain (Muller-Eberhard and Schreiber, 1980). Other, less well
Important part of host defences against microbial infection. When activation occurs the capillaries in the vicinity become dilated as certain fragments of complement components cause the release of vasoactive substances from mast cells. These substances, which include histamine, also cause the contraction of smooth muscle. The change in capillary permeability allows the movement of neutrophil polymorphs and some plasma proteins from the bloodstream into the tissue spaces. Some of these fragments can also provide chemotactic stimuli which attract the neutrophil polymorphs to the focus of complement activation. Other protein fragments produced during the activation process coat the cell or molecule which caused the reaction. The phagocytic cells bear surface receptors (Dierich and Schulz, 1983; Wilkinson, 1983; Fearon, 1984) which bind strongly to these adsorbed fragments, increasing the efficiency of phagocytosis (opsonisation). It has been claimed that the presence of the complement fragments on the surface of ingested microorganisms is important in triggering the microbicidal mechanisms of neutrophil polymorphs (Yamamura and Valdimarsson, 1977; Leigh et. al., 1979). Whatever the significance of complement-mediated lysis in the control of infectious diseases, it is clear that complement activation provides a useful means by which phagocytosis can be enhanced during an inflammatory response.

Two major mechanisms of complement activation are recognised (Figure 1-3). Others have been reported.
The so-called classical pathway was the first to be described. It requires the interaction of certain classes and sub-classes of immunoglobulin with their antigenic determinants in a suitable configuration. In contrast, the alternative pathway is activated by the binding of its early components to a variety of microorganisms, animal cells and molecules (Table 1-3). The early events in both pathways are depicted in Figures 1-4 and 1-5. They differ in a number of respects, but both deposit protein complexes which possess proteolytic activity on the activating surface. These complexes act only on the complement component C3, initiating the terminal reactions of the complement system which are common to both pathways. They are usually referred to as the classical pathway C3 convertase and the alternative pathway C3 convertase. This binding of either convertase to the antigen-antibody complex or microorganism which caused them to be produced, focusses the effects of complement activation on the undesirable intruders.

The general structure of C3, and the effects of the convertase enzymes on it are shown in Figure 1-6. The fragment C3b contains a short-lived binding site (Muller-Eberhard and Schreiber 1980) which is able to form a thioester bond with the foreign surface which caused its production. The small fragment, C3a, diffuses into the body fluids. The biological effects of this and other complement fragments are summarised in Table 1-4. Since both C3 convertase complexes act as
to C3a and C3b before activity is lost. Many of the C3b fragments will be scattered over the surface of the antigen-antibody complex or cell which activated the pathway. These C3b molecules bind strongly to the receptors on the surface of phagocytic cells, and are responsible for complement-mediated opsonisation. Other fragments such as C4b may make some contribution to this process (Holborow and Papamichael 1983).

If a single C3b fragment becomes associated with a C3 convertase complex, the enzymic specificity of the complex is modified. It now binds and cleaves the complement component C5 into two fragments C5a and C5b. The C5a peptide diffuses into the surrounding body fluids where its effects contribute to the inflammatory response (Table 1-4). C5b binds firmly to the surface which caused complement activation, at sites distinct from those occupied by C3 convertase complexes and the C3b opsonins (Muller-Eberhard, 1975). The components C6, C7, C8 and C9 associate with bound C5b to form the complex that is responsible for cell lysis. At first, it was thought that the composition of this complex was C5b6789 (Mayer, 1973) and subsequently a dimer of C5b6789 was proposed (Muller-Eberhard, 1980). The most recent work suggests a more complicated progression of the assembly of these terminal components (Lachmann, 1983; Mayer, Imagawa, Ramm and Whitlow, 1983), with a number of functional intermediates. As noted earlier, these events occur whenever complement is activated, regardless of the initiating pathway. They are often

not listed in bibliography;
Biology of the Complement System:
A Summary, Progress in Immunology, V.
445, Yamamura, Y. & Tada, T., Editors,
referred to as the common attack pathway (Figures 1-7 and 1-8).

Now that the general principles of complement activation have been described, the detailed structure of the C3 convertase complexes can be examined. When IgM, IgG1, IgG2, or IgG3 antibodies react with their antigenic determinants in a suitable configuration, they are able to bind the classical pathway component Cl (Porter and Reid, 1978; Burton et al., 1980). In essence, this requires two 7S immunoglobulin molecules to be juxtaposed. A single molecule of IgM can meet this requirement since it is composed of five 7S units. The IgG molecule consists of a single 7S unit, so that two molecules are required to bind close together for the association with Cl to occur (Figure 1-4). This juxtaposition is more probable when high concentrations of the required IgG antibody are present in the body fluids.

Cl is in fact composed of three different kinds of polypeptide subunits: Clq,Clr and Cls (Table 1-2). The complete Cl complex is thought to contain a single molecule of Clq, two molecules of Clr, and two molecules of Cls. It is the Clq subunit that binds to the antigen-antibody complex. This event induces proteolytic activity in the Cls polypeptides by a mechanism which is still the subject of some debate. The central question in this debate is whether the Clr subunits acquire proteolytic activity in the course of Cls activation. The work of Porter's group (op. cit.) suggests that this is the case. Activated Cls has the
C4 cleave the classical pathway proteins C4 and C2.

\[
\text{C4} \rightarrow \text{C4a} + \text{C4b} \\
\text{c2} \rightarrow \text{C2a} + \text{C2b}
\]

The characteristics of each fragment are given in Table 1-4. As these are enzymic reactions, several molecules of each protein will be cleaved before the activated Cl esterase molecules lose their activity. The fragments C2b and C4a diffuse into the body fluids, where they may be able to express their biological activities before they too are inactivated. The other fragments, C4b and C2a, associate on the surface of the immune complex which caused complement activation to produce the classical pathway C3 convertase C4b2a. This also has a short half-life, but should cleave several molecules of C3 before its activity is lost.

Activation of the alternative pathway contrasts sharply with that of the classical pathway. In fact, the components of the alternative pathway C3 convertase are constantly associating in normal blood plasma (Figure 1-5). The complex C3bB is acted on by the circulating enzyme, factor D. C3bBb is produced as factor D cleaves factor B into a large fragment Bb and a small fragment Ba (Table 1-4). This complex reacts with the component known as properdin to give C3bBbP. Each of these three complexes (C3bB, C3bBb and C3bBbP), have some C3 convertase activity, but C3bBbP is the most active. In the absence of any cell or molecule capable of activating the alternative pathway (Table 1-3) it will lose its activity spontaneously (Figure 1-5). Before
and factor H (Table 1-2) rapidly destroy its biological activity.

Alternative pathway activation, or more precisely amplification of alternative pathway activity, occurs when a cell or molecule capable of binding to the C3bBbP complex enters the body fluids. Once bound, the convertase is protected from the actions of factor I and factor H and is able to effect the conversion of C3 to C3a and C3b (Fearon and Austen, 1977; Schreiber et al., 1978). The C3b fragments will then cause the expression of C5 convertase activity, opsonise the activator, and feed into the reactions which produce the C3bBbP complex generating further alternative pathway activity, the so-called feedback amplification loop, (Lachmann, 1975). The appearance of C5 convertase activity will trigger the common attack pathway. While the alternative pathway can be activated in isolation, the classical pathway cannot. When C3 is cleaved during classical pathway activation, some of the C3b fragments produced combine with factor B and properdin to form alternative pathway C3 convertase complexes in the body fluids. This so-called fluid phase activation of the alternative pathway will inevitably augment the expression of classical pathway activity because of the alternative pathway feedback amplification loop (Figures 1-4 and 1-5). Thus activation of the classical pathway will always lead to activation of the alternative pathway. Even a cursory glance at the consequences of complement
response to infectious disease. Unchecked complement activity could damage host cells as well as those of the pathogen responsible for the infection. This is particularly true of the alternative pathway as it is always running, albeit at a very low level. A number of proteins control the expression of complement activity (Table 1-2). Two of these have been described already (factor I and factor H). The alternative pathway is held in check by these proteins, and it is probable that variations in the concentrations of factors I and H could allow inappropriate expression of alternative pathway activity (Lachmann and Halbwachs, 1975;).

The presence of a circulating (auto)antibody (C3 nephritic factor, C3NeF) to the alternative pathway C3 convertase is sometimes associated with complement-mediated renal damage (Habib and Levy, 1979; Sissons and Peters, 1979; Schreiber and Muller-Eberhard, 1979). The association is not complete however, and the exact causes of the renal lesions are unclear. The classical pathway generally requires immunoglobulin for its expression, although, as noted earlier Cl may be activated in the absence of antigen-antibody complexes (Loos, 1982). On the other hand, the alternative pathway requires only the pathogen or its secretions to provide a surface which can stabilise the alternative pathway C3 convertase, although not all microorganisms can provide such a surface.

The chemical nature of activating surfaces has been the
that activators (or perhaps more appropriately, protectors) of the alternative pathway are relatively hydrophobic. Mammalian cells depleted of surface sialic acid by neuraminidase treatment become activators of the alternative pathway (Kazatchkine, Fearon and Austen, 1979), while the erythrocytes of inbred mouse strains, which bear differing amounts of sialic acid, activate the human alternative pathway with a relative efficiency determined by their sialic acid content (Nydegger, Fearon and Austen, 1978). Sialic acid is a major determinant of the hydrophilic nature of animal cells. Support for this concept comes from the observation that those bacteria which secrete a sialic acid-bearing capsule do not bind the alternative pathway C3 convertase, and seemed to have gained a marked evolutionary advantage. They are usually important animal pathogens (Jacks-Weis, Kim and Cleary, 1982), such as *Streptococcus pneumonia*. There have however been reports which suggest that sialic acid content is not the sole determinant of the ability to cause alternative pathway activation (Reid and Porter, 1981). Sulphated mucopolysaccharides may also be involved. It is interesting that the recognition criteria of neutrophil polymorphs and macrophages appear to be similar to those of the alternative pathway (Wilkinson, 1976; Weir and Ogmundsdottir, 1977). Thus, in the absence of antibody, the main cellular defences and the humoral mechanisms which will recruit these cells to areas of need, have similar, relatively non-specific
some organisms. It had been tentatively suggested that phagocytes rarely ingest unopsonised particles, making the alternative pathway the key recognition mechanism (Mowbray, 1976). This suggestion may assume greater significance in the light of the work of Ezekowitz which was discussed earlier.

The advantages of specific, lymphocyte-mediated immunity are readily apparent. As noted earlier however, it can take several days for the effectors of lymphocyte-mediated immunity to reach adequate levels throughout the body fluids. The alternative pathway could provide valuable interim defences against many microorganisms (Fearon and Austen, 1980). Recently, other functions have been attributed to the complement system. These are the ability to inhibit the formation of immune complexes, and to facilitate the solubilisation of preformed complexes (Takahashi, Takahashi and Hirose, 1980; Naama et. al., 1983; Skogh and Stendahl, 1983), and the ability to suppress certain lymphocyte responses (Weiler et. al., 1982; Morgan et. al., 1983).

These properties may be distinguished from those discussed earlier since they do not exert a direct effect on the course of a microbial infection. Instead they influence the milieu in which immune responses occur. It remains to be seen whether they make a significant contribution to the control of these responses.
1.4 THE ROLE OF THE COMPLEMENT SYSTEM IN DISEASE.

Despite the diversity of cells and molecules in the immune system, the manifestations of its activity which contain, and eventually eliminate "foreign" materials are relatively few (Table 1-1). This table does however show that each manifestation, such as lysis or neutralisation, can be effected in a number of ways. The complement system is able to provide most of them. As noted earlier, the alternative pathway augments the expression of classical pathway activity, and in the absence of antibodies and activated T lymphocytes, it may provide the major vehicle of host defence. The real value of the alternative pathway can only be assessed by careful experimentation. The available evidence will now be examined.

a. In-Vitro Studies

Such studies have considered the effects of the in-vitro activation of serum complement on microorganisms and on the phagocytic cells of the immune system, as well as the lysis of suitable red cell targets. When an organism elicits antibody of the necessary class or subclasses, activation of the classical pathway is virtually inevitable, and the interaction of fresh immune sera with a given organism provides useful information on its susceptibility to complement-mediated lysis. Fine (1981) has summarised the findings of a great deal of experimental work, which used a variety of techniques. He suggests that most eukaryotic cells are readily lysed by the classical pathway, as are many microorganisms, although a number
of Gram-positive organisms, and some strains of
Gram-negative organisms are exceptions. The role of the
classical pathway-mediated lysis of viruses is unclear
particularly because it can be difficult to distinguish
between neutralisation of infectivity and physical
disruption of the virion by the lytic activity of
complement. The work of Apostolov and Sawa (1976)
indicates that complement activation is not always
beneficial. For instance, pretreatment of the Newcastle
disease virus with antibody and complement caused
disruption of the virion which facilitated subsequent
fusion with erythrocyte membranes, and hence lysis of
the erythrocytes.
The need to investigate the functional efficiency of the
whole complement system has been underlined by Porter's
speculations on the association of the genetic
polymorphism within the complement system with certain
diseases (Porter, 1983; Davis, 1983;). Although each
of the major activities of the complement system can be
measured in-vitro, none of the assays currently
available is sufficiently sensitive to test Porter's
hypothesis (Welch, 1983). Erythrocyte lysis is commonly
used in diagnostic and research laboratories to measure
the overall potency of the complement system. Serial
dilutions of each sample are tested for their ability to
lyse a suitable red cell target (Lachmann, Hobart and
Aston, 1973). The titre of lysis is taken to reflect
the complement activity of the sample. Deficiencies of
individual components may reduce this titre, but the
assay lacks sensitivity, and in any case a low value
Ideally, since the complement system is composed of a number of proteins each of which exhibit a particular biological activity, a complete analysis of the system requires the measurement of both the concentration of each protein, and its biological activity (functional level). As has been shown for several enzymes, one does not necessarily reflect the other (Landon, Carney and Langley, 1978).

The measurement of individual complement components requires specific antisera and in a single species, the production of such reagents is a major task. When several species are to be studied this type of work would assume herculean proportions. Fortunately, the structure of individual components may be sufficiently conserved between the species in question to allow the use of antisera to the components of one species to study those from other animals (Nilsson and Muller-Eberhard, 1967). If neither option is available, the investigator can only measure activity, and is unable to investigate the molecular basis of his observations.

Investigation of alternative pathway-mediated effects, requires measures to prevent classical pathway activity. These include: the use of non-immune serum as a source of complement activity, adsorption of any antibodies from the test serum, the use of sera deficient in a classical pathway component, the use of EGTA to block classical pathway activity and magnesium ions to achieve optimum alternative pathway activity (Appendix 1) and
also the reconstitution of the alternative pathway from purified components. Each approach has its merits and its drawbacks.

The work of Roberts and Phillips (1983) using a number of serum diluted in a magnesium-EGTA buffer against a number of serotypes of *E. coli* suggests that the kinetics of alternative pathway killing are slower than those of the classical and alternative pathways combined.

Muller-Eberhard and Schreiber (1980) employed the last one to study the effects of alternative pathway activity on a number of bacteria. Using scanning electron microscopy they could distinguish between microbial death and lysis of the cell. The latter required the presence of lysozyme and it was complete within 60 minutes using dilutions up to 1:16. Beyond this dilution the activity was lost. When virus-infected or malignant eukaryotic cells were treated in the same way lysis (measured by the release of $^{86}$Rb label) showed similar kinetics. If cell metabolism was intact, the cells seemed more resistant to lysis.

The properties of the peptides produced during complement activation can be demonstrated *in-vitro*. For example, histamine released from basophil polymorphs or mast cells can be measured using fluorimetric assays (Siraganian, 1976), while the chemotactic peptides will cause neutrophil polymorphs to move through support media such as agarose gels (Nelson *et. al.*, 1981) or nitrocellulose filters (Cates, 1981). Pretreatment of microorganisms with immune sera
an organism is capable of activating the alternative pathway, similar opsonisation is seen in the presence of non-immune serum. Care must be taken to exclude the effect of opsonising antibody when alternative pathway-mediated opsonisation is studied in sera whose immune status is not known. Inactivation or inhibition of the classical pathway does not prevent antibody-mediated opsonisation. If opsonisation resists heating (56°, 30 minutes) or trypan blue treatment (Guckian, Christiensen and Fine, 1978), it is probably due to antibody.

In-vitro studies appear to support the view that the complement system in general and the alternative pathway in particular are potent components of the immune system, although the evidence is not complete.

b. The Role of Complement in Diseases Associated with Inappropriate Expressions of Immunity

In 1975, Gell and Coombes proposed a classification which they hoped would facilitate the discussion and investigation of such immunological diseases. A modified version of their classification is presented in Table 1-5 as a background to this introduction. While the aetiology of many of these diseases is uncertain (Johnson, 1981), it has become clear that complement activation is a important effector mechanism in some of them. Classical pathway activation is responsible for the recruitment of neutrophil polymorphs following the binding of organ-specific autoantibodies to the components of their target organs in conditions such as
accumulation of neutrophils in tissues in which soluble immune complexes have been deposited during immune complex diseases (Table 1-5), and such deposits of immunoglobulin and complement components can be detected by immunofluorescence techniques (Jordan, 1976; Wilson, 1976).

Diseases involving the alternative pathway alone are less common, but membranoproliferative glomerulonephritis and paroxysmal nocturnal haemoglobinuria are thought to reflect inappropriate alternative pathway activation. As noted above, the first is a renal disease with characteristic clinical and laboratory findings (Habib and Levy, 1979; Sissons and Peters, 1979; Schreiber and Muller-Eberhard, 1979). In a proportion of the subjects antibody to antigenic determinants on the C3bB complex has been demonstrated, and this antibody (known as nephritic factor) protects the alternative pathway C3 convertase complex from inactivation by factor H and factor I. It has been suggested that this protection is responsible for the renal lesions but, as noted earlier, the lack of a consistent association between the renal damage and the presence of nephritic factor does not lend support to this view.

As the name implies, paroxysmal nocturnal haemoglobinuria is associated with the occasional presence of haemoglobin in early morning urine samples (Gotze and Muller-Eberhard, 1972). It is thought that changes in the body fluids which sometimes occur during
sleep favour the binding of the patients' alternative pathway C3 convertase to their own erythrocytes causing lysis. The condition appears to stem from an innate structural abnormality of the erythrocyte surface membrane in these people (Parker, Baker and Rosse, 1982).

These observations confirm the potency of the combined effects of the classical and alternative pathways, and suggest that the alternative pathway is a potent mediator of inflammation in its own right.

c. The Consequences of Innate Complement Deficiencies

Just as information on the importance of phagocytes and lymphocytes may be gained from the study of inborn and acquired deficiencies of these cells, so the investigation of subjects with complement deficiencies confirms the importance of both pathways in host defences (Agnello, 1978; Ruddy 1980). A cautionary note must be added to these observations: many of the defects are very rare, and the clinical histories recorded on these patients are sometimes incomplete. In such cases it is best to regard the clinical stigmata of a particular deficiency as uncertain, at least for the present. Some use has also been made of animal strains with innate deficiencies of complement components (Nilsson and Muller-Eberhard, 1967). The findings in humans and other species may be summarised as follows:

(i) Deficiency in C3, either a primary deficiency, or one due to a deficiency of C3b inactivator is associated with a life-threatening susceptibility to infection.

(ii) Lack of the classical pathway components, Cl, C4,
antigen-antibody complexes from the body fluids. A clinical syndrome resembling the immune complex disease, systemic lupus erythematosus (Hayward, 1977), is often present.

(iii) Deficiency of the components common to both pathways (C5 to C9) seems to cause a predisposition to infection. Infections with *Neisseria* are common in those cases so far reported, but the rarity of these conditions makes confident statements difficult at present.

(iv) Demonstrable defects of the alternative pathway components are exceedingly rare, and their clinical consequences are uncertain, but their rarity has been taken as evidence that they are incompatible with survival (Pearon and Austen, 1980). A number of reports have associated a defect in the ability of serum to opsonise yeasts with impaired alternative pathway activity (Miller, *et al.*, 1968; Soothill and Harvey, 1976; Turner, *et al.*, 1978; Candy, *et al.*, 1980; Turner, Mowbray and Robertson, 1981; Larcher, *et al.*, 1982; Wyke, Rajkovic and Williams, 1983). A precise cause has yet to be demonstrated and in contrast to the other deficiencies described above, this is an unexplained clinical syndrome. The others are clearly defined complement defects in which the clinical consequences are known to varying extents.

d. Attempts to Modify Complement Activity in-vivo.

The main research effort in this area has involved the use of cobra venom factor (Kawamoto *et al.*, 1979; Vogel,
factor B in a number of species, forming a complex with C3 convertase activity which is not inhibited by factor H and factor I. Intraperitoneal injection of cobra venom factor consumes C3 and the late complement components producing transient complement deficiency. If experiments last more than a few days further injections of cobra venom factor are required to sustain the depletion of complement. Hirsch (1982) has reviewed the use of this model, and concludes that the alternative pathway is able to control the blood-borne spread of a number of pathogens. He notes however that in the case of Sindbis virus infection in mice that while alternative pathway in serum reduces the number of lesions which appear in the brain following intravenous injection of the virus, it also appears to potentiate the subsequent brain damage. Animals treated with cobra venom factor had more infectious foci in the brain, but the development of brain damage around these foci was significantly delayed. It is important to note that the usual outcome of the infection, death, was not changed by cobra venom factor treatment.

There are some problems related to the use of cobra venom factor. For example, it produces a marked depletion of complement activity, and intermediate levels of depletion cannot be obtained. Repeated doses needed to maintain prolonged reduction in complement activity often results in the production of antibodies to cobra venom factor (Egwang and Befus, 1984). These reduce its efficacy, and introduce additional variables.
alternative pathway activation. Hence, alternative pathway components are no longer available to react with any pathogens subsequently given to these animals, but the peptide fragments are responsible for the biological activities of the complement may be present in the animals' body fluids throughout any experiments. These preformed peptides might cause "bystander" damage to injected pathogens, and their presence would modify the environment in which the animals' host defences deal with experimental infections. It may be misguided to assume that cobra venom factor treatment provides a straightforward model of complement depletion (Weiler et al., 1982).

The potency of the alternative pathway is clearly established by this work, but the extent to which differences in the rate of expression of alternative pathway activity might contribute to differences in the natural history and severity of infectious diseases remains unexplored. A more sensitive in-vitro assay of alternative pathway haemolytic activity would make it possible to answer this and other questions.

The limitations of the conventional in-vitro techniques for the measurement of haemolytic complement activity noted earlier were encountered during attempts to study the functional efficiency of the alternative pathway in serum samples which failed to opsonise baker's and brewer's yeast in-vitro (Langley and Harvey, 1980). There was considerable evidence that the underlying defect was in the alternative pathway (Turner, Mowbray and
failed to reveal consistent evidence of complement
dysfunction. It has been shown that other opsonins
which are found in blood plasma may mimic the
alternative pathway in some of the opsonisation assays
currently used (Kerr et. al., 1983). This could mean
that the yeast opsonisation defect does not reside in
the alternative pathway, making further investigation of
the complement system irrelevant. There was however
sufficient evidence at that time to suggest the need for
a modified, more informative assay of alternative
pathway lytic activity. It was therefore decided to
investigate the kinetics of red cell lysis by monitoring
the reaction continuously at a variety of serum
dilutions. When a small panel of normal human sera were
tested in this way there was considerable individual
variation in the kinetics of alternative
pathway-mediated lysis of erythrocytes (Langley,
Griffiths and Laurence, 1982; Laurence, 1982).
If these observations were confirmed, the kinetic assay
of alternative pathway activity could provide a simple
but powerful technique to compare the overall efficiency
of this pathway. In seeking such confirmation, it would
be important to characterise the phenomenon carefully,
and eliminate the influence of other plasma constituents
such as antibody, on the kinetics of complement
activity. Finally, the molecular basis of these
variations in reaction kinetics and their relevance to
the efficacy of host defences should be investigated.
The work to be described was designed to address each of
Figure 1-1: Haemopoiesis

KEY
- : Ia-like (HLA-DR) Antigen
- : Myeloid Antigen
+: Sheep Erythrocyte Rosette-Forming Protein
‖: Immunoglobulin
- : Terminal Transferase Activity in Nucleus

- Thymus Gland
- Thymocyte
- T lymphocyte
- B lymphocyte
- Plasma Cell
- Pluripotent Stem Cell
- Megakaryocyte
- Platelet
- Myeloid Stem Cell
- Myeloblast
- Monoblast
- Monocyte
- Neutrophil & Other Polymorphs
- Erythroblast
- Normoblast
- Erythrocyte
2a. Regurgitation with Feeding
Lysosomal Contents Enter Body Fluids

Mitochondrion

Lysosomes

PHAGOCYTIC CELL  Microorganisms

1. Binding & Ingestion
Increased glycolysis; RNA, Protein & Membrane Synthesis
Opsonins Enhance Efficiency of Binding & Ingestion

2. Phagosome-Lysosome Fusion
Oxygen Consumption & H₂O₂
Synthesis. Chemiluminescence

3. Killing and Digestion
Undigestable Material May be Seen as "Residual Body"
Figure 1 - 3
Complement Activation - The Conventional Diagram

Ag.Ab + Clq.Clr.Cls

Ag.Ab-Clq.Clr.Cls

C4

C2

C4b + C2a + C4b2a

C3bBb

C3bBbP

C3a

C3b

C4bC2a3b

C3bbPCC3B

C5b + C5a

(C5b6789)n

(lytic complex)

KEY:

: active component

: proteolytic enzyme activity

: protein cleavage

: protein association
Figure 1-4
Early Events in Classical Pathway Activation
Early Events in Alternative Pathway Activation

Figure 1-5

Alternative Pathway Activator

C3b → C3bBb → C3bBb

Rapid inactivation by factors I and H

C3 Convertase Stabilised

Activity Modified to C5 Convertase by C3b

C3 Cleavage
- C5 convertase
- Opsonisation
- Feedback activation
Figure 1-6

C3 and its Cleavage Products

(a) Molecular Cleavage

Prosynthetic Peptides

after Thompson (1983)

Mol. Immunol. 17: 9

after Ruddy (1980)
(b) Chemical Reactions at the Active Site of C3
Table 1-1

**Effector Mechanisms of the Immune System**

**NEUTRALISATION AND CONTAINMENT**

Neutralisation: antibody alone
antibody and complement (C4a)

Entrapment
antibody alone
antibody and complement (agglutination)
complement alone (immune adherence)

Cytotoxicity: antibody and complement
complement alone

cytotoxic T cells
K cell phenomenon
natural killer cells
macrophages

**RECRUITMENT OF PHAGOCYTES (AND OTHER EFFECTOR CELLS)**

antibody and complement
complement alone
lymphokine-producing T lymphocytes

**OPSONISATION**

antibody alone
antibody and complement
complement alone
other opsonins

**ACTIVATION OF PHAGOCYTES**

direct effects of microorganisms
complement alone (Ba)
lymphokine-producing T lymphocytes
# Proteins of the Complement System

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<th><strong>Protein</strong></th>
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<th><strong>Chains</strong></th>
<th><strong>Carbohydrate</strong></th>
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* First value, Hay and Movat, 1979; bracketed value, Brown and Frank, 1981.

**Synonyms:** B, glycine rich beta glycoprotein (GBG), C3 proactivator (C3PA); D, GBGase, C3PA convertase.


<table>
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**Control Proteins**

With clearly-defined function in vivo:

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**Synonyms:** B, glycine rich beta glycoprotein (GBG), C3 proactivator (C3PA); D, GBGase, C3PA convertase.


With uncertain role in vivo:

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<tr>
<td>D</td>
<td>25,000</td>
<td>1</td>
<td>3.0</td>
<td>alpha</td>
<td>1-5 0.04-0.20 0.6-7.0</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>200,000</td>
<td>4</td>
<td>9.8</td>
<td>5.2</td>
<td>beta</td>
<td>25 (x10^-6) 2</td>
</tr>
</tbody>
</table>

**Synonyms:** C3b inactivator (C3bINA), conglutinogen activating factor (KAF); C1 inhibitor, C1 esterase inhibitor.
Activators of the Alternative Pathway

PROKARYOTES
- whole *Escherichia coli*
- lipopolysaccharide (endotoxin) from Gram-negative bacteria
- techoic acid from Gram-positive bacteria

EUKARYOTES
- Yeast cells and the polysaccharide zymosan
- trypanosomes and other protozoa
- rabbit red cells
- neuraminidase treated sheep erythrocytes
- lymphoblastoid cell lines, e.g. Raji.
- HeLa cells
- measles virus infected cells

POLYANIONS
- dextran sulphate
- polyvinyl sulphate
- dinitrophenol/albumin conjugates

Based on Reid and Porter (1981)
## Biological Activities of Complement Fragments

<table>
<thead>
<tr>
<th>FRAGMENT</th>
<th>MOLECULAR WEIGHT</th>
<th>PROPERTIES</th>
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<tbody>
<tr>
<td>C3a</td>
<td>9000</td>
<td>spasmogenic activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>enhanced vascular permeability</td>
</tr>
<tr>
<td>C4a</td>
<td></td>
<td>immunosuppression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>granulocyte activation</td>
</tr>
<tr>
<td>C5a</td>
<td></td>
<td>spasmogenic activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>chemotactic activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>enhanced vascular permeability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>immunoenhancement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>granulocyte activation</td>
</tr>
<tr>
<td>C3e</td>
<td>ca 12,000</td>
<td>leucocytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>enhanced vascular permeability</td>
</tr>
<tr>
<td>C3d-k*</td>
<td>41,000</td>
<td>leucocytosis</td>
</tr>
<tr>
<td></td>
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<td>enhanced vascular permeability</td>
</tr>
<tr>
<td>C3b</td>
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<td>opsonic activity</td>
</tr>
<tr>
<td>C3d**</td>
<td>25,000</td>
<td></td>
</tr>
<tr>
<td>C4b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bb</td>
<td>60,000</td>
<td>monocyte activation</td>
</tr>
<tr>
<td>Ba</td>
<td>33,000</td>
<td>chemotactic activity</td>
</tr>
</tbody>
</table>

* fragment of alpha chain of C3 corresponding to C3d/g; Hugli T.E., Progress in Immunology, V : 419 (1983).

** Earlier reports: C4b able to neutralise virus infectivity, C2a possessed kinin-like activity.
<table>
<thead>
<tr>
<th>GELL &amp; COOMBS CLASSIFICATION</th>
<th>DISEASE STATES</th>
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</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td><strong>Effector Mechanism</strong></td>
</tr>
<tr>
<td>I</td>
<td>Ige-mediated degranulation of mast cells. (Overproduction Ige: ? hyperreactive mast cells)</td>
</tr>
<tr>
<td>II</td>
<td>Antigen-Antibody reaction in defined locations, often with complement activation</td>
</tr>
<tr>
<td>III</td>
<td>Antigen-Antibody reaction in body fluids (soluble antigen). Deposition in tissues may be widespread. Damage due to complement activation. (Immune Complex Disease)</td>
</tr>
<tr>
<td>IV</td>
<td>Delayed Hypersensitivity T lymphocyte response. Lymphokines recruit &amp; activate macrophages</td>
</tr>
</tbody>
</table>

Some workers have proposed a fifth category for autoantibodies such as the long acting thyroid stimulator (LATS), which enhance the activity of an organ, but they may also be regarded as a special case of Type II autoimmunity.

2.1 BLOOD SAMPLES

Sheep Erythrocytes

Blood taken as required, from a single ewe was used throughout the investigation described in Chapter 3. Each sample was collected by venepuncture from the jugular vein, into Alsever's solution (Appendix I). The erythrocytes were immediately washed three times in phosphate-buffered saline, pH 7.2 (Appendix I), then stored in Alsever's solution for no more than three days. When small quantities of sheep erythrocytes were required at other times, aliquots of a commercial preparation (Appendix I) used regularly for diagnostic serology at the Royal Surrey County Hospital were obtained.

Rabbit Erythrocytes

Rabbit blood was collected into Alsever's solution by venesection, using an ear vein. The subsequent treatment of the erythrocytes was identical to that used for sheep erythrocytes. Cells from a single collection were used for individual experiments, and single animals were used for as long as possible during the study.

Erythrocytes from Other Species

Blood samples were collected from other species by venesection (rabbit), venepuncture (ox, badger, sheep) and cardiac puncture (all other animals). Ferrets, hedgehogs and mice were bled under anaesthesia induced by ether; guinea pigs, hamsters and rabbits under barbiturate anaesthesia ("Sagatal", May & Baker), whilst
Badgers were sedated with ketamine hydrochloride ("Vetalar", Parke-Davis). Fowl, cattle and sheep were bled without anaesthesia. Once again, the treatment of the cells after collection was identical to that described for sheep erythrocytes.

Neuraminidase-Treated Erythrocytes

These were prepared using the method described in Figure 2-1.

Serum Samples

When serum was required blood was taken into a plain glass container. The samples were allowed to clot at room temperature and then separated at 4° as quickly as possible. Thereafter, they were generally stored at -70°, but liquid nitrogen was used for long-term storage, and for the work on mammalian sera described in Chapter 3.

Dialysis of Serum

10mm Visking tubing (Appendix 1) was used to dialyse 1ml aliquots of serum against an excess (>2 litres) of complement fixation test diluent (Appendix 1) overnight at 4°. After a batch of samples had been dialysed in this way, they were all made to the same volume with complement fixation test diluent. The volumes used for particular experiments will be recorded at the appropriate point.

Adsorption of Serum

Antibodies to rabbit erythrocytes were removed from serum samples using the adsorption technique described by van Djik et. al. (1980): 1 volume of packed erythrocytes was incubated at 0° (iced water bath) with
3 volumes of serum for 60 minutes or 24 hours, with occasional agitation. After incubation, the cells were sedimented by centrifugation (3000rpm, 4°, MSE "Chilspin"). The supernatant was removed carefully and generally used immediately. If storage was necessary, the conditions described above were used. Controls consisting of serum without added erythrocytes were subjected to the same treatment.

Normal Human Sera
Two members of staff (DL and PG) regularly provided serum samples and in addition, aliquots of samples taken from undergraduates for virology practicals were also used.

2.2 HAEMOLYSIN PRODUCTION

Immunisation
For the work described in Chapter 3, sheep cells collected in the way described above were taken from storage in Alsever's solution and washed once in phosphate buffered saline. A 10% (v/v) suspension of the packed erythrocytes in phosphate buffered saline was then prepared. Table 2-1 lists the animals selected for immunisation, and the protocol used. Blood samples taken from the immunised animals were separated in the usual way, and the sera stored at -70°. Antibody titres (Table 2-1) were determined using a conventional haemagglutination assay (Hudson and Hay, 1980). When rabbit antiserum to sheep erythrocytes was needed for total haemolytic complement activity assays on other occasions, a commercial preparation was used (Appendix I).
Haemagglutination Assays

The haemagglutination method cited above was used to measure the titre of rabbit erythrocyte agglutinins. Initially a 2 hour incubation period was used, but this was subsequently increased to 24 hours to increase sensitivity.

An independent measure of rabbit erythrocyte agglutinins was made using an immunofluorescence technique. Serial dilutions of each sample were made in complement fixation test diluent using 200µl volumes in LP3 tubes (Appendix 1). Then 200µl of 1% (v/v) rabbit erythrocyte suspension in the same diluent was added to each dilution. After gentle mixing, the tubes were incubated at 37° for 30 minutes, or at 4° for 24 hours. When the incubation was completed, the cells were washed 3 times with complement fixation test diluent before 1 drop of polyvalent, fluorescein-labelled antiserum to human immunoglobulins (Appendix 1) was added to each tube. After 30 minutes at 37°, the cells in each tube were washed again, and examined using a fluorescence microscope system (Appendix 1) to determine the titre of cell-bound fluorescence. Appropriate controls were included in each batch.

2.3 COMPLEMENT COMPONENT LEVELS

Imunochemical Assays

A single radial diffusion technique was used for this purpose (Dubock, 1982). Antisera to human complement components were purchased from Miles Laboratories (Appendix I). They were mixed with molten agarose at 56° in the proportions shown in Table 2-2. Individual gels were poured on alcohol-cleaned 3"x1" glass microscope
slide. When these gels had set at room temperature, they were stored at 4° in a moist chamber until required. Storage never exceeded 48 hours. Wells (3mm diameter) were cut in the agarose using a commercially-available cutter (Appendix I). A standard volume of serum (usually 4ul) was placed in each well. Once loaded, the gels were returned to a moist box and incubated at room temperature for 24 hours. Thereafter they were kept at 4° and reexamined if necessary. To ensure reliable measurement of the precipitin rings, all the gels were dried and stained with Coomassie Blue reagent (Appendix I) before the diameter of each ring was measured. A ruler supplied by Behring Diagnostics (Appendix I) was used to make these measurements. Assays were usually performed in duplicate. Each batch was standardised using serum obtained from apparently healthy subjects. These were used both singly, and as pools, according to the circumstances. The results were expressed as a percentage of the normal adult level.

Factor D Assays
These were performed using the modification of the method described by Martin et. al. (1976) shown in Figure 2-2.

2.4 COMPLEMENT ACTIVITY ASSAYS
Alternative Pathway Haemolytic Activity
Alternative pathway diluent was used to prepare rabbit erythrocyte suspensions and to make any serum dilutions. This reagent consisted of complement fixation test
bis-tetraacetic acid, EGTA, sodium salt at 0.08 mol/1, and initially, magnesium chloride at 0.04 mol/1 (Appendix 1).

Activity was measured in three ways:

a. a conventional serial dilution assay identical to that used for total haemolytic complement activity except in the use of alternative pathway diluent and rabbit erythrocytes instead of complement fixation test diluent and sheep erythrocytes.

b. an independent assessment of alternative pathway haemolytic activity was made using a modified radial diffusion technique (Lachmann, Hobart and Aston, 1973) in which target erythrocytes were incorporated in agarose gels.

c. a timed-lysis assay of alternative pathway haemolytic activity based on the method described by Jones (1979) was also used. The important modifications were the omission of gelatine from the diluent, and the use of an optimum (4 mmol/1) magnesium concentration. Rabbit erythrocytes were suspended in alternative pathway diluent to give an absorbance at 700nm in the region of 0.9, using 1 cm cuvettes. This corresponded to a red cell count circa 4 x 10^6/ml. Most of the assays were performed in a Beckman Model 24 spectrophotometer. On some occasions it was necessary to analyse large batches of samples in a single day to ensure continuity of
Perkin-Elmer "Lambda" spectrophotometer was used as these instruments made it possible to analyse four or six samples simultaneously. According to circumstances, 3000µl, 1000µl or 500µl of cell suspension was used in the assay; serum volumes were chosen to give final dilutions between 1:2 and 1:75. The chosen volume serum was warmed to 37°. At zero time the appropriate volume of rabbit erythrocyte suspension was added from a pre-warmed stock. After gentle mixing, the reaction mixture was transferred to the a warmed cuvette within 30 seconds. The progress of cell lysis was followed by measuring absorbance at 700nm, an index of light scattering, continuously using a chart recorder.

Total Haemolytic Complement Assays

Sensitised Cells

These cells were produced by mixing antiserum with a 3% suspension of sheep erythrocytes in complement fixation test diluent in proportions which corresponded to the next dilution above the haemagglutination titre of the antiserum in question (Higgins and Langley, 1985). The innate complement activity of each serum was destroyed, before use, by incubation at 56° for 30 minutes. Each mixture of sheep erythrocytes and sensitising antiserum was incubated at 37° for 15 minutes. The sensitised cells were then washed three times in complement fixation test diluent, stored at 4° in fresh diluent and used within 24 hours.

Activity Assays

a. a conventional, two-fold serial dilution test (Gewurz
In this assay, 20μl aliquots of a 3% (v/v) suspension of sensitised erythrocytes in complement fixation test diluent were added to an equal volume of serum or serum dilution. The controls consisted of an aliquot of cells mixed with an equal volume of diluent. Each microtitre plate was incubated in a moist atmosphere at 37° for 2 hours. Lysis was assessed visually, as being complete or partial. The last dilution which caused significant lysis was recorded.

b. a timed-lysis or kinetic assay performed in a Beckman Model 24 spectrophotometer using 1cm glass cuvettes held at 37°. Sensitised sheep erythrocytes were suspended in complement fixation test diluent to give an absorbance at 700nm ($A_{700}$) circa 0.9. The sample dilution and the volume of this dilution needed to give suitable activity were determined for each experiment. A fixed volume (1000μl) of the sensitised cell suspension was used throughout this investigation. The chosen volume of diluted serum was mixed with the sensitised sheep erythrocytes using the procedure described for alternative pathway assays. The lytic reaction was followed by the measurement of $A_{700}$.

2.5 STATISTICAL PROCEDURES

Basic analyses were performed using a Casio fx-180P calculator. More involved procedures were performed on the University of Surrey Prime computer system using routines from the Statistical Package for Social Sciences, SPSS, (Nie, et al., 1975). Details of the individual routines will be given at the appropriate
2.6 OTHER METHODS

The methods described above are those which were used often in this investigation. Techniques used on an occasional basis will be described at the appropriate points in the text.
Figure 2-1
Preparation of Neuraminidase-Treated Erythrocytes

Neuraminidase Type V (Sigma)

(a) Dissolved in Distilled Water at 1 unit/ml
(b) Frozen in 150μl aliquots

Sheep Red Cells
Washed 3 times in 0.85% saline; supernatant and buffy layer removed.

NEURAMINIDASE (150μl) PHOSPHATE BUFFERED SALINE (5000μl) PACKED CELLS (3000μl)

Incubate in 37° water bath for 30 minutes with gentle mixing
Wash cells twice in phosphate buffered saline
Resuspend in appropriate buffer at required concentration

(Personal Communication, Taylor, C. (1981))
Factor D Assays

FACTOR D DEPLETED SERUM (FDDS)  AGAROSE GEL (washed in EGTA)

Sephadex G200 column in PBS 2.4% solution, melt in
Exclusion peak : FDDS boiling water bath, hold
50°

Warm to 50° and add to agarose:

EGTA to 10 mmol/l
MgCl₂ to 4 mmol/l
rabbit erythrocytes to 1%
FDDS to 0.75%

Make agarose to 1% by dilution with warm CFTD

Pour gels, leave to set overnight

Cut 3mm wells, fill with 4μl appropriate serum

Incubate at 4° overnight, then 2 hours at 37° after
warming to room temperature

Read diameter of lysis

PBS : phosphate buffered saline
CFTD : complement fixation test diluent
Production of Haemolysin Antisera: Protocol and Products

Production of antisera against sheep erythrocytes

<table>
<thead>
<tr>
<th>Species</th>
<th>No</th>
<th>Breed/Strain</th>
<th>Inoculation Volume</th>
<th>Route</th>
<th>Haemagglutination titres</th>
<th>Immunized animals</th>
<th>Normal sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox</td>
<td>2</td>
<td>Jersey, Hereford</td>
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<td>IV</td>
<td>256,512</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
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<td>NZW x Lopear</td>
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<td>3</td>
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<td>Syrian</td>
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<td>IP</td>
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<td>Hartley</td>
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<td>IP</td>
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<tr>
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<td>-</td>
<td>1.0</td>
<td>IV</td>
<td>64,64,128</td>
<td>8</td>
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<td>-</td>
<td>0.5</td>
<td>IP</td>
<td>8,64,64</td>
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<tr>
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<td>White Leghorn</td>
<td>0.5</td>
<td>IV</td>
<td>256,1024</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

1 Volume (ml) of 10% SE suspension inoculated on each of days 1,2,3,5,7,9,11 and 13. Animals were bled on day 15.

2 IV, intravenous; IP, intraperitoneal.

3 Pools of two or more individuals.

Table 2-2
Proportion of Antiserum in Radial Diffusion Gels

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>% ANTISERUM</th>
<th>%PEG</th>
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<tr>
<td>C3</td>
<td>1.0</td>
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</tr>
<tr>
<td>factor B</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>properdin</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>factor I</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>factor H</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>C4</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Cl inhibitor</td>
<td>1.0</td>
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</table>

PEG: Polyethylene Glycol
3. ALTERNATIVE PATHWAY ACTIVITY IN NORMAL SERA

3.1 INTRODUCTION

The demonstration of marked differences in the kinetics of alternative pathway activation in human sera (Langley, Griffiths and Laurence, 1982; Laurence, 1982) was an unexpected finding. When sera from certain mammalian species were also shown to have distinctive reaction kinetics a detailed investigation of these phenomena was carried out. This investigation, reported below, was designed to answer certain questions, for instance, (a) what is the activity of selected animal sera in the conventional assays of complement activity? A number of publications (Rice and Crowson, 1950a,b and c; van Dijk et al. (1983) had reported work of this kind, but the present study also examined serum from species not included in earlier work. (b) what is the pattern of results obtained when the kinetics of complement activation are studied in each animal serum? Although Polhill et al. (1978b) had examined the kinetics of alternative pathway activation in mouse and chicken serum, there had been no systematic study of reaction kinetics using animal sera. It was also important to ask (c), whether the differences in reaction kinetics seen when certain human sera were used could also be found in a larger group of human sera and if so, are they reflected in the results of conventional assays of alternative pathway activity? Finally, (d) since a very simple dilution method had been used in the preliminary studies of the kinetics of alternative
more time-consuming, conventional dilution protocol was investigated.

Total haemolytic complement activity and alternative pathway activity were measured by conventional and kinetic assays. It should be remembered that the former is not a measure of classical pathway activity. Since alternative pathway activity is inevitably recruited when the classical pathway is activated, total haemolytic complement activity represents the combined activity of both pathways. Its measurement does have value nevertheless, in that a comparison of total haemolytic complement activity and alternative pathway activity gives indirect information on classical pathway activity, and on the contribution of the alternative pathway to total complement activity.

3.2 MATERIALS AND METHODS

Total Haemolytic Complement Activity

The sera were tested against sheep erythrocytes sensitised with haemolysins produced in each species. In addition, the total lytic activity was also measured using sheep cells agglutinated with a low (1:10) dilution of each antiserum. After 25μl of diluted haemolysin and sheep cell suspension had been incubated together for 60 minutes to produce agglutination, 50μl of fresh serum was added. The subsequent incubation and examination were done in the usual way.

General Screen of Alternative Pathway Activity.

1% suspensions of erythrocytes from each of the species to be studied were prepared in alternative pathway
diluent. Equal (25μl) volumes of each suspension and each test serum were mixed in a 96 well microtitre plate so that each erythrocyte suspension was tested against each serum. The plate was then incubated in the usual way. In order to eliminate the possibility that the 1:2 dilution used might reduce inhibition of the classical pathway by the alternative pathway diluent, the experiment was also done using double strength alternative pathway diluent (Appendix 1).

Interpretation of Microtitre Plate Assays
A simple criterion of variation used in clinical assays was adopted: differences in titre which exceeded one dilution step were regarded as significant.

3.3 RESULTS
3.3.1 ANIMAL SERA
Total Haemolytic Complement Activity Against Sensitised Cells
The control titre in each experiment (Figure 3.1 to 3.10, left-hand plot) represents the alternative pathway activation by the sheep erythrocytes, while the difference between the titre of total haemolytic complement activity and the control titre indicates the amounts of classical and alternative pathway activity which are expressed after activation of the classical pathway by the sensitised sheep erythrocytes. Chicken serum did not cause a significant increase in lysis, while hedgehog serum gave a 2-3 well increase in the titre of lysis with most cells and hamster serum did so with some: those sensitised with rabbit, guinea pig and bovine haemolysins. Guinea pig complement also caused
substantial increases in the titre of lysis with some antibodies (rabbit, hamster, guinea pig and bovine). Despite the marked lysis of the control cells by badger serum, the combination of badger antibody and badger serum produced the strongest reaction seen in the homologous reactions.

**Total Haemolytic Complement Activity Against Agglutinated Cells**

Interaction of agglutinated erythrocytes with complement from the same species as the antibody causing agglutination produced a spectrum of results (Figures 3.1 to 3.10, right-hand plot). There was little difference in the extent of lysis of sensitised and agglutinated erythrocytes in the hedgehog, guinea pig and rabbit systems, although complete lysis was seen in each well with the sensitised cells, the equivalent wells tended to show only partial lysis in the test using agglutinated cells. A marked increase in total haemolytic complement activity against agglutinated cells was seen with the hamster and bovine systems. The effect was most marked in the case of bovine antibody and complement. In contrast, the use of agglutinating concentrations of antibody abolished total lytic activity in the badger and ferret systems.

In the heterologous reactions most antibodies appeared capable of reacting with most complement systems to the extent shown by the autologous mixtures of antibody and complement. Notable exceptions were the enhanced reactivity of hamster complement with sheep erythrocytes sensitised with rabbit, guinea-pig and bovine
with hamster antibodies, while hamster complement failed to react with hedgehog antibody. Human serum reacted well with most of the sensitised cells, whereas badger and ferret serum reacted poorly.

In some wells showing partial lysis of agglutinated cells there was dissociation of the unlysed agglutinates.

**Kinetics of Total Haemolytic Complement Activity Against Sheep Cells Sensitised With Rabbit Haemolysin**

At a serum dilution of 1:40, complete lysis of was achieved within 4 minutes using ferret, guinea pig badger and hedgehog serum (Table 3.1). It was completed within 7 minutes by human serum and 11 minutes by hamster serum. In all these examples, the lag time was less than 2 minutes, and in the case of the badger and the hedgehog, it was less than 1 minute. Chicken, bovine and murine sera did not show lytic activity at this dilution. Both chicken and bovine serum were able to lysis at a dilution of 1:2, but each showed distinctive kinetics. The reaction curve for chicken serum at this dilution resembled that obtained with the other sera at a 1:40 dilution. While the bovine serum showed a similar lag phase (2.2 minutes), the time to complete lysis was markedly extended (14 minutes). Mouse serum failed to cause lysis of the sensitised cells at either dilution (Table 3.2) and also failed to produce lysis of sheep erythrocytes sensitised with murine antibodies, even at the lowest possible dilution, 1:2. Conversely, the mouse antibodies did fix guinea
cells. Since the main thrust of this work was concerned
with the alternative pathway, this line of investigation
was not pursued further.

Alternative Pathway Activity

General Screen

Rabbit erythrocytes were lysed by all the sera tested
(Table 3.3), so were guinea pig erythrocytes. Hamster
cells reacted with all except human serum, while mouse
serum was activated by all except hamster erythrocytes.

In the assays of total haemolytic complement activity
the control (sheep) cells were lysed to some extent by
all except bovine serum, but this experiment, done in
alternative pathway diluent, only ferret, chicken, and
badger serum lysed the sheep erythrocytes. Apart from
these exceptions, the general pattern was that
erthrocytes from most species reacted with ferret,
chicken, badger and bovine complement, although badger
and ferret erythrocytes showed some additional
reactions. The results obtained using double strength
diluent were identical to those described above.

Comparison of Alternative Pathway Activity

Rabbit erythrocytes, sheep erythrocytes, and where
possible, neuraminidase-treated sheep erythrocytes were
used in a selection of conventional assays. All three
were used in serial dilution assays, while only rabbit
and sheep erythrocytes were used for radial diffusion
assays. Figure 3.11 gives the results of the serial
dilution tests and Table 3.4 the results of the radial
diffusion assays.
The relative activities in the sera tested were graded (Table 3.5), badger serum produced only partial lysis of rabbit erythrocytes at the higher dilutions, while chicken serum did so at all dilutions. When sheep erythrocytes were used, chicken serum showed partial lysis at the higher dilutions. Rabbit serum was not active against sheep erythrocytes.

When neuraminidase-treated sheep erythrocytes were used, the relative activities were similar to those with rabbit cells, but the titres were slightly lower. Finally, each serum was mixed with suspensions of the three types of erythrocyte prepared in complement fixation test diluent rather than the alternative pathway diluent (Table 3.6). Certain anomalies can be seen when these results are compared with those from the initial screen and those of the radial diffusion assay. Human serum exhibited low activity in all analytical systems.

**Kinetics of Alternative Pathway Activity**

A range of serum dilutions was tested against rabbit erythrocytes (Table 3.7). It was necessary to perform the assays in two batches on successive days. Fresh reagents were used on each day and those made for the second day gave slightly lower figures for t50. The values of tL and t50 for the second batch have been weighted to compensate for this variation. When the results of the two batches are considered together, the activities obtained using the reaction mixture 600μl serum : 3000μl red cell suspension may be ranked (Table 3.8).
both $t_\text{L}$ and $t_{50}$ were greater than those seen at a dilution of 1:5 in other species. The extent to which activity could be detected in dilutions greater than 1:5 correlated broadly with the conventional assays of alternative pathway activity, whose endpoints are the limiting dilutions for key components (Table 3.9)

3.3.2 HUMAN SERA

Survey of Activity

Normal human sera were assayed for alternative pathway activity using the conventional titre and radial diffusion assays, and the kinetic method. The reaction mixture for the latter experiment was 100µl serum and 900µl rabbit erythrocyte suspension. The kinetics of alternative pathway lysis varied significantly between samples (Table 3.10) but there was little difference in the results of the conventional assays.

Comparison of Dilution Protocols

The protocols used to assay the human sera DL and PG are given in Table 3.11. The differences in the alternative pathway activity of the two sera (Figure 3.12) were less marked when the total volume of reactants was kept constant. Since the simple method of varying the volume of serum assayed (mixing fixed volume of red cell suspension with different volumes of serum) appeared to have no disadvantages, and was indeed more informative, it was used throughout the subsequent studies.

3.4 DISCUSSION

The wider implications of these results will be examined
made at this point. Most sera gave a sigmoid curve when the lytic reaction in total haemolytic complement activity or alternative pathway haemolytic activity assays were monitored continuously. In practice the lag phase was not always distinctive, especially when activity was low at the chosen dilution. This is not unexpected since the dilutions in question often corresponded to those near the endpoint in serial dilution tests where the statistical probability of protein-protein interactions is minimal. It had been hoped that the rate of lysis (change in $A_{700}$/minute) could be calculated from all the reaction curves obtained in this investigation. In fact, the course of the lytic reaction was seldom linear and the calculation of reaction rates was difficult or impossible. To overcome this problem the derived value ($t_{50}-t_{L}$), the difference between the time to 50% lysis and lag time, was adopted as an index of the efficiency of the lytic reaction. Other workers who have used kinetic assays (Polhill et al., 1978a,b) have restricted themselves to $t_{50}$, but as this is a composite value (lag phase and early lytic reaction) its use can be misleading. In those reactions which gave a poorly defined lag phase and a slow lytic reaction however it was necessary to rely on $t_{50}$ or even the rate of reaction. Fortunately such instances were rare. The time to 100% lysis ($t_{100}$) was not regularly used because of the time involved in measuring it, especially in sera with low complement activities.
The data on the reaction of immune complexes, in this case, sensitised and agglutinated erythrocytes, in which antibodies from one species with complement from a number of other species in assays of total haemolytic complement activity does confirm the feasibility of the traditional complement fixation test. In the second stage of complement fixation tests for the detection of antibodies, immune complexes formed from these antibodies are usually reacted with guinea pig serum complement. When the various immunoglobulins tested were used to sensitise sheep erythrocytes, the lysis which occurred was never very marked, even when guinea pig haemolysin was used to sensitise the sheep erythrocytes. When, however, agglutinated sheep erythrocytes were used instead of sensitised cells, more lysis was seen in each serum. These agglutinated cells were arguably more like the complexes produced in the first stage of a complement fixation test than sensitised cells, since the antigen:antibody ratio in the latter is unusually high. None of the sera examined exhibited more complement activity than guinea pig serum. In the final stage of a complement fixation test, the residual guinea pig complement is titrated using sheep erythrocytes sensitised with rabbit haemolysin. These reacted most effectively with guinea pig serum.

The dissociation of unlysed agglutinates in the presence of complement is reminiscent of the dissociation of immune complexes described in earlier (Takahashi, Takahashi and Hirose, 1980).
Figures 3-1/10
Animal Sera: Total Haemolytic Complement Activity

------ = complete lysis in well

-------- = partial lysis in well
Figure 3-1

Figure 3-2
FERRET SERUM

HAMSTER SERUM

Figure 3-3

Figure 3-4
**BOVINE SERUM**

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>HUMAN</th>
<th>CHICKEN</th>
<th>MOUSE</th>
<th>RABBIT</th>
<th>BADGER</th>
<th>FERRET</th>
<th>HAMSTER</th>
<th>GUINEA PIG</th>
<th>HEDGEHOG</th>
<th>BOVINE Jersey</th>
<th>BOVINE Hereford</th>
<th>CONTROL</th>
</tr>
</thead>
</table>

**RABBIT SERUM**

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>HUMAN</th>
<th>CHICKEN</th>
<th>MOUSE</th>
<th>RABBIT</th>
<th>BADGER</th>
<th>FERRET</th>
<th>HAMSTER</th>
<th>GUINEA PIG</th>
<th>HEDGEHOG</th>
<th>BOVINE Jersey</th>
<th>BOVINE Hereford</th>
<th>CONTROL</th>
</tr>
</thead>
</table>

Figure 3-5: SENSITISED CELLS vs AGGLUTINATED CELLS

Figure 3-6: SENSITISED CELLS vs AGGLUTINATED CELLS
SENSITISED CELLS: not tested
See Kinetic Data, Table 3-1
CHICKEN SERUM

HUMAN SERUM

Figure 3-9

Figure 3-10
Figure 3-11

Animal Sera: Alternative Pathway Activity

NORMAL SERUM vs RABBIT RED CELLS

HUMAN
CHICKEN
RABBIT
BADGER
FERRET
GUINEA PIG
HEDGEHOG
BOVINE Jersey

NORMAL SERUM vs SHEEP RED CELLS

HUMAN
CHICKEN
RABBIT
BADGER
FERRET
GUINEA PIG
HEDGEHOG
BOVINE Jersey

RE & SEC(N) vs NORMAL SERA

HUMAN
CHICKEN
MOUSE
RABBIT
BADGER
FERRET
GUINEA PIG
HEDGEHOG
BOVINE Jersey
Comparison of Dilution Protocols for Human Sera DL & PG

![Comparison of Dilution Protocols for Human Sera DL & PG](image)

- Varying total volume DILUTIONS
- Fixed total volume

- $t_{50}$
- $t_L$
Animal Sera: Kinetics of Total Haemolytic Complement Activity

<table>
<thead>
<tr>
<th>Complement source</th>
<th>Reaction volume (µl)</th>
<th>Time (min) of</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum:SEA</td>
<td>LP</td>
<td>t50</td>
</tr>
<tr>
<td>Human</td>
<td>25:1000</td>
<td>1.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Ox</td>
<td>25:1000</td>
<td>— no activity—</td>
<td>2.2</td>
</tr>
<tr>
<td>Mouse</td>
<td>25:1000</td>
<td>— no activity—</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500:500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster</td>
<td>25:1000</td>
<td>1.8</td>
<td>5.3</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>25:1000</td>
<td>1.1</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>25:250</td>
<td>1.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Ferret</td>
<td>25:1000</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Badger</td>
<td>25:1000</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>25:1000</td>
<td>0.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Fowl</td>
<td>25:1000</td>
<td>— no activity—</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>500:500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SEA: sensitized erythrocytes; LP: lag phase; t50: time to 50% lysis; t100: time to 100% lysis.

Table 3-2

Mouse Serum: Kinetics of Total Haemolytic Complement Activity

Target Cells: Sheep Erythrocytes Sensitised with Mouse Haemolysin. Reaction Mixture: 250µl serum, 250µl cell suspension.

<table>
<thead>
<tr>
<th>Serum</th>
<th>tL</th>
<th>t50</th>
<th>t100 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>C57Black</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>C3H</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>CBA</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>1.7</td>
<td>5.4</td>
<td>10.5</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All mouse sera showed alternative pathway activity.
### Interaction of sera and erythrocytes in alternative pathway diluent (APD)

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Human</th>
<th>Ox</th>
<th>Rabbit</th>
<th>Mouse</th>
<th>Hamster</th>
<th>Guinea-pig</th>
<th>Ferret</th>
<th>Badger</th>
<th>Hedgehog</th>
<th>Fowl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (Group A)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Human (Group B)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ox</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sheep</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mouse</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hamster</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ferret</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Badger</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fowl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*: lysis; +: partial lysis; -: no lysis.
### Lysis of unsensitized erythrocytes in agarose gels

<table>
<thead>
<tr>
<th>Serum</th>
<th>Diameter of lysis (mm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sheep erythrocytes in CFTD</td>
<td>Rabbit erythrocytes in CFTD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>APD</td>
<td>APD</td>
</tr>
<tr>
<td>Species</td>
<td>Volume (μl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Badger</td>
<td>2</td>
<td>7.8</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.3</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.0</td>
<td>7.6</td>
</tr>
<tr>
<td>Ferret</td>
<td>2</td>
<td>7.4</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.3</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.1</td>
<td>8.5</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2</td>
<td>6.6</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.6</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.7</td>
<td>+</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>2</td>
<td>5.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.0</td>
<td>-</td>
</tr>
</tbody>
</table>

CFTD: complement fixation test diluent; APD: alternative pathway diluent.

1Weak positive results; also seen for human and hedgehog against SE in both diluents. Negative results against SE with ox, fowl, hamster and mouse. Against rabbit erythrocytes weak positive results were obtained with human serum (in both diluents) and hedgehog serum (in CFTD only). Negative results with ox, fowl, hamster and mouse.
Animal Sera: Comparison of Activity in Alternative Pathway Serial Dilution Assays

vs RABBIT ERYTHROCYTES

hedgehog

Badger > ferret > chicken > guinea pig > human

ox

vs SHEEP ERYTHROCYTES

Badger ox human

Ferret > chicken > hedgehog > guinea pig
Animal Sera: Haemolytic Activity in Complement Fixation

Test & Alternative Pathway Diluents

vs RABBIT ERYTHROCYTES:
Increase in titre of lysis:
human
rabbit
ferret
hedgehog
bovine
No change in titre of lysis:
badger
chicken
Decrease in titre of lysis:
guinea pig

vs SHEEP ERYTHROCYTES:
Increase in titre of lysis:
human
rabbit
badger
ferret
guinea pig
No change in titre of lysis:
hedgehog
bovine
chicken (1 dilution)

vs NEURAMINIDASE-TREATED SHEEP ERYTHROCYTES:
Increase in titre of lysis:
human(*)
chicken
badger
ferret
guinea pig
hedgehog
bovine(*)
Decrease in titre of lysis:
mouse(**)
rabbit(**)

* no activity in alternative pathway diluent
** activity abolished in complement fixation test diluent
Animal Sera: Kinetics of Alternative Pathway Haemolytic Activity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Species</th>
<th>Volume (μl)</th>
<th>LP</th>
<th>Time (min) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Human female</td>
<td>750</td>
<td>1.7</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>2.5</td>
<td>3.8</td>
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<td>7.0</td>
</tr>
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<td></td>
<td>600</td>
<td>3.5</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>Fowl</td>
<td>750</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>2.2</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>6.0</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>Ferret</td>
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<td>2.3</td>
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<tr>
<td></td>
<td></td>
<td>750</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>0.9</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>1.7</td>
<td>5.4</td>
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</tr>
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<td></td>
<td></td>
<td>75</td>
<td>3.2</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>Guinea-pig</td>
<td>1000</td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750</td>
<td>1.8</td>
<td>4.0</td>
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<td></td>
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<td>600</td>
<td>2.3</td>
<td>6.0</td>
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<td></td>
<td>Mouse</td>
<td>500</td>
<td>3.0</td>
<td>10.5</td>
</tr>
<tr>
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<td>Human female</td>
<td>1000</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750</td>
<td>2.7</td>
<td>4.9</td>
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<td>600</td>
<td>3.7</td>
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<td></td>
<td>Human male</td>
<td>1000</td>
<td>2.2</td>
<td>4.4</td>
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<td></td>
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<td>750</td>
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<td></td>
<td>600</td>
<td>4.0</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>Badger</td>
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<td>1.0</td>
<td>3.0</td>
</tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>4.7</td>
<td>10.3</td>
</tr>
</tbody>
</table>

LP: lag phase; \( t_{50} \): time to 50% lysis; \( t_{100} \): time to 100% lysis.

1 Volume of serum added to 3 ml rabbit E suspension; except for mouse where 500 μl serum was mixed with 500 μl rabbit E suspension.
Table 3-8

Animal Sera: Relative Alternative Activity in Kinetic Assay

<table>
<thead>
<tr>
<th>Animal</th>
<th>BY t VALUE:</th>
<th>BY t50-t VALUE:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ferret</td>
<td>chicken</td>
<td>bovine</td>
</tr>
<tr>
<td>badger</td>
<td>ferret</td>
<td>hedgehog</td>
</tr>
<tr>
<td>hedgehog</td>
<td>bovine</td>
<td>chicken</td>
</tr>
<tr>
<td>bovine</td>
<td>hedgehog</td>
<td>guinea pig</td>
</tr>
<tr>
<td>chicken</td>
<td>badger</td>
<td>hamster</td>
</tr>
<tr>
<td>guinea pig</td>
<td>human</td>
<td></td>
</tr>
</tbody>
</table>

**HIGHEST:LOWEST**

- t VALUE: = 3.8
- t50-t VALUE: = 4.8

**SPAN BETWEEN THEM**

- = 2.5 min.
- = 4.5 min.
<table>
<thead>
<tr>
<th>RECIPROCAL TITRE</th>
<th>ALTERNATIVE PATHWAY HAEMOLYTIC ACTIVITY</th>
<th>DIAMETER (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>log₂ titre</td>
<td>by serial dilution by radial diffusion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vs RE vs SE vs SE(N) vs RE</td>
<td></td>
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<tr>
<td>10</td>
<td>2048</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>1024</td>
<td>9</td>
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<tr>
<td>8</td>
<td>256</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
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<td>7</td>
</tr>
<tr>
<td>6</td>
<td>64, ferret, badger ferret</td>
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</tr>
<tr>
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</tr>
<tr>
<td>3</td>
<td>8, guinea-pig, hedgehog guinea-pig</td>
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</tr>
<tr>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>2, human guinea-pig mouse rabbit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minimal Activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No Activity</td>
<td></td>
</tr>
</tbody>
</table>

- CFTD only

Animal Sera: Comparison of Activity in Alternative Pathway Serial Dilution & Radial Diffusion Assays
## Table 3-10

Human Sera: Comparison of Activity in Alternative Pathway Serial Dilution, Radial Diffusion & Kinetic Assays

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>KINETIC ASSAY</th>
<th>TITRE</th>
<th>RID (mm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>tL</td>
<td>t50</td>
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</tr>
<tr>
<td><strong>FEMALES</strong></td>
<td></td>
<td></td>
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<tr>
<td>PG</td>
<td>1.3</td>
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<td>4</td>
</tr>
<tr>
<td>ST1</td>
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<td>4.2</td>
<td>4</td>
</tr>
<tr>
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<td>4</td>
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<td>ST3</td>
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<td>7.3</td>
<td>4</td>
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<td>4</td>
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<td>ST6</td>
<td>3.6</td>
<td>8.4</td>
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<tr>
<td>ST7</td>
<td>*</td>
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<td>ST8</td>
<td>*</td>
<td>16.2</td>
<td>2</td>
</tr>
<tr>
<td>ST9</td>
<td>0.5</td>
<td>2.7</td>
<td>4</td>
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<tr>
<td>ST10</td>
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<td>4</td>
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<tr>
<td><strong>MALES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL</td>
<td>3.1</td>
<td>6.5</td>
<td>4</td>
</tr>
<tr>
<td>ST11</td>
<td>1.1</td>
<td>3.2</td>
<td>4</td>
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<tr>
<td>ST12</td>
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<td>ST13</td>
<td>4.3</td>
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<td>2</td>
</tr>
<tr>
<td>ST14</td>
<td>*</td>
<td>6.7</td>
<td>4</td>
</tr>
</tbody>
</table>

**MEAN (STANDARD DEVIATION)**

<table>
<thead>
<tr>
<th></th>
<th>Whole Group</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tL</td>
<td>t50</td>
<td>TITRE</td>
</tr>
<tr>
<td></td>
<td>(1.3)</td>
<td>(3.2)</td>
<td>(0.9)</td>
</tr>
<tr>
<td>Heated Serum Caused No Lysis.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Titre:** titre in serial-dilution assay.

**RID:** radius of ring in radial diffusion assay.

* : not discernable
### VARYING TOTAL VOLUME

<table>
<thead>
<tr>
<th></th>
<th>(A)</th>
<th>(B)</th>
<th>(C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM (μl)</td>
<td>500</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td>TARGET CELL SUSPENSION (μl)</td>
<td>500</td>
<td>750</td>
<td>900</td>
</tr>
<tr>
<td>EFFECTIVE DILUTION</td>
<td>1:2</td>
<td>1:4</td>
<td>1:10</td>
</tr>
</tbody>
</table>

### FIXED TOTAL VOLUME

<table>
<thead>
<tr>
<th></th>
<th>(A)</th>
<th>(B)</th>
<th>(C)</th>
<th>(D)</th>
<th>(E)</th>
<th>(F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM (ml)</td>
<td>1.2</td>
<td>0.9</td>
<td>0.6</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>DILUENT (ml)</td>
<td>-</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>DILUTION</td>
<td>-</td>
<td>1:2.7</td>
<td>1:4</td>
<td>1:8</td>
<td>1:12</td>
<td>1:24</td>
</tr>
</tbody>
</table>

Each dilution mixed with 1.2 ml target cell suspension for assay.
4.1 INTRODUCTION

The work described in the previous chapter showed that serum samples from a number of vertebrate species exhibited significant differences in the kinetics of alternative pathway-mediated red cell lysis. These differences could be demonstrated at dilutions which are in the range used for other functional tests of the complement system such as opsonisation assays (Levinsky, Harvey and Paleja, 1978). Before attempting to demonstrate the clinical significance of these observations, an attempt was made to investigate their molecular basis. In the light of present knowledge, a number of possibilities exist:

a. that the phenomena are analytical artefacts or biological artefacts due to the biochemistry of the rabbit erythrocyte surface membrane.

b. that they represent real differences in the efficiency of complement activation which result from differences in the concentration of key complement components. The concentration of each component depends on the balance of synthesis, consumption in immune responses and catabolism by the reticuloendothelial system. Each could be varied by genetic influences, or by environmental factors such as diet. Straightforward techniques for the measurement of biosynthetic and catabolic rates are not available at present, and for this most investigators rely on the measurement of component levels as a general pointer. The relationship
levels of key components was explored with sera from two specific sources each known to be associated with changes in component levels and haemolytic activity. These were patients with rheumatoid arthritis and a group of premature and full-term neonates. Rheumatoid arthritis is an immune complex disease associated with complement consumption and an acute phase reaction (that is, increased production of a number of proteins, including several complement components; Whicher and Chambers, 1984). Thus a variety of changes in the complement system should be found if sera from a group of patients with this disease are examined.

In the case of premature and full-term neonates, a number of reports (Sawyer et. al., 1971; Mills, Bjorksten and Quie, 1979; Strunk, Fenton and Gaines, 1979) have indicated that complement activity at full term (i.e. those infants born at 38 weeks gestation) was between 60% and 90% of adult levels. Furthermore, it was found that premature infants showed a statistically significant correlation between birth weight or gestational age and total haemolytic complement activity. Therefore this study offered a unique opportunity to investigate the relationship between component levels and alternative pathway activity.

c. that the differences in reaction kinetics reflect the presence of differing amounts of "naturally-occurring" antibody to rabbit erythrocytes. While antibody is not an essential requirement for alternative pathway activation, it has been shown to play a part in the
and to modify the kinetics of red cell lysis. Oldstone and his colleagues (Joseph, Cooper and Oldstone, 1975; Perrin et al., 1976) demonstrated that the specific binding of large amounts of antibody to a selection of virus infected cells made possible alternative pathway activation not seen in the absence of antibody, while Tarr et al. (1982) demonstrated a role for antibody in the alternative pathway-mediated killing of Haemophilus influenzae. Moore, Fearon and Austen (1981) came to similar conclusions using mouse erythrocytes as a target of complement activity. In another study, Polhill et al. (1978b) used a kinetic assay of alternative pathway activity to study the effects of antibody on the rate of red cell lysis by the alternative pathway. They demonstrated that a gamma globulin component of serum was able to enhance alternative pathway haemolytic activity, and produced evidence that the component was immunoglobulin. In view of these observations a number of experiments were performed to test the hypothesis that such antibodies might be responsible for the distinctive reaction kinetics of the human sera DL and PG.

First these two sera, and a number of other sera were tested for the presence of antibody. Next, the sera were adsorbed with rabbit erythrocytes to determine the effect of removing any antibody present on alternative pathway activity. The effect of sensitising sheep erythrocyte with antibodies from each of the species studied in the previous chapter on the reaction of the
cells with the alternative pathway in serum from normal animals was also studied.

Generalised immunoglobulin deficiency (hypogammaglobulinaemia) in humans and chickens is said to be associated with a defect in alternative pathway activity which can be reversed by the addition of purified immunoglobulin to the serum **in-vitro** (Polhill et al., 1978b; Johnston, Polhill and Pruitt, 1978). These authors claimed that their observations confirmed the role of antibody in governing the rate at which alternative pathway activity is expressed. The complement system in sera from a number of patients with various forms of hypogammaglobulinaemia was examined extensively to test this hypothesis. Finally an attempt to eliminate the effects of antibodies in human sera during haemolytic assays was made by modifying the surface of human erythrocytes so that they would activate the alternative pathway.

d. that the differences in activity result from differing levels of the low molecular weight inhibitor of complement activity reported by Baker, Parker and Osofsky (1984). This hypothesis was tested by examining the effect of dialysis on the complement activity of the human sera DL and PG.

e. that they result from the presence of allelic forms of key components which impart a distinctive level of activity to the individual's alternative pathway. The outbred nature and varied lifestyle of human populations made the definitive investigation of genetic variants
opportunities to study the products of defined gene pools. The serum alternative pathway activity of selected strains was investigated.

f. the possibility that other mechanisms governed alternative pathway activity remained. In a final attempt to define the basis of the variation in reaction kinetics more clearly, the response of alternative pathway activity in sera DL and PG to a number of dilution procedures was investigated.

4.2 MATERIALS AND METHODS

Rheumatoid arthritis sera

These were provided by the Pathology Department at the Royal Surrey County Hospital, Guildford.

Neonatal Sera

Serum samples were collected from 38 premature and full-term neonates in the Special Care Baby Unit at the Royal Free Hospital London when they were bled for other essential investigations. Gestational ages at birth ranged from 27 to 41 weeks. Where more than one sample was collected from an infant, the ages at which the second and subsequent samples were taken were expressed as a gestational age. Thus a sample taken two weeks after the birth of an infant judged to have been born at 36 weeks gestation was referred to as the 38 week sample.

Clinical Condition of Infant.

Because some diseases cause an acute phase response or complement consumption in-vivo the child’s condition was assessed using the scale shown in Table 4.1 whenever a sample was taken.
Sixteen samples were obtained from the Immunodeficiency Referral Clinic at Northwick Park Hospital, London.

**Standard Sera**

A panel of "normal" adult sera which included PG and DL were used as standards and controls for both the activity assays and the measurement of component levels.

**Standardisation of Activity Assays on Neonatal Sera**

Since the samples were collected over several months and analysed in two large batches, there was some "between-batch" variation in the reactivity of the rabbit erythrocytes used on each occasion with the standard sera. To overcome this the lag time (tL) and (t50-tL) were expressed as functions of those shown by the standard sera. The sensitivity of two functions was compared:

a. % normal adult = \( \frac{\text{lysis time of standard serum (min)}}{\text{lysis time of test serum (min)}} \times 100 \)

b. Difference in Activity = \( \text{lysis of test (min)} - \text{lysis time of standard (min)} \)

**Tanned Red Cells**

Human group 0 negative erythrocytes were treated with tannic using the method described by Hudson and Hay (1982).

**Functional Test for Antibodies to Rabbit Erythrocytes**

Sera were tested for haemolytic activity against rabbit erythrocytes, first in alternative pathway diluent, then in complement fixation test diluent.

**Use of Haemolysins**

The titre of alternative pathway-mediated haemolytic
activity against sheep erythrocytes sensitised with
haemolysins produced in chickens, rabbits, ferrets,
hedgehogs, guinea pigs and cows using the microtitre
plate assay. Each plate was read after incubation at
37° for 2 hours.

4.3 RESULTS
Component Levels and Complement Activity in Rheumatoid
Arthritis Sera
In these rheumatoid sera the properdin levels were
generally reduced, while factor B levels were above the
standard normal adult level (Table 4.2). Few samples
however showed any associated changes in activity. One
serum showed a marked decrease in alternative pathway
activity, but the levels of each of the components
measured fell into the general pattern seen in the rest
of the group. A more detailed statistical evaluation of
this limited study was not considered appropriate.

Neonatal Sera
Preliminary tests indicated that 100ul of neonatal serum
and 300ul of erythrocyte suspension gave suitable
reaction curves. A summary of the results is presented
in Table 4-3, with the appropriate statistical analyses.
Lag time (tL) and (t50-tL) generally parallel each other
between 27 and 41 weeks (Figure 4.1). The earliest
results (27 weeks) for tL and (t50-tL) were both between
20 and 40% of adult levels, but they reached the levels
shown for most of pregnancy (tL ca. 60% ; (t50-tL) ca.
50%) by 30 weeks. Generally speaking the ± two standard
deviation limit was still below 100% adult levels
although sometimes close to it. One individual, PCI,
The levels of properdin (Figure 4.3), factor I and factor H (Figure 4.4) and Cl-esterase inhibitor (Figure 4.5) changed relatively little, whatever the clinical condition of the infants. In contrast, the levels of C3 (Figure 4.2), factor B (Figure 4.3) and C4 (Figure 4.5) showed considerable variations between samples and between individuals. The changes in C3 and factor B levels did not appear to have a great deal of effect on total alternative pathway activity. It was apparent that the clinical classification used in this study did not correlate with the perturbations in C3, factor B and C4 levels, nor with the less dramatic changes in activity. There was no significant pattern in clinical grades of the infants recorded as each sample was taken (Table 4.3).

The Effects of Antibody

Examination of the human sera used for the survey of alternative pathway activity described in section 3.3 failed to reveal significant amounts of haemagglutinin in any of the samples. When however, the indirect immunofluorescence technique was used, it became apparent that some immunoglobulin had bound to the cells at low dilutions (1:2 to 1:16).

In the functional test for antibody the human sera DL and PG showed enhanced activity against rabbit erythrocytes in complement fixation test diluent (Table 4.4). After adsorption with rabbit erythrocytes at 4°C, both exhibited reduced alternative pathway activity
The titres to which alternative pathway activity was expressed against sheep erythrocytes sensitised with each of the haemolysin antisera are shown in Figures 4.8 to 4-16. They may be summarised as follows:

a. HUMAN SERUM (Figure 4.6): this only showed activity against sheep erythrocytes at a dilution of 1:2. When the cells were sensitised with chicken antiserum this activity was unaltered. Sensitisation with all the other antisera abolished lysis.

b. CHICKEN SERUM (Figure 4.7): this lysed unsensitised sheep cells to a titre of 1:16. All the sensitised cells gave the same result.

c. RABBIT SERUM (Figure 4.8): this did not lyse sheep erythrocytes. Only cells sensitised with hamster antibodies induced alternative pathway activity, to a titre of 1:2.

d. BADGER SERUM (Figure 4.9): unsensitised sheep cells were lysed to a titre of 1:64. Cells sensitised with badger antiserum increased this titre to 1:128, as did those sensitised with chicken, mouse, ferret and hedgehog antisera. Guinea pig antibodies did not affect the activity of badger serum, while the others used (rabbit, hamster and bovine antibodies), increased the titre of activity to 1:256.

e. FERRET SERUM (Figure 4.10): the control cells were lysed to a serum titre of 1:64. Badger and hamster antibodies did not alter this result, but those raised in guinea pig reduced it to 1:32. The remaining sensitised cells (including those produced using ferret
f. HAMSTER SERUM (Figure 4.11): the control cell titre was 1:64. Chicken, guinea pig and hedgehog antibodies decreased it to 1:8, while those from mouse, ferret and hamsters themselves decreased it to 1:4. Badger antibodies only produced lysis at 1:2, while the rabbit antiserum abolished activity. Only the bovine antiserum failed to affect activity.

g. HEDGEHOG SERUM (Figure 4.12): in this instance sheep cell lysis occurred to a titre of 1:32. The only deviation from this value by the sensitised cells was in the case of those prepared with the antiserum raised in a hedgehog, as well as those from ferret and guinea pig. Each reduced the titre of activity to 1:16.

h. GUINEA PIG SERUM (Figure 4.13): a variety of results was obtained. The unsensitised sheep cells were lysed to a serum dilution of 1:512. The presence of badger antibodies on the cells failed to alter this result, while those from the hamster and Jersey cow serum caused a marked reduction in activity (titre 1:2). Guinea pig antibodies were less inhibitory (titre 1:8). Cells sensitised with mouse, ferret and hedgehog antisera were lysed to a titre of 1:16, while those treated with the antisera from the rabbit and the Hereford cow lysed to 1:64.

i. BOVINE SERUM (Figure 4.14): this was not active against unsensitised sheep cells, and none of the sensitised cells elicited alternative pathway activity.

Hypogammaglobulinaemic Sera
No gross deficiencies of haemolytic activity were seen
individual components were rare, marked increases were common. Antibodies to rabbit erythrocytes were common; titres ranged between 0 and 1:256. There was no obvious relationship between these titres and total alternative pathway activity.

Use of Modified Human Erythrocytes

Neuraminidase treated human erythrocytes failed to activate the alternative pathway. Tanned red cells were lysed very slowly by serum at a 1:2 dilution and did not provide the basis of an assay for alternative pathway activity.

Low Molecular Weight Inhibitor

Dialysis reduced total haemolytic complement activity and alternative pathway haemolytic activity (Table 4.8)

Inbred Mouse Serum

Significant differences (Table 4.9) were demonstrated in the alternative pathway activity of Balb/c, C57 black, C3H and CBA mice of both sexes.

Other Factors

Dilution of the human sera DL and PG with each other, and with a variety of diluents used in complement studies (Table 4.10) produced a number of changes in alternative pathway haemolytic activity (Figures 4-15 and 16). When either serum was diluted with complement fixation test diluent (Figure 4.16a) there was a steady decrease in the alternative pathway activity. The lag time (tL) shown by undiluted DL was greater than that of PG. Dilution increased tL in both sera, but the change was less marked in serum PG. In
measured by the difference between the time to 50% lysis and lag time (t50-tL), was more susceptible to dilution in serum PG. When each individual's serum was diluted with autologous serum which had been heat inactivated (Figure 4.15b), there was little effect on the response of tL to dilution. While lytic activity was still abolished at the same dilution (1:2), heated serum diluent caused smaller changes in (t50-tL) in lower dilutions of DL than the complement fixation test diluent. When serum PG was diluted with heated serum both tL and (t50-tL) were little changed at the lower dilutions and some activity was detectable in all dilutions. When serum PG was used to dilute serum DL (Figure 4.15b), enhanced activity was seen in all mixtures. The use of heat inactivated PG to dilute serum DL protected alternative pathway activity. and this phenomenon was also seen when heated DL was used to dilute serum PG (Figure 4.16a). Finally, diluents produced by warming the sera to 50° for 10 minutes (nominally factor B depleted sera), generally enhanced activity (Figure 4.16b) in the way that native serum had done (Figure 4.15b). The curves showing the effect of each dilution protocol on lag time (tL) are very similar, and only the dilution of native DL with factor B depleted DL gives a distinctive pattern for the variation of (t50-tL) with dilution.

4.4 DISCUSSION

The differences which had been observed between the
associated with different concentrations of certain components, but it is seldom possible to measure all the components in each sample because of limits to the availability of samples or reagents, especially if feral animal sera are studied. This investigation was not exempt from such restrictions, and some compromises had to be made. In the sera from patients with rheumatoid arthritis the number of components measured was deliberately limited. For the work on neonatal serum samples C3, factor B and properdin together with the control proteins factor I and factor H were chosen as determinants of alternative pathway activity; a reliable factor D assay was not available at this time. C4 and Cl esterase inhibitor levels were used to give some indication of the state of the classical pathway in particular and of complement protein metabolism in general. The limited amount of serum obtained from many infants made further investigations impractical. In any event, measurement of the remaining components would have caused an unacceptable increase in the cost of the work.

As noted earlier the significance of titrations of total haemolytic complement activity and alternative pathway haemolytic activity as indices of complement activity can be questioned. These techniques determine the titre of the component or components whose individual functions can be diluted out first; the relative activity of each stage of complement activation cannot be determined. In addition, the commonly used two-fold
for a relatively insensitive analytical technique. The kinetic assays do however offer greater sensitivity. The efficiency with which the lytic reaction is initiated and completed can be compared at a single dilution, so that differences in activity which would not be detected by the serial dilution methods can be studied with ease. Furthermore, if activity is studied at two or more dilutions, the effect of this progressive dilution on activity can also be studied.

Rheumatoid arthritis subjects were chosen because this disease is associated with an acute phase reaction that is increased synthesis of some complement components, and the activation of complement in diseased tissues, but the results suggested that there was no consistent effect on alternative pathway activity.

In neonates it had been shown (Sawyer, et al.; Mills, Bjorksten and Quie; Strunk, Fenton and Gaines; op. cit.) that complement component levels and haemolytic activity were 60 to 100% of adult levels in infants born "at term", that is 40 weeks gestation, and somewhat less in premature infants. Such infants should therefore provide samples with an interesting range of activities which could be compared with the levels of key components and inhibitors. The results presented here confirmed the earlier observations, and indicated that the use of kinetic assays did provide a more sensitive index of alternative pathway haemolytic activity and made it possible to compare the maturity of the reaction to assemble C5 convertase (lag time, tL) and those which
The expression "% standard serum" proved more sensitive than "difference in activity" (Figure 4.1). Inevitably, there was a bias to the selection of unhealthy subjects in this group of infants, but the use of a clinical grading system went some way towards allowing for the effects of disease on the complement system. It can be seen however, that a healthy infant, as defined by this classification was not exempt from perturbations in the complement system, nor did all sick infants always have abnormal results.

A small but significant number of premature infants develop a life-threatening bowel disorder known as necrotising enterocolitis (British Association for Perinatal Paediatrics and Public Health Laboratory Service, Communicable Disease Surveillance Centre, 1983). Since the aetiology of this syndrome remains unclear it was hoped that this prospective study of neonatal complement might throw some light on this important clinical problem. The acute phase responses noted earlier were not unique to those infants who developed necrotising enterocolitis, and the condition was not associated with a unique pattern of complement activity.

Finally, the experiment in which two human serum samples were subjected to a number of dilution procedures did yield some information on the determinants of alternative pathway activity. Points of particular interest were (i) the enhanced activity seen when the two sera were mixed, (ii) the way in which serum PG
during the course of dilution with heat-inactivated PG, and (iii) the decrease in \( (t_{50} - t_L) \) seen when DL was diluted with heat-inactivated DL was not apparent when factor B depleted DL was used, suggesting the involvement of factor B in this phenomenon. The addition of purified components to serum samples might be a valuable development of this procedure.
Figure 4- 1/5

Neonates: Results of Complement Assays
4-1 ALTERNATIVE PATHWAY ACTIVITY

4-2 C3 LEVELS
4-5 C4 & C1INH LEVELS

% NA

0 100 200 300

C1INH

C4

30 35 40
Figures 4-6/14

Animal Sera: Alternative Pathway Activity Against Sensitised Cells

THCA : Total Haemolytic Complement Activity

APA  : Alternative Pathway Haemolytic Activity
HEDGEHOG SERUM vs SENSITISED CELLS

GUINEA PIG SERUM vs SENSITISED CELLS

Diagram showing the comparison of HEDGEHOG and GUINEA PIG SERUM against Control, Mouse, Rabbit, Badger, Ferret, Hamster, Guinea Pig Hedgehog, Bovine Jersey, and Bovine Hereford. The graph displays the log reciprocal titre values.
BOVINE SERUM vs SENSITISED CELLS

Figure 4-14
Figures 4-15/16

Human Sera: Dilution with Selected Reagents
Neonates: Criteria for Each Clinical Grade

GRADE 0
Infants were well/very well, had rickets or apnoea when less than 28 weeks gestation requiring no more than minimal intervention.

GRADE 1
Included infants with apnoea when less than 28 weeks gestation requiring mild intervention (i.e. stimulation), or apnoea when more than 28 weeks gestation. Also uncomplicated bradycardias of any nature; respiratory distress grade 1; loose stools including those secondary to phototherapy, or mild tachyopnoea of the newborn.

GRADE 2
Included bradycardia secondary to apnoea; idiopathic apnoea requiring ventilation; uncomplicated consolidation of one lobe of lung and respiratory distress syndrome grade 2. Also, loose stools for reasons other than phototherapy, other causes of transient tachyopnoea of the newborn and minor skin inflammations.

GRADE 3
Infants with secondary apnoea requiring ventilation, and those with more than one lung lobe consolidated. Respiratory distress syndrome greater than grade 2.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Activity tL t50 (min)</th>
<th>Component Levels (% Standard Adult)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1.7 2.4</td>
<td>C3 115 B 148 P 60 H 117 C4 98</td>
</tr>
<tr>
<td>A2</td>
<td>4.2 10.2</td>
<td>C3 116 B 128 P 71 H 112 C4 60</td>
</tr>
<tr>
<td>A3</td>
<td>1.8 2.6</td>
<td>C3 113 B 140 P 54 H 115 C4 97</td>
</tr>
<tr>
<td>A4</td>
<td>1.0 1.3</td>
<td>C3 125 B 145 P 54 H 129 C4 103</td>
</tr>
<tr>
<td>A5</td>
<td>1.6 2.4</td>
<td>C3 118 B 158 P 63 H 112 C4 90</td>
</tr>
<tr>
<td>A6</td>
<td>0.9 1.3</td>
<td>C3 108 B 147 P 54 H 93 C4 123</td>
</tr>
<tr>
<td>A7</td>
<td>&lt;0.4 0.5</td>
<td>C3 83 B 141 P 71 H 93 C4 96</td>
</tr>
<tr>
<td>A8</td>
<td>1.6 2.8</td>
<td>C3 136 B 125 P 74 H 134 C4 113</td>
</tr>
<tr>
<td>A9</td>
<td>0.9 1.4</td>
<td>C3 130 B 196 P 77 H 131 C4 103</td>
</tr>
<tr>
<td>A10</td>
<td>1.8 3.2</td>
<td>C3 78 B 101 P 83 H 100 C4 22</td>
</tr>
<tr>
<td>A11</td>
<td>1.0 2.4</td>
<td>C3 106 B 98 P 91 H 107 C4 60</td>
</tr>
<tr>
<td>A12</td>
<td>2.8 3.5</td>
<td>C3 125 B 120 P 57 H 122 C4 102</td>
</tr>
<tr>
<td>A13</td>
<td>2.2 4.0</td>
<td>C3 125 B 120 P 57 H 129 C4 61</td>
</tr>
<tr>
<td>A14</td>
<td>1.4 1.9</td>
<td>C3 120 B 140 P 34 H 124 C4 #</td>
</tr>
</tbody>
</table>

STANDARD SERA

| DL     | 1.2 1.6               | C3 100 B 100 P 100 H 100 C4 100   |
| PG     | <0.2 0.3              | C3 - B - P - H - C4 -             |

*Reaction Mixture: 150μl serum, 500μl cell suspension

# : result not available
<table>
<thead>
<tr>
<th>No.</th>
<th>Age (days)</th>
<th>IL</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>C4 ClqClh</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>10</td>
<td>0239</td>
<td>05</td>
<td>02</td>
<td>01</td>
<td>02</td>
<td>03999999</td>
<td>226208</td>
</tr>
<tr>
<td>16</td>
<td>9</td>
<td>0371</td>
<td>02</td>
<td>05</td>
<td>02</td>
<td>06</td>
<td>05999999</td>
<td>290682</td>
</tr>
<tr>
<td>17</td>
<td>9</td>
<td>0391</td>
<td>02</td>
<td>05</td>
<td>02</td>
<td>06</td>
<td>06999999</td>
<td>390682</td>
</tr>
<tr>
<td>18</td>
<td>9</td>
<td>0392</td>
<td>02</td>
<td>05</td>
<td>02</td>
<td>06</td>
<td>06999999</td>
<td>390682</td>
</tr>
<tr>
<td>19</td>
<td>9</td>
<td>0393</td>
<td>02</td>
<td>05</td>
<td>02</td>
<td>06</td>
<td>06999999</td>
<td>390682</td>
</tr>
<tr>
<td>20</td>
<td>9</td>
<td>0394</td>
<td>02</td>
<td>05</td>
<td>02</td>
<td>06</td>
<td>06999999</td>
<td>390682</td>
</tr>
</tbody>
</table>

Table 4.3
Neonates: Results of Complement Assays
(a) Data
<table>
<thead>
<tr>
<th>tL</th>
<th>t50</th>
<th>t100</th>
<th>B</th>
<th>P</th>
<th>C3</th>
<th>H</th>
<th>I</th>
<th>C4</th>
<th>CLNH</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>92</td>
<td>54</td>
<td>70</td>
<td>71</td>
<td>78</td>
<td>78</td>
<td>79</td>
<td>77</td>
<td>41</td>
</tr>
</tbody>
</table>

**VALID OBSERVATIONS**

**MISSING OBSERVATIONS**

**MEAN**

**STANDARD ERROR**

**STD. DEVIATION**

**KURTOSIS**

**SKEWNESS**

**CORRELATION COEFFICIENTS vs GESTATIONAL AGE**

<table>
<thead>
<tr>
<th>FACTOR</th>
<th>COEFFICIENT</th>
<th>SLOPE</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>-0.28</td>
<td>-0.017</td>
<td>0.016</td>
</tr>
<tr>
<td>P</td>
<td>0.25</td>
<td>0.045</td>
<td>0.018</td>
</tr>
<tr>
<td>C3</td>
<td>-0.11</td>
<td>-0.013</td>
<td>0.177</td>
</tr>
<tr>
<td>H</td>
<td>0.30</td>
<td>0.044</td>
<td>0.004</td>
</tr>
<tr>
<td>I</td>
<td>-0.17</td>
<td>-0.037</td>
<td>0.076</td>
</tr>
<tr>
<td>C4</td>
<td>-0.29</td>
<td>-0.025</td>
<td>0.006</td>
</tr>
<tr>
<td>C1 INHIBITOR</td>
<td>-0.03</td>
<td>-0.0059</td>
<td>0.435</td>
</tr>
</tbody>
</table>

**CORRELATION COEFFICIENTS vs tL**

| B      | 0.37        | 0.15  | 0.00099      |
| P      | 0.09        | 0.10  | 0.34         |
| C3     | 0.34        | 0.26  | 0.0017       |
| H      | 0.14        | 0.13  | 0.121        |
| I      | 0.42        | 0.59  | 0.00006      |
| C1 INHIBITOR | 0.33 | 0.53  | 0.018        |

**CORRELATION COEFFICIENTS vs t50**

| B      | 0.41        | 0.20  | 0.00026      |
| P      | 0.14        | 0.20  | 0.12         |
| C3     | 0.41        | 0.39  | 0.00012      |
| H      | 0.06        | 0.07  | 0.29         |
| I      | 0.28        | 0.49  | 0.006        |
| C4     | 0.35        | 0.25  | 0.00099      |
| C1 INHIBITOR | 0.34 | 0.62  | 0.015        |

**SPEARMAN CORRELATION COEFFICIENT (no assumptions on distribution)**

Significant associations (p = 0.001)

| tL with: C3, 0.44; t50, 0.77 |
| t50 with: C3, 0.47; t100, 0.88; B, 0.55; I, 0.39 |
| t100 with: C3, 0.50; C4, 0.47 |
| B with: C4, 0.42; grade: 0.37 |
| P with: H, 0.51 |
| C3 with: C4, 0.46; CLNH, 0.64 |
| C4 with: CLNH, 0.64 |

**Table 4.3**

Neonates: Results of Complement Assays

**(b) Statistical Analyses**
### Human Sera DL & PG: Functional Tests for Antibody Activity

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ACTIVITY IN APD</th>
<th>ACTIVITY IN CFTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM DL</td>
<td>tL 2.5</td>
<td>tL 0.6</td>
</tr>
<tr>
<td></td>
<td>t50 5.5</td>
<td>t50 0.8</td>
</tr>
<tr>
<td>SERUM PG</td>
<td>tL 1.4</td>
<td>tL 0.2</td>
</tr>
<tr>
<td></td>
<td>t50 3.2</td>
<td>t50 0.4</td>
</tr>
</tbody>
</table>

#### Table 4-5

Human Sera DL & PG: Effect of Adsorption on Complement Activity

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tL</td>
</tr>
<tr>
<td>SERUM DL - UNTREATED</td>
<td>0.7</td>
</tr>
<tr>
<td>ADSORBED</td>
<td>0.9</td>
</tr>
<tr>
<td>SERUM PG - UNTREATED</td>
<td>0.3</td>
</tr>
<tr>
<td>ADSORBED</td>
<td>0.4</td>
</tr>
</tbody>
</table>
# Hypogammaglobulinaemic Sera: Results of Complement & Other Assays

<table>
<thead>
<tr>
<th>ALTERNATIVE PATHWAY ACTIVITY (min)</th>
<th>THCA (min)</th>
<th>COMPONENT LEVELS (% normal adult)</th>
<th>OTHER RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td>tL t50 (t50-tL) t50</td>
<td>C3 B P I H D C4 CLINH</td>
<td>anti-RE IgG IgA IgM</td>
</tr>
<tr>
<td>1 1.1 3.1 2.0 3.0</td>
<td>102 139 205 106 123 122 131 124</td>
<td>8 # # #</td>
<td>g/l</td>
</tr>
<tr>
<td>2 1.5 5.1 3.6 4.4</td>
<td># 140 94 103 99 111 152 109</td>
<td>128 4.8 &lt;0.2 0.5</td>
<td></td>
</tr>
<tr>
<td>3 2.5 5.3 2.8 3.5</td>
<td>91 138 186 110 122 120 168 109</td>
<td>128 # # #</td>
<td></td>
</tr>
<tr>
<td>4 1.5 1.8 0.3 2.7</td>
<td>100 133 198 75 108 116 160 109</td>
<td>256 10.3 3.4 1.1</td>
<td></td>
</tr>
<tr>
<td>5 1.0 2.4 1.4 4.0</td>
<td>105 124 # 98 124 120 71 98</td>
<td>256 9.4 &lt;0.2 1.1</td>
<td></td>
</tr>
<tr>
<td>6 0.9 2.0 1.1 2.5</td>
<td>106 125 155 97 104 96 162 #</td>
<td>8 2.2 0.1 0.4</td>
<td></td>
</tr>
<tr>
<td>7 0.5 4.4 3.9 2.8</td>
<td>112 147 # # 133 122 281 #</td>
<td>64 # # #</td>
<td></td>
</tr>
<tr>
<td>8 1.2 2.4 1.2 4.0</td>
<td>100 113 155 # 106 113 77 142</td>
<td>64 2.6 &lt;0.2 0.3</td>
<td></td>
</tr>
<tr>
<td>9 0.4 4.2 3.8 3.3</td>
<td>121 197 105 78 132 113 270 91</td>
<td>2 &lt;1.2 &lt;0.2 0.2</td>
<td></td>
</tr>
<tr>
<td>10 1.1 2.8 1.7 2.8</td>
<td>96 119 136 146 103 116 167 98</td>
<td>2 &lt;1.5 &lt;0.2 &lt;0.1</td>
<td></td>
</tr>
<tr>
<td>11 1.0 2.5 1.5 2.8</td>
<td>115 222 136 # 123 109 256 100</td>
<td>256 2.4 &lt;0.1 0.3</td>
<td></td>
</tr>
<tr>
<td>12 1.2 4.3 3.1 4.6</td>
<td>86 111 155 103 100 98 95 138</td>
<td>256 &lt;4.8 0.3 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>13 0.5 5.8 5.3 3.4</td>
<td>85 121 102 111 113 100 92 91</td>
<td>NIL # #</td>
<td></td>
</tr>
<tr>
<td>14 1.1 4.1 3.0 3.8</td>
<td>82 93 125 110 88 104 33 93</td>
<td>NIL # # #</td>
<td></td>
</tr>
<tr>
<td>15 1.5 1.9 0.4 3.5</td>
<td>86 121 214 199 123 113 129 198</td>
<td>256 9.9 &lt;0.1 0.6</td>
<td></td>
</tr>
<tr>
<td>16 0.5 4.4 3.9 3.8</td>
<td>109 114 # # 166 84 72 #</td>
<td>256 # #</td>
<td></td>
</tr>
<tr>
<td>SERUM DL 100/500: 1.1 4.8 3.7 10ul 3.9</td>
<td>100 100 100 100 100 100</td>
<td>256 - - -</td>
<td></td>
</tr>
<tr>
<td>50/500 2.5 9.1 6.6 20ul 4.7</td>
<td>- - - - - -</td>
<td>- - -</td>
<td></td>
</tr>
<tr>
<td>SERUM PG 100/500: 0.9 2.3 1.4</td>
<td>- - - - - -</td>
<td>- - -</td>
<td></td>
</tr>
<tr>
<td>50/500 1.1 5.3 4.2</td>
<td>- - - - - -</td>
<td>- - -</td>
<td></td>
</tr>
</tbody>
</table>

Key: THCA - Total Haemolytic Complement Activity.
anti-RE - Titre of Rabbit Erythrocyte Agglutinins.

Reaction Mixtures: Alternative Pathway, 100µl serum + 500µl cell suspension
THCA, 10µl serum + 1000µl cell suspension

# : result not obtained
- : assay not performed
Table 4-8

Human Sera DL & PG: Effect of Dialysis on Complement Activity

(A) ALTERNATIVE PATHWAY ACTIVITY.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>tL</th>
<th>t50</th>
<th>t100 (MIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM DL - UNTREATED</td>
<td>0.7</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>DIALYSED</td>
<td>&gt;10.0</td>
<td>&gt;10.0</td>
<td></td>
</tr>
<tr>
<td>SERUM PG - UNTREATED</td>
<td>0.3</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>DIALYSED</td>
<td>&gt;10.0</td>
<td>&gt;10.0</td>
<td></td>
</tr>
</tbody>
</table>

(B) TOTAL HAEMOLYTIC COMPLEMENT ACTIVITY

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>tL</th>
<th>t50</th>
<th>t100 (MIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERUM DL - UNTREATED</td>
<td>2.5</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>DIALYSED</td>
<td>6.3</td>
<td>&gt;20.0</td>
<td></td>
</tr>
<tr>
<td>SERUM PG - UNTREATED</td>
<td>1.8</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>DIALYSED</td>
<td>5.0</td>
<td>9.1</td>
<td></td>
</tr>
</tbody>
</table>
Table 4-9
Inbred Mouse Sera: Kinetics of Alternative Pathway
Haemolytic Activity

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>tL</th>
<th>t50</th>
<th>t50-tL (MIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c, male</td>
<td>2.7(0.28)</td>
<td>12.8(3.20)</td>
<td>10.1</td>
</tr>
<tr>
<td>Balb/c, female</td>
<td>6.5(0)</td>
<td>12.7(2.33)</td>
<td>6.2</td>
</tr>
<tr>
<td>C3H/He, male</td>
<td>4.0(0.07)</td>
<td>9.0(1.34)</td>
<td>5.0</td>
</tr>
<tr>
<td>C3H/He, female</td>
<td>7.0(0.71)</td>
<td>12.0(0.78)</td>
<td>5.0</td>
</tr>
<tr>
<td>C57Bl., male</td>
<td>2.8(0.28)</td>
<td>9.4(2.83)</td>
<td>6.6</td>
</tr>
<tr>
<td>C57Bl., female</td>
<td>6.8(1.06)</td>
<td>11.4(0.64)</td>
<td>4.6</td>
</tr>
<tr>
<td>CBA, male</td>
<td>3.2(0.50)</td>
<td>8.3(1.41)</td>
<td>5.1</td>
</tr>
<tr>
<td>CBA, female</td>
<td>7.7(0.28)</td>
<td>11.8(0.28)</td>
<td>4.1</td>
</tr>
</tbody>
</table>

6 mice of each sex & strain bled, 2 pools of sera, each from 3 animals, prepared. Each pool assayed in duplicate. Value in brackets is Standard Deviation.

Reaction Mixture: 500µl serum + 500µl rabbit erythrocyte suspension.
Human Sera DL & PG: Protocol for Dilution with Selected Diluents

<table>
<thead>
<tr>
<th>MIXTURE (SAMPLE/DILUENT)</th>
<th>SAMPLE (μl)</th>
<th>DILUENT (μl)</th>
<th>TUBE NUMBERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL/PG</td>
<td>400 300 200 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL/CFTD</td>
<td>1-1 1-2 1-3 1-4</td>
<td>100 200 300</td>
<td></td>
</tr>
<tr>
<td>DL/HDL</td>
<td>2-1 2-2 2-3 2-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL/WDL</td>
<td>3-1 3-2 3-3 3-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG/CFTD</td>
<td>4-1 4-2 4-3 4-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG/HPG</td>
<td>5-1 5-2 5-3 5-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG/WPG</td>
<td>6-1 6-2 6-3 6-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG/HPG</td>
<td>7-1 7-2 7-3 7-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL/WPG</td>
<td>8-1 8-2 8-3 8-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG/HDL</td>
<td>9-1 9-2 9-3 9-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG/WDL</td>
<td>10-1 10-2 10-3 10-4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

KEY TO DILUENTS:
- CFTD: complement fixation test diluent
- HDL or HPG: serum heated at 56°C for 30 minutes
- WDL or WPG: serum heated at 50°C for 10 minutes
5.1 INTRODUCTION

Inbred mice are frequently used in attempts to elucidate the genetic basis of immune responsiveness to purified antigens and microorganisms (Gasser and Silvers, 1974; Krco and David, 1980; Rosentreich, Weinblatt and O´Brien, 1982). The majority of these studies examine the role of lymphocyte-mediated responses such as antibody production, or delayed hypersensitivity reactions. Skamene and his colleagues have made extensive use of inbred mice in their investigations of host responses to a variety of microorganisms (Forget, et. al., 1981; Gros, et. al., 1981; Stevenson, et. al., 1981; Pelletier, et. al., 1982; Skamene, et. al., 1982). Their work with the vaccine strain of *Mycobacterium bovis* (BCG), defined each strain of mice examined as resistant or susceptible to BCG on the basis of the number of organisms which could be recovered from the spleen of each animal after intravenous doses of BCG (Gros, Skamene and Forget, 1981). Their data suggested that a component of the immune system acting early in the infection determined the extent of spleen colonisation. The investigation of the kinetics of alternative pathway-mediated erythrocyte lysis by sera from a selection of inbred mouse strains described in the previous chapter revealed significant differences between some of the strains, and between the sexes of each strain. It can be argued that such variations in alternative pathway activity are responsible for the patterns of spleen colonisation reported by Skamene's
Studies of this kind are rarely possible in humans. Instead, much use is made of in-vitro correlates of immunological functions such as the opsonisation tests described earlier. Abnormal opsonisation associated with the immunodeficiency syndrome seen in some neonates is thought to be due to a defect in the alternative pathway yet the conventional tests of alternative pathway haemolytic activity are seldom abnormal. This may reflect the insensitivity of these tests, but the defect in opsonisation may not interfere with the assembly of lytic complexes. Serum samples from patients with one of a number of primary and secondary liver diseases also show a significant incidence of opsonisation defects (Larcher et. al., 1983). Table 5-1 summarises these findings. A selection of sera taken from children with such defects were analysed for alternative pathway activity using the kinetic method to establish whether defective opsonisation was associated with a distinctive change in the kinetics of alternative pathway haemolytic activity.

5.2 THE ALTERNATIVE PATHWAY IN MYCOBACTERIAL INFECTIONS IN MICE.

5.2.1 MATERIALS AND METHODS

Bacterial Suspensions

BCG suspensions were prepared from a commercially
The contents of 10 ampoules were suspended in sterile saline, pooled, and made to 40ml with sterile saline. *Mycobacterium fortuitum* was obtained from a culture collection held at the Central Veterinary Laboratory. It was grown in liquid culture (Chadwick 1982), and the harvested slurry of microorganisms washed and diluted in sterile saline to give the required suspensions. The actual number of organisms present in all the suspensions was determined by titration.

**Mice**

All mice were purchased from Olac Ltd (Bicester, Oxon, U.K.) when 8-9 weeks old. Males and females from each of the following strains were used: Balb/c, C3H/He, C57Black, and CBA.

In the first experiment (subcutaneous injection of BCG in each sex and strain), the members of each sex and strain were divided into 5 groups of 6 mice designated A, B, C, D and E. Group A, the control animals received 0.1ml sterile saline in the left shoulder; all other groups were given 0.1ml BCG suspension in the same site. Control animals were then housed separately from the test animals. The experimental groups from each sex and strain were examined on the following days after injection: A (controls), day 4; B, day 1; C, day 3; D, day 7; and E, day 28 (Table 5.2).

In the second experiment (intravenous challenge with BCG or *M. fortuitum*), males and females from the C57Black and C3H strains were used. In this case the experimental groups were: F, (controls); G, (BCG,
(BCG examined at 12h); M. fortuitum examined at 12h; J, M. fortuitum examined at 36h). The mice in each group were given 0.1ml of the appropriate suspension in a caudal vein (Table 5.3).

**Collection of blood and tissue samples.**

On the day of sampling, each animal was anaesthetised with ether and weighed. Blood was collected by cardiac puncture. In the subcutaneous challenge experiment, the lymph nodes which drained the injection site and the spleen were removed from each animal and weighed. The spleen alone was removed from the mice which received intravenous challenges. Within each group of animals, blood samples, lymph nodes and spleens were examined in pools of three for ease of handling, e.g. mice 1-3, 4-6, and so on.

**Culture of lymphoid tissue pools.**

Each group of organs was homogenised in sterile saline using Griffiths tubes. Ten-fold serial dilutions of each homogenate were made in sterile saline over the range 1:10 to 1:10^6. Duplicate 0.2ml aliquots of each dilution were then cultured on Lowenstein-Jensen slopes enriched with pyruvate (Chadwick, 1982). All cultures were set up on the day of sampling, incubated at 37° and examined at regular intervals for 6 weeks.

**Serum samples**

Blood samples were kept on iced water until the collection of specimens was completed. They were allowed to clot at room temperature. After centrifugation at 4°, the serum was separated and stored at -70°. Total alternative pathway activity was
measured using the kinetic assay; 500 µl 38% 500µl rabbit erythrocyte suspension). The readings were taken until the lytic reaction was complete, or 20 minutes had elapsed without evidence of cell lysis.

5.2.2 RESULTS

Subcutaneous Challenge

The results are given in Figures 5.1 to 5.4. Replicate cultures of lymphoid organs showed poor agreement. Nevertheless, certain clear differences were seen, and when these are examined in conjunction with the data on organ weights it is possible to compare the kinetics of the inflammatory response in each sex and strain. In most cases lymph node size increased steadily; the smallest change at 28 days were seen in C3H/He (male and female) and in CBA females. Intermediate weights were by Balb/c females and CBA males, while C57Black males and females responded most markedly. The Balb/c males showed a distinctive peak of lymph node enlargement at 3 days and a steady decline thereafter.

In all cases there was significant lymph node colonisation in days 1-7, and a decline in the number of organisms by day 28. Early spleen colonisation, cleared by day 28, was seen in CBA males and females, while the spleens of C57Black females were colonised throughout the experiment. Late colonisation was seen in the Balb/c male.

There was no obvious correlation between total alternative pathway activity and the time-course of spleen and lymph node infection, although all female mice showed lag time (tL) values which were longer than
the time to 50% lysis (t50) was also larger (extended times indicate lower complement activity). Low alternative pathway activity was not necessarily associated with spleen colonisation. In the Balb/c male, significant spleen counts were seen when the serum alternative pathway activity was greatest. The most striking feature of these results is the marked reduction in t50 seen in the serum of Balb/c and CBA males. In both cases this change in t50 is due to a marked decrease in (t50-tL). The CBA male also showed a small reduction in tL, but this is not significant. The kinetic assay is a simple bioassay which indicates relative complement activity. Therefore complex statistical analyses of the data could be considered inappropriate. It is possible to regard the differences in alternative pathway activity as significant if they exceed the 95% confidence limits, that is + two standard deviations (Strike, 1981). The results were however also analysed using SPSS programmes (Table 5.4).

Intravenous Challenge

Although the culture method lacked some precision, the differences in alternative pathway activity seen in control animals did not affect the magnitude of spleen colonisation. In the C3H/He males, the presence of M. fortuitum and BCG in the spleen had little effect on alternative pathway activity (Figures 5.5 - 5.6). The C3H/He females however, showed transient changes in t50. Associated changes in tL were not considered significant. In the C57Black males, there was a
marginal decrease in t50 values during \textit{M. \textit{fortuitum}} infection, while BCG appeared to cause complement consumption (increased t50). \textit{M. \textit{fortuitum}} also produced a transient change in t50 in C57Black females, but BCG produced a marked and continuing effect. Spleen weights showed little variation in any group. Detailed statistical analysis was not considered appropriate for these results.

5.3 ALTERNATIVE PATHWAY LYtic ACTIVITY IN SERA SHOWING DEFECTIVE OPSONISATION

5.3.1 MATERIALS AND METHODS

Serum Samples:

Childhood Liver Disease

Samples were obtained from the Liver Unit at King's College Hospital, London. They were grouped as follows:

a. children without demonstrable complement defects - (childhood controls, 5 subjects).

b. geriatric adults without demonstrable complement defects - (geriatric controls, 4 subjects).

c. children with sickle cell disease - (5 Subjects).

d. children with chronic active hepatitis - (3 subjects).

e. children with an innate deficiency of alpha-1-antitrypsin deficiency - (5 subjects).
Neonatal Opsonisation Defect

One of the sera examined in the work described in the previous chapter showed abnormal opsonisation activity. It was included in this investigation as a positive control.

Adult Liver Disease

These were obtained from patients awaiting surgery for obstructive jaundice due to malignant disease or gallstones at the Royal Surrey County Hospital, Guildford.

5.3.2 RESULTS

Alternative Pathway Activity:

Childhood Liver Disease

The reaction mixture used for neonatal sera (100μl serum + 300μl cell suspension) proved unsuitable, higher dilutions were required because of the higher alternative pathway activity in children and adults. These were: A, 50μl serum + 500μl cell suspension (effective dilution 1:11); B, 50μl serum + 1000μl cell suspension (effective dilution 1:21); C, 25μl serum + 1000μl cell suspension (effective dilution 1:41).

Results obtained using mixture A are shown in Figure 5.7 (time to 50% lysis, t50) and Figure 5.8 (lag time, tL, and (t50-tL)). The sera from children with biliary atresia caused instantaneous lysis, but the activity of the other sera could be measured at this dilution. Furthermore, when the sera from children with these
and C, the alternative pathway activity was lost (Figure 5.9). Only sera from patients with severe liver damage showed decreased lytic activity. Reaction mixture B proved suitable for the analysis of serum from children with extra-hepatic biliary atresia, and many still exhibited activity when reaction mixture C was used (Figure 5.10). The spectrum of activity seen using reaction mixture B is compared with the clinical condition of the children in Table 5.5.

**Neonatal Opsonisation Defect**

Alternative pathway activity was normal in this serum (Table 5.6)

**Adult Liver Disease**

Adults with obstructive jaundice also showed increased alternative pathway activity (Table 5.7)

### 5.4 DISCUSSION

In the mouse experiments, the poor reproducibility of bacterial counts was at first attributed to the tendency of BCG suspensions to aggregate (Brown and Brown, 1982). Hall's recent comments (1985) on the dangers of making assumptions about pathways of lymphoid drainage may also have some bearing on this matter.

Only in C57black mice was there a marked difference in the early uptake of organisms between males and females after subcutaneous injection of BCG. This difference was not seen when mice of this strain were given an intravenous dose of BCG. Although the complement activity of male and female mice in each strain is different, the natural affinity of mycobacteria for the
would seem to ensure that entry into these target cells precedes effective complement activation. The report by Johnson et al. (1980) is of interest in this respect.

In a study of 92 human subjects with active tuberculosis, they found that sera from 13 (14%) exhibited defective opsonic activity. Unlike those with normal activity, none of these patients developed extra-thoracic foci of infection. These results would suggest a role for complement-mediated opsonisation in the spread of mycobacteria. Discussion of the role of the alternative pathway in the development of the later stages of inflammatory responses (that is, 7 days and after) since lymphocyte activation would then be established. More intricate experiments would be needed to discern the role of alternative pathway activity.

The results obtained from the analysis of sera from human neonates suggested that acute phase responses (which involve enhanced production of complement components) do not affect alternative pathway haemolytic activity. Nevertheless, it could be suggested that the differences in alternative pathway activity seen in mice given BCG reflect differences in the response of the macrophage-interleukin 1-hepatocyte axis to BCG infection. Measurement of the murine acute phase protein SAP (Pepys, 1982) could be used to test this hypothesis. It has, however, been suggested (Pepys, 1985) that a much larger experiment would be needed to ensure reliable results because of the variability of SAP results in mouse populations. Analysis of the serum
provide reliable data on SAP levels. Since the effects of BCG infection differ so markedly according to strain of BCG used (Orme and Collins, 1983a), it would also be interesting to repeat this work using other strains of this organism.

Impaired opsonisation was not associated with retarded red cell lysis. Where however, the sera possessed increased opsonic activity (Larcher, et al., 1983) a marked acceleration of alternative pathway-mediated haemolysis was seen. All the subjects suffered from some form of biliary obstruction. The significance of these observations is underlined by the fact that these sera caused lysis at dilutions which abolished the lytic activity of normal sera. Morrison et al (1984) reported markedly increased concentrations of alternative pathway components in these circumstances. More recent work in this Department (Ahmed and Langley, 1985) suggests that only modest increases in C3 and factor B occurs in the serum of adults with obstructive jaundice. As this work also suggests that in-vivo activation of C3 occurs in some of these patients, it can be argued that the enhanced lysis was a bystander effect due to pre-existing alternative pathway activation. Increased levels of endotoxin from Gram-negative gut flora are often found in sera from these patients (Pain 1985). As noted earlier, endotoxins are potent activators of the alternative pathway.

A programme of further investigations is in progress. Three important questions are:
b. do bile, and its principal components such as bilirubin and the bile salts, affect macrophage function?
c. do C3d levels reflect an enhanced turnover of the alternative pathway in-vivo?

Other findings include the demonstration of normal alternative pathway activity in sickle cell disease, in contrast to earlier reports of deficiencies (Koethe, Casper and Rodey, 1976; Wilson, Thompson and Sissons, 1979; Larcher et al., 1982). In the other liver diseases studied alternative pathway activity was generally similar to that shown by the control sera, although severe loss of hepatocellular function caused reduced alternative pathway haemolytic activity.
Effects of Subcutaneous BCG in Mice

Inoculum: $10^5$ organisms
ORGAN WEIGHT INDICES & CULTURES

Figure 5-3

BALB/c

log c.f.u.

male

weight indices

Figure 5-4

C3H

c.f.u.: colony forming units

(i.e. colony count).

C57 BLACK

log c.f.u.

male

weight indices

CSA

Days after Injection

0 1 3 7 28

Days after Injection

0 1 3 7 28

Days after Injection

0 1 3 7 28

Days after Injection

0 1 3 7 28
Effects of Intravenous BCG & M. fortuitum in Mice

Inoculum: $10^4$ organisms

In Figure 5-5, the graph shows the spleen weight index and $t_{50}$ (time to 50% of the original inoculum) for both male and female C57 black and C3H mice at different time points (0, 12, 36 hours). The c.f.u.: colony forming units (i.e. colony count).
c.f.u.: colony forming units
(i.e. colony count).
Childhood Liver Disease: Alternative Pathway Activity
(As Time to 50% Lysis, t50)), Mixture A

[Diagram with data points and labels]

- Young Children
- Elderly
- Controls

Time (min) categories: <1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, >20
Childhood Liver Disease: Alternative Pathway Activity
(As Lag Time (tL) & (t50-tL)), Mixture A

Figure 3-9

- EHBA
- CAH
- AAD
- SCD
- CHILDREN
- GERIATRIC ADULTS
- YOUNG ADULTS

Time (min):

< 1  2  4  6  8  10  12  14  16  18  20  > 20

139
Childhood Liver Disease: Alternative Pathway Activity
(As Lag Time (tL) & (t50-tL)). Mixtures B & C
Figure 3-10
Biliary Atresia: Alternative Pathway Activity, Mixtures

B & C
## Liver Disease: Changes Reported in Complement System

<table>
<thead>
<tr>
<th>RESULT</th>
<th>FHF</th>
<th>CAH(U)</th>
<th>CAH(T)</th>
<th>INH</th>
<th>AAD</th>
<th>EHBA</th>
<th>NORMAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opsonisation Index</td>
<td>1.6 (0.6)</td>
<td>2.5</td>
<td>3.0</td>
<td>4.3</td>
<td>5.4</td>
<td>5.6</td>
<td>4.0 (1.2)</td>
</tr>
<tr>
<td>C3 level</td>
<td>52% (16%)</td>
<td>50%</td>
<td>down</td>
<td>normal↑ normal normal normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4 level</td>
<td>39%</td>
<td>-</td>
<td>down</td>
<td>normal↑ normal normal normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3d level</td>
<td>nil</td>
<td>nil</td>
<td>↑</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td></td>
</tr>
<tr>
<td>THCA</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APA</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td></td>
</tr>
<tr>
<td>functional B</td>
<td>&lt;45%</td>
<td>&lt;25%</td>
<td>&lt;50%</td>
<td>normal</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>:</td>
<td>:</td>
<td>opsonisation normal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>opsonisation normal</td>
</tr>
</tbody>
</table>

**KEY:**

*(After Lewis et al. 1983)*
Mice of both sexes from each strain (Balb/c, C3H/He, C57black and CBA) divided into 5 groups of 6 mice:

<table>
<thead>
<tr>
<th>Injection</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline:</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCG</td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Control animals housed separately after injection

Examined on

Day: 4 1 3 7 28

Blood taken by cardiac puncture under ether anaesthesia,
Lymph nodes draining injection site (2), and spleen weighed & cultured

---

**Table 5-3**

Intravenous BCG and M. fortuitum in Inbred Mice:

Mice of both sexes from two strains (C57black and C3H) divided into 5 groups of 12 mice

<table>
<thead>
<tr>
<th>Injection</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline:</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCG</td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Control animals housed separately after injection

Examined at

(hours) 24 12 36 12 36

Blood taken by cardiac puncture under ether anaesthesia,
Spleen weighed & cultured
**Table 5-4**

**Statistical Analysis of Data from Inbred Mouse Experiments**

Subcutaneous Injection of BCG

The data were analysed using the SPSS analysis of variance programme on the University of Surrey computer (Significant results p < 0.001).

<table>
<thead>
<tr>
<th>COMPLEMENT DATA</th>
<th>tL</th>
<th>Significance of F</th>
<th>t50</th>
<th>Significance of F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degrees of Freedom</td>
<td>F</td>
<td></td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>One-Way Interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>breed</td>
<td>3</td>
<td>42.5</td>
<td>0.000</td>
<td>26.6</td>
</tr>
<tr>
<td>sex</td>
<td>1</td>
<td>723.9</td>
<td>0.000</td>
<td>30.8</td>
</tr>
<tr>
<td>day</td>
<td>3</td>
<td>14.4</td>
<td>0.000</td>
<td>3.5</td>
</tr>
<tr>
<td>Two-Way Interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>breed-sex</td>
<td>3</td>
<td>44.4</td>
<td>0.000</td>
<td>7.5</td>
</tr>
<tr>
<td>breed-day</td>
<td>9</td>
<td>7.6</td>
<td>0.000</td>
<td>5.4</td>
</tr>
<tr>
<td>sex-day</td>
<td>3</td>
<td>7.0</td>
<td>0.001</td>
<td>5.3</td>
</tr>
<tr>
<td>Three-Way Interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>breed-sex-day</td>
<td>9</td>
<td>5.9</td>
<td>0.000</td>
<td>3.8</td>
</tr>
</tbody>
</table>

**OTHER DATA** (not given in detail; see page 131)

Organ Cultures:
- Lymph Nodes: significant F values in all except sex-day two-way interaction.
- Spleen: results not significant.

Spleen Weights: Only showed significant F value in one-way interaction with breed.

Lymph Node Weights: Significant F value with breed and day in tests of one- and two-way interaction.

**FOOTNOTE**

The data from exploratory experiments such as these can be analysed very effectively using the methods of Dunnett and Tukey (Denenberg, 1976). This was not possible with these results because the way in which the experiment had been designed.

* : especially chapter 8.
<table>
<thead>
<tr>
<th>SUBJECT NUMBER</th>
<th>CORRECTIVE SURGERY ON BILIARY TRACT?</th>
<th>CLINICAL CONDITION.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No Surgery</td>
<td>Very jaundiced, died</td>
</tr>
<tr>
<td>2</td>
<td>Yes, postoperative sample</td>
<td>No longer jaundiced</td>
</tr>
<tr>
<td>3</td>
<td>Yes, postoperative</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Yes, postoperative sample</td>
<td>Poor, but not jaundiced</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Yes, sample taken immediately after operation</td>
<td>Good</td>
</tr>
<tr>
<td>7</td>
<td>No surgery</td>
<td>Reassessed, not biliary atresia</td>
</tr>
<tr>
<td>8</td>
<td>Yes, preoperative sample</td>
<td>Poor, but did well after surgery</td>
</tr>
<tr>
<td>9</td>
<td>No surgery</td>
<td>Poor, died</td>
</tr>
</tbody>
</table>


Method of Levinsky, Harvey and Paleja (1978)

Infant PC1  (Results of Complement Assays, Table 4.2)

(a) Screening Test

Opsonisation Indices (mean number of yeasts per neutrophil)

At recommended dilution:

- Normal Adult (DL) 2.4
- Infant PC 1.4
- No Serum 1.0

(b) Opsonisation Indices: Range of Serum Dilutions

<table>
<thead>
<tr>
<th>DILUTION</th>
<th>SERUM DL</th>
<th>INFANT PC</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>nil</td>
<td>3.7</td>
<td>3.0</td>
<td>-</td>
</tr>
<tr>
<td>1:5</td>
<td>3.1</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>1:10</td>
<td>2.9</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>no serum</td>
<td>-</td>
<td>-</td>
<td>1.03</td>
</tr>
</tbody>
</table>
Obstructive Jaundice in Adults: Alternative Pathway

Activity

<table>
<thead>
<tr>
<th>Subject</th>
<th>tL</th>
<th>t50</th>
<th>t50-tL</th>
</tr>
</thead>
<tbody>
<tr>
<td>50µl serum/1000µl cell suspension (1:21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JE</td>
<td>2.0</td>
<td>2.7</td>
<td>0.7</td>
</tr>
<tr>
<td>TA</td>
<td>4.5</td>
<td>6.1</td>
<td>1.6</td>
</tr>
<tr>
<td>BR</td>
<td>6.3</td>
<td>7.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Normal Adult (DL)</td>
<td>&gt;15.0</td>
<td>&gt;15.0</td>
<td>-</td>
</tr>
<tr>
<td>Normal Adult (PG)</td>
<td>9.0</td>
<td>11.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>tL</th>
<th>t50</th>
<th>t50-tL</th>
</tr>
</thead>
<tbody>
<tr>
<td>100µl serum/1000µl cell suspension (1:11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Adult (PG)</td>
<td>1.5</td>
<td>7.0</td>
<td>5.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>tL</th>
<th>t50</th>
<th>t50-tL</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>JE</td>
<td>2.0</td>
<td>2.7</td>
<td>0.7</td>
</tr>
<tr>
<td>TA</td>
<td>4.5</td>
<td>6.1</td>
<td>1.6</td>
</tr>
<tr>
<td>BR</td>
<td>6.3</td>
<td>7.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Normal Adult (DL)</td>
<td>&gt;15.0</td>
<td>&gt;15.0</td>
<td>-</td>
</tr>
<tr>
<td>Normal Adult (PG)</td>
<td>9.0</td>
<td>11.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>tL</th>
<th>t50</th>
<th>t50-tL</th>
</tr>
</thead>
<tbody>
<tr>
<td>100µl serum/1000µl cell suspension (1:11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Adult (PG)</td>
<td>1.5</td>
<td>7.0</td>
<td>5.5</td>
</tr>
</tbody>
</table>
It is important to set the relevance of kinetic analyses in perspective. The haemolytic activity of complement is commonly measured by making serial dilutions of each sample and finding the highest dilution at which lysis occurs after a fixed incubation period. This is usually fifteen to sixty minutes. A general indication of the total haemolytic activity or alternative pathway haemolytic activity is obtained in this way, and gross deviations from normal are easily detected. Essentially however, the analyst only measures the titres of components whose biological activities are most susceptible to dilution. The concentrations and identities of these limiting components are not revealed.

This approach is adequate for the preliminary assessment of residual complement activity in diseases associated with complement consumption \textit{in-vivo}. If however, the investigator wishes to explore the functional efficiency of the complement system in greater detail, then dilution techniques have little value.

The need to establish the nature of complement consumption in diseased subjects is important, but are comparisons of the efficiency of complement activation also worthwhile? The case for such investigations was presented in the introduction to this work, and the subsequent demonstration of a spectrum of activation within human populations and between a variety of animal populations confirms its relevance.

It must be stressed that complement generates
case of the alternative pathway can substitute for lymphocyte-mediated immunity in the early stages of infection. Both classical and alternative pathways work together to provide a powerful extension of antibody function once classical pathway activation has occurred. Even though deficiencies of many complement components are not life-threatening, they do have a significant effect on the quality of life. Yet is it reasonable to argue that delay in the expression of complement activity, particularly that of the alternative pathway, in individuals with "normal" titres of haemolytic activity would have an effect on the course of an inflammatory reaction? Situations in which alternative pathway activity would have a vital role have been described already, but why should a few minutes delay in its expression matter?

Firstly, the ability of the alternative pathway to contain the replication of a dividing inoculum of bacteria could be influenced by the rate at which activation occurred. Also, since the blood volume of most animals passes around the vascular system in a few minutes (Strand, 1983), failure to express adequate alternative pathway activity against a blood-borne pathogen within that time would allow secondary infection of vital organs to occur unchecked. This kind of spread is a common feature of virus infections (Taussig, 1984b). Conversely, the ease of expression of complement activity in response to the deposition of immune complexes in key organs may determine the extent...
immune complex disease. Indeed, the recent reports that complement may inhibit (Naama, et. al., 1983) or reverse (Takahashi, Takahashi and Hirose, 1980) the formation of immune complexes may mean that the system plays a more fundamental role in the pathogenesis of immune complex disease - that is making it possible for the damaging complexes to form in significant amounts in susceptible individuals. Finally, convincing reports that some product of C3 activation can inhibit a number of lymphocyte responses have appeared in recent years (reviewed by Weiler, et. al., 1982), thus an individual whose complement system activated rapidly might generate localised levels of this product which would reduce the extent of lymphocyte proliferation. Furthermore, since the alternative pathway is now thought to turn over continuously in normal individuals, held in check by the inhibitory factors I and H, it can be argued that a particularly reactive pathway might produce a environment richer in the inhibitory fragment of C3, with generalised effects on the kinetics of lymphocyte responses. The fragment of C3 known as C3d provides a useful index of complement consumption in-vivo since it is catabolised slowly (Vergani, et. al., 1983). The spread of C3d levels in normal subjects reported by these workers lends support to the suggestion that the rate of alternative pathway turnover can vary. Although it may be simplistic to consider the alternative pathway acting alone in the control of blood-borne infections, a strong case emerges for the investigation of the kinetics of complement activation.
The growing awareness of a dynamic equilibrium between microorganisms and their vertebrate hosts has replaced the more rigid concept of pathogenic and non-pathogenic organisms. The clinical spectra of diseases such as tuberculosis and leprosy (Lenzini, Rottoli and Rottoli, 1977) suggest a spectrum of immune responsiveness within apparently normal populations, and close examination of many of the more common infectious diseases reveals a similar pattern. A careful characterisation of the general kinetics of immune responsiveness might enable us to speak with greater confidence on resistance and susceptibility to infectious diseases and diseases of the immune system. An examination of the kinetics of complement activation is an important part of this work. Which of the biochemical reactions or biological activities of the complement pathways should be studied in this way? At this stage a firm answer is not possible, but since the haemolytic assays are easy to perform, and lysis involves the whole pathway in both alternative and classical pathway activation they were thought to be a good starting point.

In the kinetic assays of haemolytic complement activity lag time was thought to reflect the efficiency of the reaction which assembles effective C5 convertase complexes on the cell surface, at the dilution in question. On the other hand, \((t_{50}-tL)\) was thought to be a measure of the ability to sustain this convertase activity and the efficiency with which the terminal components \((C5b, C6, C7, C8 \text{ and } C9)\) associate to form the lytic complex and damage the cell membrane.
If the kinetics of haemolysis are studied at a number of dilutions the effects of dilution on lag time and (t50-tL) can be studied. These dose-response curves proved useful in general comparisons of alternative pathway kinetics, while assays at a single dilution were sometimes used to compare the effects of a particular treatment or disease on alternative pathway activity. The traditional serial dilution assays look at complement activity in discontinuous steps. A study of the kinetics of alternative pathway-mediated haemolysis at various dilutions provides a way of removing this discontinuity and increasing the sensitivity of haemolytic assays.

Reports of earlier work on the kinetics of alternative pathway activation (Gallin, Clark and Frank, 1975; Polhill et al., 1978a,b) were found during the course of this work, but they did not investigate the effects of dilution on reaction kinetics nor did they attempt a comprehensive investigation of the phenomena reported. The data on the kinetics of alternative pathway activity against rabbit erythrocytes revealed the interesting fact that differences in (t50-tL) were most marked in human sera. The relevance of these observations might be questioned because they are only apparent when the sera were diluted 1:10 or more; at a 1:2 dilution most sera gave similar results. Perhaps they were merely in-vitro artefacts, but such dilutions are used in tests of accepted clinical significance such as the opsonisation assays (Levinsky, Harvey and Paleja, 1978). The fact that conditions have to be manipulated to
invalidate the results of these tests and such differences may be of greater significance in the study of the alternative pathway in tissue fluids. Although the recent work of Ezekowitz and his colleagues (1984) offered the model for the distribution of complement activity in the tissues described in the first chapter, the traditional view is that the greater part of this activity arises by transudation of complement components from the blood plasma. Most of the components are thought to be at significantly lower levels in the tissues. It is known that the ratio of large to small proteins is lower in tissue fluids than in blood plasma (Hay, 1979), so that there may be differential accumulation of complement components in the tissue fluids according to their molecular weight. This adds an interesting new dimension to the question of complement activity in the tissues. If molecules such as immunoglobulin or low molecular weight inhibitor do modify complement activity, their differential passage across capillary walls might accentuate any differences in complement activity in the blood plasma and the tissue fluids. The spread of molecular weights seen in the complement components may also affect this distribution.

Both Ezekowitz (Ezekowitz, et. al., 1984) and Whaley (1980) have shown that cells of the monocyte-macrophage system are able to secrete the components of the alternative pathway. This led Ezekowitz to suggest that these cells use the alternative pathway proteins which they produce to generate opsonins, and possibly
surrounded by a halo of enhanced alternative pathway availability. Activation of these components would produce local vascular changes and cause exudation of plasma proteins, including complement components and other immunological effectors. In this model, the tissue fluids may be seen as containing a background of relatively low complement activity with areas of higher activity around the macrophages, and in areas of complement activation. These proposals raise the interesting question of the origins of the alternative pathway components found in the blood plasma. Are they derived from tissue macrophages, hepatocytes (Ramađori, et. al., 1984), other tissues (Thompson 1983) or a combination of sites? Since activated macrophages secrete interleukin 1 which enhances the secretion of acute-phase proteins by hepatocytes (Dinarello, 1984), macrophages may have a fundamental role in the control of serum complement levels.

In any event, the final test of the hypothesis that the differences in alternative pathway haemolytic activity detected in-vitro are expressed in the body fluids in-vivo would involve a comparison of the inflammatory responses to alternative pathway activators in-vivo. Ideally, family studies should be made to ascertain the genetic element in these observations but the ethical and logistical constraints on such exercises in human populations would make it impossible to include a systematic study of this kind in the work described here.
Recent discussions of the potential protective immunity and the result of skin tests in diseases such as tuberculosis (Orme and Collins, 1983b) point to the practical benefits of such an investigation. A significant part of the skin test reaction may have more to do with complement activation than with delayed hypersensitivity reactions to tuberculin (Black, Niven and Humphrey, 1963).

In the study of the kinetics of total haemolytic complement activity and haemolytic alternative pathway activity in animal sera (Higgins and Langley, 1985), the benefits of these simple in-vitro assays were readily appreciated. A substantial body of information was obtained on the relative activity of the complement system in each of the species examined. As noted earlier, a complete investigation of the complement system would require antisera to individual components to measure their concentrations and erythrocytes bearing partially assembled complement complexes to study the biological activity of each component. Although they did not provide the same degree of insight, these kinetic assays did give information on the assembly and activity of functional complement complexes.

Measurement of total haemolytic complement activity and alternative pathway haemolytic activity on the same sample also proved informative. Effective lysis in the former argued against any defects in the terminal complement components (C5 to C9) which are common to both pathways. This clarified the interpretation of alternative pathway kinetics.
The results might be challenged because of the possibility that some species are phylogenetically close to the rabbit, while others are more distant. In other words, the distinctive reaction profiles reflect evolutionary differences in the extent to which the rabbit erythrocyte can bind to the alternative pathway C3 convertase of each species rather than absolute differences in alternative pathway activity. The expression of alternative pathway activity against the panel of cells used in the general screen, and the spread of results for the titre of total alternative pathway activity argue against this suggestion, particularly when the results obtained with neuraminidase-treated sheep erythrocytes are considered. Analysis of the alternative pathway haemolytic activity in rat serum (Langley and Bach, 1985) has also helped to refute this suggestion. Despite the close phylogenetic relationship between the rabbit, the rat and the mouse, rat sera showed similar activity to human serum in the kinetic assay, whereas mouse serum displays poor activity.

The limited number of individual samples examined in the screen of alternative pathway activity in various animal sera was unavoidable, but when larger numbers of badger (Heath, 1984) and bovine sera (Langley and Ray, 1984) were examined a remarkable similarity was seen in the results within each group. This contrasts with the spectrum of activity found in human sera. This difference might reflect the geographical constraints on wild and domesticated animals which would make them
a diet which along with many other environmental factors, varied little. External influences on complement metabolism would therefore be limited. In contrast, humans have greater mobility and ingenuity, and hence a much greater scope for outbreeding and for producing varied environments. Modern medicine has also reduced the constraints on genetic diversity which operate in feral populations.

A clear distinction between genetic and environmental influences could not be made although the data obtained from inbred mice of both sexes suggest a powerful genetic influence: lag time (tL) varied to some extent between the strains examined, while (t50-tL) showed marked differences. The differing activities shown by sera from male and female mice within each strain raise the interesting possibility of genetic differences which are not primarily concerned with the complement system.

Hormonal differences have been shown to determine the levels of certain classical pathway components in mouse serum (Ferreira, Weisz-Carrington and Nussensweig, 1978), but their relevance to the control of alternative pathway activity remains to be established. It has been reported recently that the properdin gene is X-linked in humans (Lachmann, 1982). A number of authors have discussed the implications of genetic variants of complement components (Rittner and Bertrams, 1981; Hauptmann, 1982; Davis, 1983; Porter, 1983), and the recent advances in genetic manipulation offer an exciting new approach to research in this area (Carroll,
post-translational processes can produce protein variants (Williamson, Salamari and Kreth, 1973). Speculations on the effects of environmental factors on complement activity could take many forms. One intriguing possibility is raised by the apparent association between a primarily carnivorous diet and a potent alternative pathway. Badgers, ferrets and hedgehogs showed this pattern of activity, although there were some differences between them. Could it be that some component of the carnivorous diet modifies the activity of the macrophage-interleukin 1-hepatocyte-complement axis? For the present this must remain mere conjecture.

Some incidental and relevant information was obtained from these experiments on badger serum. Epidemiological evidence has suggested that badgers were responsible for outbreaks of bovine tuberculosis in certain parts of the United Kingdom (Wilesmith, et. al., 1982; Little, et. al., 1982a,b), and the idea had developed that the badger was in some way susceptible to Mycobacterium bovis and perhaps immunodeficient (Barrow, 1982). Indeed, Higgins and Gatrill, (1984) showed that the badger responded poorly to a number of antigens which elicited high antibody titres in the rabbit, although there is at present, no firm evidence of defective T cell function (Higgins, 1984). During the preparation of the haemolysin reagents used in this work, both badgers and ferrets produced low titres of antibodies in response to immunisation with sheep erythrocytes. The similarity of these results and that
is intriguing. Perhaps the erythrocytes were rapidly destroyed by the combined actions of the alternative pathway and the reticuloendothelial system. On the other hand, this phenomenon may have been the result of complement-mediated suppression of B lymphocyte response in the badger and the ferret. The ferret might provide a valuable model of the badger's immune system. Since the alternative pathway is so potent in an animal which, if not excessively susceptible to *M. bovis*, certainly contracts the infection, one might ask if the alternative pathway offers any protection against mycobacteria. As noted earlier, several members of this bacterial genus activate the alternative pathway, but if they were susceptible to serum-mediated killing, the blood-borne spread of the organisms should be minimal. Conversely, complement-mediated opsonisation of mycobacteria could hasten their entry to their main "target tissue", the cells of the macrophage-monocyte system. Thorns et al. (1982) have reported that when hedgehogs are infected with *M. bovis*, the organisms become disseminated through the body fluids without adverse effects on the animals. In the kinetic assay of alternative pathway haemolytic activity, hedgehog serum was as potent as that of badgers and ferrets.
human infant is capable of mounting an acute phase reaction. In these reactions, C3, factor B, and C4 levels can increase dramatically, whereas properdin, factor I and factor H levels remain unchanged. The increased availability of C3 and factor B had little effect on alternative pathway activity. It may be inferred that these components are not key determinants of activity once adequate levels are achieved, since their levels increased when inhibitor levels were unchanged or decreased, without significant effects on haemolytic activity. This observation prompted consideration of the relative concentration of complement components. In biochemical studies this would involve calculation of molar concentrations. Such an analysis of the complement system is not apparent in the literature. In Table 1-6 published values for molecular weight and component concentrations in human serum were used to calculate molar concentrations. This approach shows the complement system in a new light. Potential rate-limiting steps can be recognised, while many components are present in substantial amounts. Interestingly, careful examination of the results obtained from the analysis of neonatal sera suggests that increases in C3 and factor B may reduce alternative pathway activity in the kinetic assay. So, it was not possible to identify the effect of individual components had on alternative pathway activity, since in general the levels of individual components develop together. While the statistical
the concentration of a number of components, they cannot pinpoint limiting components. It would seem that the analysis of any clinical samples is unlikely to define the determinants of differences in reaction kinetics - it may suggest contenders but the complexity of the samples analysed militates against more precise observations. In retrospect, the idea that it could do so seems naive. Perhaps the approach used by Muller-Eberhard and Schreiber (1980) offers the only effective course. Purification of the components and the analysis of the activity of the pathway reconstituted from these purified components in-vitro. In this way permutations of component concentrations could be tested. Because of the possibility that allelic forms of each component exist within the population, it would be necessary to have a panel of donors to provide starting material for the purification. It is clear that this would be a major undertaking, and as described, would not take account of the effects of other serum components on complement activity. Lachmann, Hobart and Aston (1973) have described a variety of techniques to deplete the concentration of individual components from serum samples, and these are widely used in clinical investigations and basic research. Some of them were used in this project, but they were not totally effective. For example, the technique for factor B depletion (50° for 10 minutes), left residual alternative pathway activity, while longer periods at
complement components. Complete removal or inactivation of a component by methods of this type is unlikely, and the extent to which the other components are affected is uncertain. In addition, some, such as the method for factor D depletion, involve dilution of the sample or modification of its general composition (ionic strength, pH). This must introduce uncontrollable, hence undesirable, changes which could easily affect activity assays.

The availability of monoclonal antibodies to complement proteins appears to offer a more reliable method of neutralising or removing selected components. To avoid the effects on the general composition of samples just described, the use of monoclonal antibodies bound to some solid-phase such as the side of a microtitre well or the surface of polystyrene beads is attractive. Addition of monoclonal antibodies to serum samples should also be avoided because although the selected component may be neutralised, the formation of immune complexes in-situ will affect the complement system via the classical or alternative pathways. Even the use of solid-phase bound antibodies may cause problems since some antibody-coated surfaces activate the alternative pathway (Joseph, Cooper and Oldstone, 1975; Perrin et. al., 1976, Polhill et. al., 1978a,b; Moore, Fearon and Austen, 1981; Tarr et. al., 1982).

Oldstone's group clearly showed a role for antibodies in alternative pathway activation by virus-infected cells in-vitro. Similarly, Polhill et. al. (op. cit.)
"naturally-occurring antibody" to rabbit red cells affected the kinetics of alternative pathway activity in an assay similar to the one described here. Antibodies to rabbit erythrocytes is certainly present in many human sera, although the titre can vary markedly. Adsorption of this antibody did reduce the efficiency of alternative pathway activation in the human sera DL and PG. This would point to some role for antibody, but it could be postulated that some alternative pathway C3 convertase was also removed during the adsorption process. Recently, analysis of C3, factor B and properdin levels in adsorbed sera has supported this view (Ahmed and Langley, 1985). In this context, the study on the effects of antibody from a number of species on the expression of alternative pathway activity against sheep erythrocytes was a most illuminating exercise. It showed that the effect of antibodies on the extent of lysis varied according to the species from which the antibodies came, and the serum from which the species providing the complement activity were taken. Given the diversity of immunoglobulin classes (isotypes) within and between the vertebrate species examined this seems a realistic finding.

None of the sera from hypogammaglobulinaemic subjects exhibited the degree of alternative pathway deficiency described by Polhill and his colleagues. In general, the levels of each complement component were high. Complement consumption may occur in the subjects receiving intravenous immunoglobulin therapy, as a
All the subjects in this study had received intramuscular injections of immunoglobulin. The possibility of using modified human erythrocytes as a target for human alternative pathway activity seem to offer a valuable method of excluding the effects of antibodies on the expression of alternative pathway activity. It was particularly disappointing that the attempts to achieve it should prove unsuccessful. The use of alternative pathway activators such as inulin, zymosan or E. coli endotoxin (Table 1-3) to provide independent demonstrations of differences in the kinetics of alternative pathway reactivity was considered. Natural products such as inulin or endotoxins have no advantage over rabbit erythrocytes since the occurrence of antibodies against them in human sera is almost inevitable (Gallin, Clark and Frank, 1975). The soluble activators, whatever their origins, present an additional problem. While the initial incubation of the chosen activator with serum is straightforward, the subsequent back-titration of residual complement, by whatever method, is done in the continued presence of the soluble activator (Riches and Stanworth, 1980). This must have an effect on the indicator reaction, and make the results of kinetic methods meaningless. Despite the technical difficulties in studying the effects of antibodies on the kinetics of alternative pathway haemolytic activity, it would seem that some isotypes have a significant effects, but they are not the only explanation of the differences in manifestation of serum sickness (Gall, Coli and Mann, 1984).
The examination of sera from inbred mouse strains did lend some evidence for their existence. Schreffler and his colleagues have described congenic mouse strains which exhibit functional variants of factor B (Atkinson et. al., 1982; Paolucci and Shreffler, 1983), but efforts to obtain serum samples derived from these animals proved unsuccessful. Since the genes for C3, factor B, and the classical pathway components C2 and C4 lie within the major histocompatibility complex (Carroll, et. al., 1984), variants such as these would provide opportunities to test the hypothesis that innate differences in complement activity play a part in the aetiology of diseases such as rheumatoid arthritis and juvenile onset diabetes mellitus which are associated with the expression of certain products of the major histocompatibility complex in cell surfaces (Bodmer, 1980).

Complement-mediated killing of microorganisms relies on the same reaction sequence as complement-mediated haemolysis. If the differences observed in the alternative pathway activity of inbred mice have any significance, it had seemed most likely that it would be seen in an in-vivo test of microbial killing. In this respect, the lack of potency in the murine classical pathway added to the value of the inbred mouse as a model to test alternative pathway effects. The choice of mycobacteria in these experiments reflected local interests and the availability of resources - the cost of such experiments is high. In retrospect, the use of
pharmacologically active components of these bacteria had a profound effect on the complement system in the mouse when injected by the subcutaneous route. While such effects are interesting, and may offer new models of altered alternative pathway activity, they did confuse the interpretation of the experiments with BCG. The alternative pathway does not appear to be responsible for the phenomena reported by Skamene's group (Gros, Skamene and Forget, 1981). Recently, their findings have been challenged by other workers (Orme, 1984). Nevertheless, the observations on the alternative pathway haemolytic activity of inbred mice seem to support the concept of genetically determined differences in complement activity.

This investigation had arisen from observations made during attempts to elucidate the molecular basis of defects of opsonisation seen in some infants with recurrent infections. An important test of these differences in the kinetics of alternative pathway-mediated haemolysis was to establish whether they correlated with opsonic activity. Impaired opsonisation was not associated with retarded red cell lysis, but serum samples from children with biliary atresia showed enhanced opsonic and lytic activity. Renal failure is a complication of obstructive jaundice in children and adults. The endotoxin which appears in the bloodstream as a result of biliary obstruction is said to damage the kidney directly (Pain, 1985), but these observations might indicate complement-mediated
ability to clear radio-labelled albumin aggregates from the bloodstream (Pain, op. cit.), and while disordered Kupffer cell function is thought to be responsible, the phenomenon might be caused by the abnormality of the complement system (Skogh and Stendahl, 1983).
The kinetic method offers a sensitive means of comparing complement activity in samples from a variety of species. Naturally occurring differences demonstrated between certain species appear to offer a new range of experimental models in which the role of alternative pathway activity can be assessed. Jones (1979) originally proposed his method for use in diagnostic laboratories. The variations in alternative pathway haemolytic activity seen when human sera are diluted make the method unreliable for this purpose (Langley, Griffiths and Laurence, 1982), but they do open up new areas of research.

Apart from the low molecular weight inhibitors, most of the factors examined played a part in producing such variations. While these may be analysed in further studies, there is a case for examining the significance of the alternative pathway activity exhibited by given sera without attempting to determine its origins - it reflects the functional capabilities of a part of host defences. In wider terms, investigations of the kinetics of the expression of opsonic, chemotactic, and complex solubilising activity are now indicated. Existing complement technology is still relatively crude.
These are seldom complete, and often too long. One might simply say:
To those who made it possible: my special thanks.
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10. APPENDICES
1. Buffers and Diluents

Phosphate Buffered Saline
Phosphate Buffered Salts, Dulbecco Formula (modified);
Flow Laboratories, Irvine & Uxbridge;
Catalogue Number 28-103-05.
One tablet added to 100ml distilled water, autoclaved 10
minutes at 115°.

Complement Fixation Test Diluent
Complement Fixation Diluent Tablets; Oxoid, Basingstoke;
Product Code BR16;
One tablet added to 100ml warm distilled water, autoclaved
before use, final pH 7.2.

Alternative Pathway Diluent
Constituents:
(A) complement fixation test diluent (CFTD), as above.
(B) ethylene glycol bis(beta-amino ethyl ether) N, N', tetra
acetic acid (EGTA), molecular weight 380.4;
Sigma Chemical Company, Poole; Product Number E4378.
0.08mol/L
(C) magnesium chloride hexahydrate, molecular weight 203.3
Hopkins & Williams, Chadwell Heath;
Product Number 533000 2.0 mol/L

Standard Formulation ("single-strength"):
9 volumes A + 1 volume B + 0.02 volumes C
"Double - Strength" Reagent:
9 volumes "double-strength" CFTD (2 tablets/100ml water)
+ 2 volumes B + 0.02 volumes C.

Alsever's Solution
Flow Laboratories, Irvine & Uxbridge;
Catalogue Number 28-011-49.
2. Other Chemicals

Coomassie Blue

12.5g Brilliant Blue R (Sigma Chemical Company Poole, Product Number B0630) dissolved in a mixture of 50ml glacial acetic acid and 185ml distilled water. After staining, gels were washed glacial acetic acid/water mixture to remove excess dye.

Agarose

Sigma Chemical Company, Poole; Product Number A6013.

3. Antisera

Goat Antisera to Human Complement Components

Miles Laboratories, Stoke Poges:

C3 proactivator
(factor B) 68-020-1 C3b inactivator 68-020-1
properdin 68-030-1 beta-1-H 68-032-1
C3 68-004-1 C4 68-005-1

Rabbit Antiserum to Cl-inhibitor

Behring Diagnostics, Hounslow; Product Code OTNK 05

Other Antisera

Sheep antiserum to human immunoglobulin, fluorescein-labelled; Code MF01.

Rabbit Haemolytic Serum Code VDl5.

Wellcome Reagents Limited, London.

4. Sheep Erythrocytes

Sheep blood in Alsever's solution;
Tissue Culture services, Slough.
6. Disposable Items

Microtitre Plates
microwell plates, 96 U well, with lids;
Nunc, Kamstrupje, Denmark; Product Number 2-62170.

Microtitre Plate Sealers
Titertek plate sealers;
Flow laboratories, Irvine & Uxbridge;
Catalogue number 77-400-05.

Plastic Test Tubes
LP3 tubes; Luckham Ltd, Burgess Hill.

Dialysis Tubing
Dialysis tubing, Visking, 0.32mm thick, flat width 10mm;
molecular weight cut-off 12,000-14,000
Gallenkamp, London; Catalogue Number PJC-400-030R.

7. Fluorescence Microscope
Leitz Dialux 20 microscope, with incident light fluorescence
and manufacturers' filter system for use with fluorescein.
A COMPARATIVE STUDY OF COMPLEMENT ACTIVATION

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ABSTRACT


Antisera to sheep erythrocytes (E) were raised in cattle, rabbits, mice, hamsters, guinea-pigs, ferrets, badgers, hedgehogs and fowls. Cross activation of total haemolytic complement (THCA) examined all combinations of sensitized sheep E and normal sera (including human); kinetic assays examined the lysis of E sensitized with rabbit antibodies. From the same species, all combinations of normal serum and xenogeneic E were used to measure total alternative pathway activity (TAPA); TAPA was also activated by rabbit and sheep E in titrations and in agarose gels, and examined kinetically against rabbit E.

Ox, rabbit and fowl sera were low in THCA, guinea-pig complement was universally active, while human complement showed marked selectivity; ferret, badger and hedgehog sera were activated to high titres but probably via the alternative pathway. In studies of TAPA an inverse relationship existed between serum complement activities and the activating abilities of E from the same species. The most efficient activators of alternative pathway were E from rabbits and laboratory rodents, while the sera with broadest response were badger, ferret and fowl. Kinetic studies of TAPA showed that initiation of lysis and subsequent completion of lysis could occur with different efficiencies, suggesting these events reflected separate events in complement activation.

INTRODUCTION

The technology of complement fixation (CF) has been important in diagnostic bacteriology and virology for many years. The initial recognition of CF as a sequel to the interaction of antibody and antigen (classical pathway, CP) has recently been somewhat overshadowed by interest in the direct activation of complement by a variety of substances including heterologous
cells and the surface antigens of many pathogens (alternative pathway, AP). Both pathways play important roles in the induction and suppression of immune responses, opsonisation, inactivation of viruses, lysis of cells and bacteria, immunopathology and the outcome of host-parasite relationships (Mueller-Eberhart and Schreiber, 1980; Fine, 1981; Weiler et al., 1982).

It is well known that antibodies from different species do not activate CP from a single source with equal efficiency (Rice, 1980; Rice and Crowson, 1980; Jankovic and Isakovic, 1960; Cigli and Austen, 1971); some antisera actually inhibit CP fixation (anticomplementarity) (Orlans et al., 1962). Whether this is an absolute deficiency of the antibodies or a reflection of species' preferences is largely unknown. The serological solution to these problems has been to supplement the xenogeneic complement with fresh serum allogeneic or syngeneic to the antibody (Hoffman and Mayer, 1977; Marquardt, 1977). This has assumed that homology between antibodies and Cl could initiate the complement cascade, whereafter interaction with subsequent complement components would be unhindered by species restrictions. Such mixtures are difficult to standardize and are conceptually and practically undesirable. A better approach would be to define which species' complement interact efficiently with which species' antibodies, and to utilize the appropriate combinations. Similarly, the relative efficiencies of AP activation require study. A common method of activation is to use xenogeneic E (Platts-Mills and Ishizaka, 1974) but the species spectrum of interaction requires examination. The work described here is a preliminary study of the cross activation of CP and AP by antibody and E between nine species.

MATERIALS AND METHODS

Production of antisera to sheep erythrocytes (SE)

A single healthy ewe was used as a source of SE. Blood was collected into an equal volume of sterile Alsever's solution, pH 6.2. The SE were washed three times in 0.1M phosphate buffered saline, pH 7.2 (PBS), then stored in Alsever's solution at 4°C for up to 3 days. Just prior to inoculation, the cells were washed again in PBS and a 10% (v/v) suspension was prepared in PBS. The animals used and the protocols for immunization (Campbell et al., 1970) are described in Table I. Ferrets, hedgehogs and mice were bled under anaesthesia induced by ether, guinea-pigs, hamsters, mice and rabbits under anaesthesia induced by barbiturates ('Sagatal', May and Baker), whilst badgers
were anaesthetized with ketamine hydrochloride ("Vetalar", Parke Davis). Fowl and cattle were bled without anaesthetic. Sera were separated, frozen at -70°C, and later transferred to liquid nitrogen. Antibody titres (Table 1) were assayed by doubling-dilution direct haemagglutination tests against a 3% suspension of SE.

**Normal sera (complement)**

Bloods were collected from unimmunized animals and allowed to clot at 0°C for up to 1 hr. Sera were clarified by centrifugation at 0°C and stored in the same way as the antisera.

**Titration of total haemolytic complement activity (THCA)**

Antisera were inactivated at 56°C for 30 min and mixed with a 3% suspension of SE in CF test diluent (CFTD) (Oxoid), to reproduce the mixture one dilution above the HA titre. The mixtures were incubated at 37°C for 15 min and the sensitized cells (SEA) washed 3 times in CFTD before being resuspended at 3% in CFTD. Equal volumes (20 µl) of SEA and serum or serum dilution were incubated in microtitre trays in a moist atmosphere at 37°C for 2 hr. Lysis was assessed visually.

**Titration of total alternative pathway activity (TAPA)**

Serial two-fold dilutions of each serum were made in microtitre plates using AP diluent (APD). This consisted of CFTD supplemented with 8mM ethylene glycol bis-tetraacetic acid, sodium salt (Sigma) and 20 mM MgCl₂. An equal volume (20 µl) of rabbit (R) E or SE suspension in APD was added to each well. Lysis was assessed visually after 2 hr incubation at 37°C in a moist atmosphere.

**Radial haemolysis**

TAPA was also assessed against SE and RE by radial haemolysis in agarose gel (Lachmann et al., 1973).
TABLE I
Production of antisera against sheep erythrocytes

<table>
<thead>
<tr>
<th>Species</th>
<th>No</th>
<th>Breed/Strain</th>
<th>Inoculation Volume</th>
<th>Route</th>
<th>Haemagglutination titres</th>
<th>Immunized animals</th>
<th>Normal sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox</td>
<td>2</td>
<td>Jersey, Hereford</td>
<td>3.0 IV</td>
<td></td>
<td>256,512</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>4</td>
<td>NZW x Lopear</td>
<td>1.0 IV</td>
<td></td>
<td>128,128,256,256</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>15</td>
<td>CFLP</td>
<td>0.25 IP</td>
<td></td>
<td>128,256,512^3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Hamster</td>
<td>10</td>
<td>Syrian</td>
<td>0.25 IP</td>
<td></td>
<td>128,128,256,256^2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>5</td>
<td>Hartley</td>
<td>0.5 IP</td>
<td></td>
<td>128,128^3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Ferret</td>
<td>3</td>
<td>Polecat</td>
<td>1.0 IP</td>
<td></td>
<td>32,32,64</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Badger</td>
<td>3</td>
<td>-</td>
<td>1.0 IV</td>
<td></td>
<td>64,64,128</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Hedgehog</td>
<td>3</td>
<td>-</td>
<td>0.5 IP</td>
<td></td>
<td>8,64,64</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Fowl</td>
<td>2</td>
<td>White Leghorn</td>
<td>0.5 IV</td>
<td></td>
<td>256,1024</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

^1Volume (ml) of 10% SE suspension inoculated on each of days 1, 2, 3, 5, 7, 9, 11 and 13. Animals were bled on day 15.

^2IV, intravenous; IP, intraperitoneal.

^3Pools of two or more individuals.

Timed-lysis assays

These were based on methods described previously (Jones, 1979). They were performed on a Beckman model 24 spectrophotometer using 1 cm glass cuvettes held at 37°C. THCA was studied using SEA suspended in CFTD, TAPA using RE in APD. Cell suspensions were adjusted to give an absorbance at 700 nm (A_700) of 0.9. Sera and E suspensions were warmed separately to 37°C, mixed in the chosen proportions and quickly transferred to the spectrophotometer where lysis was followed at 700 nm.

Graphs of A_700 versus time were sigmoidal. Three values from these plots could be used to describe the time-course of lysis: the lag phase (LP; time from mixing the reactants to commencement of lysis); the time to 50% lysis (t_50); and the time to complete lysis (t_100). LP was considered an index of
the time required to produce C3 convertase, while $t_{50}^{LP}$ or $t_{100}^{LP}$ reflected the efficiency of interaction of the membrane attack complex, C5 to C9.

RESULTS

THCA

Cross activation and titrations (Table II). There was considerable variation between species in the titres of serum complement. In general, variation was a property of the complement, not the antibody. Thus ox, rabbit and chicken complement were low irrespective of the antibody source and even against homologous antibody. Ferret, badger and hedgehog sera had high titres of haemolytic activity, though the reaction against unsensitized SE was also high suggesting the activation was via the AP. The greatest variation occurred with human complement which was activated efficiently by ox, rabbit, hamster and badger, moderately by mouse, guinea-pig and ferret, and poorly by hedgehog and fowl antibodies. Guinea-pig was universally active to high titres, with little activation of AP by unsensitized SE.

Timed-lysis assays (Table III). When 25 µl serum was added to 1 ml SEA suspension, complement from guinea-pig, hedgehog, ferret and badger initiated lysis within 1 min, and the process was complete within 3 min. Hamster and human complement required 2-3 min to produce detectable lysis, and 7-12 min to complete it. Ox and fowl sera did not show any lytic activity at this dilution; when equal volumes (250 µl) of serum and SEA were mixed, ox complement caused lysis with the same kinetics as hamster and human complement at the lower concentration, while chicken complement gave similar results to guinea-pig complement at the lower concentration. Mouse complement did not induce lysis at either concentration. Even when SE were sensitized with mouse antibody and exposed to mouse complement at a dilution of 1:2, lysis did not occur, although these cells could be lysed by guinea-pig complement.

TAPA

Cross activation and titrations. The pattern of activation of AP in normal sera by E from each species is shown in Table IV. Table V shows the titre of AP in each species' serum against RE and SE and Table VI the activity in agarose gels. Fowl, badger, ferret and ox AP were activated by a wide range of E. Human, rabbit, mouse, guinea-pig and hedgehog AP were more selective;
### TABLE II

Total haemolytic complement activity (THCA) of normal sera titrated against sheep erythrocytes sensitized with antiserum

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Titre of normal serum giving complete (partial) lysis&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>Ox</td>
<td>64(256)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>64(0)</td>
</tr>
<tr>
<td>Mouse</td>
<td>4(16)</td>
</tr>
<tr>
<td>Hamster</td>
<td>64(128)</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>8(16)</td>
</tr>
<tr>
<td>Ferret</td>
<td>16(128)</td>
</tr>
<tr>
<td>Badger</td>
<td>64(128)</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>0(16)</td>
</tr>
<tr>
<td>Fowl</td>
<td>0(32)</td>
</tr>
<tr>
<td>None</td>
<td>2(0)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mouse complement used against SEA in kinetic studies only.
TABLE III
Timed-lysis assays of total haemolytic complement activity (THCA) using sheep erythrocytes sensitized with rabbit antibody

<table>
<thead>
<tr>
<th>Complement source</th>
<th>Reaction volume (µl)</th>
<th>Time (min) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum:SEA</td>
<td>LP</td>
</tr>
<tr>
<td>Human</td>
<td>25:1000</td>
<td>1.9</td>
</tr>
<tr>
<td>Ox</td>
<td>25:1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500:500</td>
<td>6.3</td>
</tr>
<tr>
<td>Mouse</td>
<td>25:1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500:500</td>
<td>5.3</td>
</tr>
<tr>
<td>Hamster</td>
<td>25:1000</td>
<td>1.1</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>25:1000</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>250:250</td>
<td></td>
</tr>
<tr>
<td>Ferret</td>
<td>25:1000</td>
<td>1.0</td>
</tr>
<tr>
<td>Badger</td>
<td>25:1000</td>
<td>0.5</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>25:1000</td>
<td>0.9</td>
</tr>
<tr>
<td>Fowl</td>
<td>25:1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500:500</td>
<td>2.5</td>
</tr>
</tbody>
</table>

SEA: sensitized erythrocytes; LP: lag phase; t_{50}: time to 50% lysis; t_{100}: time to 100% lysis.

Activation occurred most often with rabbit, mouse, hamster and guinea-pig E (though not, of course, within the homologous system) and to some extent with badger E. Hamster AP was activated only by rabbit and guinea-pig E. Ox and fowl E (three in each case) and badger, hedgehog and ferret E (four in each case) activated the smallest number of sera, while rabbit, mouse, hamster and guinea-pig E activated AP of all or most sera.

Timed-lysis assays (Table V). The relationship between the square root of LP, t_{50} or t_{100} was linear to a characteristic point in each case, e.g. 600 µl for chicken, 150 µl for ferret. With lower concentrations of serum there was a marked increase in time requirement, suggesting the concentration of one or more components was below a critical concentration. LP and t_{50} - LP (or t_{100} - LP) were not necessarily equally efficient; e.g. with 150 µl ferret serum and 600 µl ox serum LP was 2.1 min, but t_{50} - LP was 4.4 min for ferret and only 1.0 min for ox. LP was shortest with ferret, hedgehog and badger sera, but t_{50} - LP was shortest with ox, fowl and human sera.
### TABLE IV

**Interaction of sera and erythrocytes in alternative pathway diluent (APD)**

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Human</th>
<th>Ox</th>
<th>Rabbit</th>
<th>Mouse</th>
<th>Hamster</th>
<th>Guinea-pig</th>
<th>Ferret</th>
<th>Badger</th>
<th>Hedgehog</th>
<th>Fowl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (Group A)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Human (Group B)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ox</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sheep</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mouse</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hamster</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ferret</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Badger</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fowl</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*: lysis; +: partial lysis; -: no lysis.
### TABLE V
The titre of total alternative pathway activity (TAPA) in sera from various species

<table>
<thead>
<tr>
<th>Serum</th>
<th>RE</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0(4)</td>
<td>0(4)</td>
</tr>
<tr>
<td>Ox</td>
<td>2(16)</td>
<td>0(8)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0(0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Mouse</td>
<td>0(2)</td>
<td>0(2)</td>
</tr>
<tr>
<td>Hamster</td>
<td>4(8)</td>
<td>0(2)</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>0(8)</td>
<td>0(4)</td>
</tr>
<tr>
<td>Ferret</td>
<td>64(0)</td>
<td>8(64)</td>
</tr>
<tr>
<td>Badger</td>
<td>8(128)</td>
<td>8(64)</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>8(16)</td>
<td>0(8)</td>
</tr>
<tr>
<td>Fowl</td>
<td>4(32)</td>
<td>2(16)</td>
</tr>
</tbody>
</table>

RE: rabbit erythrocytes; SE: sheep erythrocytes.

**DISCUSSION**

The variables in complement fixation systems are many. Optimum activation of CP in serum of a given species depends upon the source and number of E, the source of antibody, the concentration of essential minerals in the environment, and the temperature, time and volume of reaction (Barta and Barta, 1975; Berden et al., 1978). Differences exist even between phylogenetically close species (Barta and Klei, 1978). It cannot be concluded therefore that the THCA results obtained in this study employing a single set of physical conditions were optimum, but some conclusions can be drawn regarding the interaction of various combinations of antibody and complement.

The low haemolytic activity of ox, mouse and fowl complement confirmed previous reports (Rice, 1950; Orlians et al., 1962; Berden et al., 1978). The low titres of rabbit complement were unexpected. The rabbit is a common source of complement in clinical immunology and tissue typing, though admittedly against human antibodies, which we did not employ, and with well-recognized individual variation. Human complement was surprisingly selective.
TABLE VI

Lysis of unsensitized erythrocytes in agarose gels

<table>
<thead>
<tr>
<th>Serum</th>
<th>Diameter of lysis (mm)</th>
<th>Sheep erythrocytes in CFTD (μl)</th>
<th>Sheep erythrocytes in APD (μl)</th>
<th>Rabbit erythrocytes in CFTD (μl)</th>
<th>Rabbit erythrocytes in APD (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Volume (μl)</td>
<td>2</td>
<td>7.8</td>
<td>0.4</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>8.3</td>
<td>6.9</td>
<td>8.8</td>
</tr>
<tr>
<td>Badger</td>
<td></td>
<td>10</td>
<td>10.0</td>
<td>7.6</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>7.4</td>
<td>7.5</td>
<td>7.2</td>
</tr>
<tr>
<td>Ferret</td>
<td></td>
<td>5</td>
<td>8.3</td>
<td>7.7</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>9.1</td>
<td>8.5</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>6.6</td>
<td>+1</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td>5</td>
<td>8.6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>8.7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>5.8</td>
<td>-</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>6.0</td>
<td>-</td>
<td>6.0</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td></td>
<td>10</td>
<td>9.0</td>
<td>-</td>
<td>9.0</td>
</tr>
</tbody>
</table>

CFTD: complement fixation test diluent; APD: alternative pathway diluent.

1Weak positive results; also seen for human and hedgehog against SE in both diluents. Negative results against SE with ox, fowl, hamster and mouse. Against rabbit erythrocytes weak positive results were obtained with human serum (in both diluents) and hedgehog serum (in CFTD only). Negative results with ox, fowl, hamster and mouse.

The basis of species selectivity in CP activation is poorly understood. The sera of several species displaying poor haemolytic activity have been examined for the presence of complement components; the most common deficiency in these species was of C4 (Rice and Crowson, 1950; Barta et al., 1976). This does not disprove the concept of incompatibilities between certain combinations of Clq and immunoglobulin. Clq of various species are chemically and structurally quite similar (Hoffken et al., 1978). However, charge and accessibility of the C2 domain of IgG affect the interaction with Clq (Burton et al., 1980) and these properties probably vary from species to species. It remains to be explained why guinea pig complement was universally acceptable to other species' antibodies. The intensive immunization schedule adopted was typical of that used previously by many workers to stimulate rabbit antibodies to SE. However, it is probable that other species would respond with antibodies of different isotypes and subclasses, which would affect CP activation (Feinstein and Hobart, 1969; Klaus et al., 1979), and of different affinities and avidities. Genetic as well as species differences should be considered.
TABLE VII

Timed-lysis assays of alternative pathway activation by rabbit erythrocytes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Serum</th>
<th>Volume (μl)</th>
<th>Time (min) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Species</td>
<td></td>
<td>LP</td>
</tr>
<tr>
<td>1</td>
<td>Human female</td>
<td>750</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Human male</td>
<td>750</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Fowl</td>
<td>750</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Ferret</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Guinea-pig</td>
<td>1000</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>500</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>Human female</td>
<td>1000</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Human male</td>
<td>1000</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
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<td>750</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Badger</td>
<td>1000</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Hedgehog</td>
<td>600</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>1000</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Ox</td>
<td>600</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>4.7</td>
</tr>
</tbody>
</table>

LP: lag phase; t<sub>50</sub>: time to 50% lysis; t<sub>100</sub>: time to 100% lysis.

1Volume of serum added to 3 ml rabbit E suspension; except for mouse where 500 μl serum was mixed with 500 μl rabbit E suspension.
It seems unlikely that a biological pattern exists between these xenogeneic combinations. Why should antibodies of one species need to interact with complement of another species in nature? Equally, however, the relatively poor reactions often elicited by the syngeneic or allogeneic systems are perhaps surprising. We agree with Hildemann (1978) concerning "...the artificiality of most in vitro tests of complement-dependent cytotoxicity..." and that we should "...use only syngeneic antibodies and complement to measure the functional activities of either, if the results are intended to reveal probable interactions in the living organisms..." for "...in...life... every vertebrate... must depend on its own serum complement components to act in concert with its own antibodies." But does the apparent weakness of the syngeneic or allogeneic reaction indicate that the in vitro cytotoxic effects of complement activation are weaker than we have previously inferred from xenogeneic combinations?

It is generally believed that AP is a mechanism of in vivo destruction of cells bearing "foreign" antigens and some pathogens (McConnell et al., 1981). The range of AP recognition and functions remains to be defined, and its activation by xenogeneic E must be acknowledged as a tool with numerous limitations. Nevertheless, optimum combinations should be established for this assay also, and an overview of the phylogenetic distance required for activation might help us understand the specificity and evolution of this system. Our results indicate a considerable species variation in the activation of AP by xenogeneic E. To a large extent this was a property of the E, some activating widely, some rarely. In general, species whose E stimulated many other species' AP possessed AP of only limited responsiveness. This might be an important cause and effect relationship, suggesting that E which activate AP efficiently are undesirable, or even self-eliminated, in a host with avid AP. Van Dijk et al. (1983) examined the cross activation of AP by E in all combinations of 13 species, 7 of which were also examined by us. They too noted an inverse relationship between AP responses and the stimulation capabilities of E. However, in their hands rabbit AP was not activated by any E; in our study mouse and guinea-pig E activated rabbit AP. It is interesting that the sera with broadest AP response were among the most primitive of the species examined, i.e. badger, ferret and fowl; the exception was the hedgehog, a species perhaps requiring unique conditions for optimum activation. Conversely, the most efficient activators of AP were E from rabbits and laboratory rodents. Thus, this initial study suggests
phylogenetic groupings worthy of further investigation. An unresolved possibility is the involvement of non-specific antibodies to E in the activation of AP. Some antibodies enhance AP (Nelson and Ruddy, 1979; Moore et al., 1981), and species differences in the immunoglobulin classes and subclasses of heterophile antibodies could contribute to differences in AP activation. This is a difficult problem to overcome, for absorption of serum in the cold has some effects on complement levels and rarely completely removes antibodies.

While groups of related species show greater or lesser complement efficiencies, an overall developmental phylogenetic pattern remains to be seen (Gigli and Austen, 1971; Ballow, 1977). A more diverse approach than activation of CP by a single xenogeneic source of antibody, or of AP by a single xenogeneic source of E, will assist in the evaluation of this phylogeny, and our study is offered as a step in such direction. Our choice of species was influenced by our interest in the natural occurrence of some diseases. Whether there is a relationship, direct or indirect, between the avidity of CP or AP and susceptibility or resistance to certain infectious diseases remains to be seen. Nevertheless, it is particularly interesting that badger, ferret and hedgehog, animals with high susceptibility to mycobacteria, had high levels of AP. This suggests that AP activation, which almost certainly is induced in vivo by mycobacteria, plays little or no part in protection. Indeed, it might be speculated that AP activation, with consequent generation of opsonins, is to the biological advantage of a facultative intracellular parasite; phagocytosis and hence the attainment of the desirable intracellular environment might be more efficient of organisms possessing appropriate activators of AP and in host species with avid AP.

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REFERENCES


