11-25-1992

Evidence for the alternative pathway of complement activation in the nurse shark

Lieneke Cecile Culbreath
Florida International University

DOI: 10.25148/etd.FI14061562

ABSRACT OF THE THESIS

Evidence for the alternative pathway of complement activation in the nurse shark

by

Lieneke C. Culbreath
Florida International University, 1992
Miami, Florida
Professor Sylvia Smith, Major Professor

Complement is activated via two pathways: classical (CCP) and alternative (ACP). The CCP has been demonstrated in the nurse shark. The ACP has not been demonstrated in any cartilagenous fish. Nurse shark serum was evaluated for complement activity by its ability to lyse heterologous erythrocytes. As CCP activity requires calcium and magnesium, activity of shark serum chelated with EGTA (a selective calcium chelator) or EDTA (a chelator of calcium and magnesium) was assessed. Activity remained in serum chelated with EGTA but not EDTA. Furthermore, activity of chelated serum was enhanced by added magnesium. Activation of shark complement by activators of mammalian ACP (zymosan, LPS, inulin, CVF) was assessed. Complement was activated by zymosan and LPS. Immunoblots were employed, with limited success, to demonstrate complement

proteins in nurse shark serum. This study unequivocally demonstrates that the ACP is present in the primitive nurse shark.

FLORIDA INTERNATIONAL UNIVERSITY
Miami, Florida

Evidence for the Alternative Pathway of Complement Activation in the Nurse Shark

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Medical Laboratory Sciences

by

Lieneke Cecile Culbreath

1992

To Professors: Sylvia Smith, Ph.D.
Steven Obenauf, Ph.D.
Patrick Shen, Ph.D.
Charles Bigger, Ph.D.

This thesis, having been approved in respect to form and mechanical execution, is referred to you for judgement upon its substantial merit.

Dean William Keppler
College of Health

The thesis of Lieneke Cecile Culbreath is approved.

Steven Obenauf

Patrick Shen

Charles Bigger

Sylvia Smith, Major Professor

Date of Examination:

November 25, 1992

Dean Richard Campbell
Division of Graduate Studies

Florida International University, 1992

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Sylvia Smith, for the original idea for this study and for her constant guidance and encouragement throughout. I would like to thank my committee members for their guidance and suggestions, particularly Dr. Steve Obenauf for his helpful suggestions, technical assistance and support throughout the course of this study. Thanks are due to the Keys Marine Laboratory, Long Key, FL for maintaining the nurse sharks. The technical assistance of John Swanson, Bill Gibbs and Ed Seymour in bleeding the animals is gratefully acknowledged. I would like to thank my husband and my family for their support and patience without which this work would not have been possible. This study has been supported, in part, by a grant from the Lerner Gray Fund for marine research from the Museum of Natural History, New York, NY and a grant from the Sigma Xi Society. Both grants were awarded to the author.

Table of Contents

List of Tables

List of Figures

INTRODUCTION

The discovery of a protein in human serum termed properdin led Pillemer and his colleagues to propose the discovery of a new system for natural resistance to infection in which properdin activated the complement component C3 when complexed with certain substances (Pillemer et al., 1954; Pillemer et al., 1955). Pillemer's properdin system was thought to consist of properdin, magnesium and the four complement components known at that time (C1, C2, C3 and C4). His experimental results determined that the system required a heat labile factor and a hydrazine sensitive factor which was interpreted as indicating a requirement for C1, C2 and C4. The properdin system was proposed as a mechanism by which substances such as dextrans, levans, bacterial cell walls and other bacterial carbohydrates could directly activate complement via properdin; therefore bypassing the need for specific antigen/antibody complexes which were required to activate the classical pathway of complement. Nelson (1958) later suggested that the depletion of C3 activity by zymosan (a substance prepared from the cell walls of yeast and used extensively, although not exclusively, by Pillemer) could be explained by the presence of natural antibodies to zymosan in human serum which, in complex with zymosan, could activate the classical pathway of complement. This study,

along with others, (Cowan, 1958; Toussaint and Muschel, 1959) brought a skepticism to the theory of the properdin pathway as a newly discovered mediator of natural immunity. Pillemer's theory fell into disfavor as a result of these publications. It was not until the early 1970's that experimental evidence began to accumulate which strongly supported the original concept of Pillemer (Sandberg et al., 1970; Sandberg and Osler, 1971; Sandberg et al., 1971; Gotze and Muller-Eberhard, 1971; Brade et al., 1971) although properdin was not as central to the system as originally proposed (Medicus et al., 1976). The apparent discrepancy arising from Pillemer and Nelson's studies was resolved by studies showing that the hemolytic activity of the alternative pathway, as the properdin pathway is now called, is enhanced by antibody, however antibody is not essential for the activation of the pathway (Schenken and Ruddy, 1981; Nelson and Ruddy, 1976; Polhill et al., 1978). In addition, these later studies have shown that the enhancement of alternative pathway activity by antibody is mediated by the Fab portion of the antibody molecule rather than the Fc portion which activates the classical pathway via its interaction with C1q (Sandberg et al., 1971).

The classical and the alternative complement pathway have been well characterized in mammals (Reid, 1976). The two pathways have requirements for activation that are

distinct from each other. With the exception of C3, which is common to both pathways, the serum protein components generating and controlling the C3 and C5 convertases of the classical pathway are separate from those of the alternative pathway. Activation of the classical pathway is initiated mainly by interaction of the C1q subunit of C1 with the Fc regions of IgG or IgM in complex with specific antigen. Activation of the classical pathway can however occur in the absence of specific antibody, for example on the surface of certain microorganisms and by polyanionic molecules like DNA, carrageenan and heparin. The significance of this type of activation in a physiological system is unknown (Abbas et al., 1991). Once bound to the activating substance, a change in the conformation of the C1q molecule occurs which then allows activation of C1r which in turn activates C1s. The integrity of the $C1r_2C1s_2$ tetramer is dependent on calcium ions, as in the absence of these ions the tetramer dissociates into a $C1r_2$ dimer and 2 C1s chains (Reid, 1986). Activated C1s, a serine protease, within the C1 complex cleaves C4 into C4a and C4b. This is an amplification step as activated C1 can cleave many C4 molecules. The C4b molecule, via its reactive thiolester, then becomes bound to the surface of the activator and the proenzyme C2 associates with C4b in a magnesium-dependent reaction to be cleaved by C1s to form C4bC2b, the C3 convertase of the classical pathway. C2b is a serine protease and is responsible for

the subsequent cleavage of C3 by the classical pathway C3 convertase.

The alternative pathway is activated directly on the surface of activating particles such as lipopolysaccharides, pure polysaccharides, bacteria, fungi, tumor cells, red blood cells, parasites and aggregates of immunoglobulins in particulate form without requiring specific antibody (Gotze and Muller-Eberhard, 1976). A low level of C3b must be available in serum in order to initiate the formation of the alternative pathway C3 convertase i.e. C3bBb. This is provided by the continuous generation of a C3b-like molecule, $C3(H_2O)$, by slow hydrolysis of the internal thiolester bond in the alpha chain of C3. This molecule can then complex with factor B in a magnesium-dependent reaction to form $C3(H_2O)B$. Factor B in this complex now becomes susceptible to cleavage by the enzyme, factor D, to form a C3 convertase, $C3(H_2O)Bb$ which is capable of generating a low level of C3b (Pangburn and Muller-Eberhard, 1980). Factor B subsequently binds to true C3b in a magnesium-dependent reaction where it is again susceptible to cleavage by factor D. If this occurs on the surface of a suitable activator of the pathway a stable C3 convertase, C3bBb, is formed. The alternative pathway activator is thought to provide a site at which C3b and C3bBb are protected from rapid decay by the endogenous control proteins factor I (C3b

inactivator) and factor H (beta 1 H) (Fearon and Austen, 1977). Bb is a serine protease and is responsible for the C3 cleaving activity of the alternative pathway C3 convertase. The hydrazine-sensitive factor and the heat-labile factor originally described by Pillemer are now known to be C3 and factor B respectively (Medicus et al., 1976). Once the C3 convertase is formed, the reaction becomes amplified whereby large amounts of C3b become deposited on the surface of the activator. Properdin is now known to act late in the activation pathway as a stabilizer for the already formed C3 and C5 convertases (Medicus et al., 1976). The binding of a molecule of C3b to either C4bC2b or C3bBb changes the specificity of the convertases from C3-cleaving activity to C5-cleaving activity generating identical fragments, C5a and C5b. Once C5 has been cleaved by either of the two convertases, both pathways share the common terminal components C6, C7, C8 and C9 which form the membrane attack complex (MAC).

Thus, activation of the classical pathway is dependent on the presence of calcium and magnesium ions whereas activation of the alternative pathway requires only magnesium ions. This difference in divalent cation requirement can be used to selectively inhibit classical pathway activity by removal of calcium ions while demonstrating complement activity exclusively by the

alternative pathway. The C1 complex of the classical pathway is easily dissociated in the presence of calcium chelators such as ethylene diaminetetra acetic acid (EDTA) and ethyleneglycol-bis-(beta-amino ethyl ether) N,N,N,N-tetra acetic acid (EGTA) (Reid, 1986). EDTA is a strong chelator of calcium, magnesium and other metal ions, and therefore will prevent activation of both complement pathways. EGTA is a selective chelator of calcium ions as the avidity of EGTA for calcium is about 100,000 times greater than its avidity for magnesium (Bryant and Jenkins, 1968) and therefore will prevent activation of the classical but not the alternative pathway.

The complement system is responsible for the generation of anaphylatoxins, C3a and C5a, which are powerful chemotaxic and inflammatory effectors. The potent opsonin, C3b, is also generated by the activation of complement. Lysis of foreign erythrocytes is perhaps not the most important physiological function of complement, however, in vitro, hemolytic assays have been used routinely to measure complement activity because cell damage is easily detected and quantitated by measurement of hemoglobin released into the medium (Muller-Eberhard, 1969). Chelation of calcium and subsequent demonstration of lytic activity (i.e. in the presence of EGTA) is an indication of the functioning of the alternative pathway since only magnesium is required for

this pathway to proceed. EDTA will abolish hemolytic activity by either pathway as it is a strong chelator of both calcium and magnesium ions.

In hemolytic assays employing sensitized sheep or unsensitized rabbit erythrocytes as targets, both human and guinea pig serum showed ability to lyse these target cells, although activity against sensitized sheep was greater than against rabbit cells (Platts-Mills and Ishizaka, 1974). The lysis of sheep erythrocytes, sensitized with either 7s or 19s hemolysin, by human or guinea pig serum is completely blocked in the presence of 8 mM EGTA. Conversely, the same serum shows greater than 90% hemolysis of unsensitized rabbit cells in the presence of 8 mM EGTA and 2 mM Mg++ (Platts-Mills and Ishizaka, 1974). These results demonstrate that in mammalian serum the lysis of sensitized sheep erythrocytes is mediated primarily by the classical pathway whereas the lysis of rabbit erythrocytes is mediated by the alternative pathway.

The abrogation of hemolytic activity of serum by incubation with alternative pathway activators such as zymosan, Cobra venom factor (CVF), lipopolysaccharide (LPS) and inulin reflects activation of complement with consumption of the terminal complement components. As these components are common to both pathways, activation of either

pathway results in consumption of the terminal components such that they are no longer available to effect the lysis of target cells regardless of the presence of early acting components of the other pathway. The ability of alternative pathway activators to abolish the hemolytic activity of serum in the absence of specific antibody is considered to be a criterion for demonstration of alternative pathway activity in a serum sample. These functional characteristics have been used to evaluate complement activity in mammals as well as lower vertebrate species.

Ohta et al. (1984) reported the lysis of sheep erythrocytes, sensitized with rabbit antibody (hemolysin), by chicken complement was blocked by 4 mM EDTA or 4 mM EGTA and this activity was not restored by the addition of magnesium ions. From these observations they concluded that calcium ions were required for the lysis of sensitized sheep erythrocytes by chicken complement. However, lysis of unsensitized horse erythrocytes was blocked only at a concentration of 12 mM EGTA and was completely restored by the addition of 4-6 mM magnesium indicating that a calcium independent lytic pathway was responsible for the lysis of horse erythrocytes by chicken complement. Absorption of serum with horse erythrocytes and employing serum of bursectomized chickens (Bursa of Fabricius is the site of antibody production in birds) showed that the lysis of horse

erythrocytes by chicken serum was antibody-independent. Depletion of serum hemolytic activity by alternative pathway activators and selective inhibition of activity by chelators was reported (Ellis et al., 1989) for turkey serum. Results obtained were similar to those seen with chicken serum. Thus, activity of the classical and the alternative pathway can be demonstrated in birds using the same functional criteria used to distinguish the activity of the two complement pathways in mammals. Additionally, horse erythrocytes appear to be efficiently lysed via the alternative pathway in these birds.

Complement activity has been demonstrated in ectothermic vertebrates using functional assays similar to those described for mammals and birds. To date, the presence of alternative pathway activity has been established in representatives of the reptiles, the amphibians, the bony fish and the agnathans. The serum of Naja naja kaouthia, the Indian cobra, can lyse mammalian erythrocytes in the presence of Mg-EGTA but not in the presence of EDTA (Vogel and Muller-Eberhard, 1985). In the presence of Mg-EGTA, the hemolytic activity was greatest against rabbit erythrocytes although, a low level of activity could be detected against sheep erythrocytes. The alternative pathway activators inulin, zymosan, and LPS, were able to deplete cobra serum of its hemolytic activity

indicating an alternative pathway of complement activation in this species. The serum of five Japanese species of snakes have been tested for lytic activity against sheep erythrocytes (Kawaguchi et al., 1978). The degree of hemolysis was seen to be dependent on the titre of natural antibody to sheep cells, perhaps indicating a classical pathway-like activation mechanism in the lysis of these target erythrocytes. Koppenheffer (1986) reported the lysis of rabbit erythrocytes by the serum of turtles, Psudemys scripta, in the presence of magnesium alone. Sekizawa et al. (1984) reported classical and alternative pathway activity in the serum of clawed toads, Xenopus laevis. The lysis of sheep erythrocyte targets by toad serum was found to require both calcium and magnesium ions whereas the lysis of rabbit erythrocytes required only magnesium. Additionally, toad serum absorbed with sheep erythrocytes did not lyse sheep cell targets indicating the need for toad natural antibody for the lysis of sheep cell targets.

Alexander and Steiner (1985) isolated from the serum of Rana catesbieans, the bullfrog, what appears to be the bullfrog analog of classical complement pathway component C1. C1 from bullfrog serum can replace guinea pig C1 in hemolytic assays using C1-deficient guinea pig serum. A comprehensive study of complement activity in the rainbow trout, Salmo gairdneri, (Nonaka et al., 1981) demonstrated

that lysis of sheep red blood cells by trout serum was dependent on trout anti-sheep erythrocyte IgM and was blocked by both EDTA and EGTA indicating a calcium-dependent hemolytic system which requires antibody, thus resembling the classical pathway of mammals. Alternatively, the lysis of rabbit erythrocytes proceeded in the absence of calcium ions and without trout anti-rabbit erythrocyte antibody indicating the presence of alternative pathway activity. Furthermore, incubation of trout serum with zymosan, LPS and inulin depleted the serum of its hemolytic activity. Functional assays of carp, Cyprinus carpio, complement activity (Matsuyama et al., 1988) indicate both classical and alternative pathway activity in this teleost fish. Assay of carp serum for hemolytic activity, in the presence of EGTA and magnesium ions showed a higher degree of lysis of rabbit cells under these conditions than other target cells. A low level of lytic activity however, did remain against both sheep and bovine erythrocytes. Horse erythrocyte targets were not used in this study. Jensen and colleagues (Jensen et al.,1968; Jensen, 1969; Jensen et al.,1973; Ross and Jensen, 1973; Jensen et al.,1981; Hyder-Smith and Jensen, 1986) in an extended study of the complement system of the nurse shark, Ginglymostoma cirratum, were able to functionally isolate six components which act sequentially to bring about the lysis of sheep erythrocytes sensitized with nurse shark natural antibody.

The lesions produced by shark complement on sheep erythrocytes were indistinguishable from those produced by mammalian complement (Jensen et al., 1981). The complement system in the nurse shark is functionally analogous to the classical pathway of mammals and no evidence for alternative pathway activity in this species was presented. Results of these studies have lead several reviewers to state that the classical pathway of activation alone operates in sharks (Koppenheffer, 1987; Day and Dodds, in press).

The serum of the lamprey, Lampetra japonica, one of the most primitive of vertebrates, has been shown to strongly promote phagocytosis (Nonaka et al., 1984). This activity was attributed to a C3-like molecule which in the presence of lamprey serum (source of other needed serum factors) and divalent cations, bound to the surface of zymosan, a good activator of the mammalian alternative pathway. The activity of lamprey serum was thought to be due to an alternative pathway-like system. This activity was also sensitive to heat and to the presence of EDTA, two characteristics essential for activity to be attributable to complement. The lytic activity of lamprey serum against rabbit erythrocytes did not meet these criteria and therefore could not be attributed to complement (Gigli and Austen, 1971).

Studies in invertebrates have provided functional evidence for the existence of complement-like activity in these primitive animals (Gigli and Austen, 1971). Day et al., (1970) found that cobra venom factor, an activator of mammalian alternative complement pathway and an analog of mammalian C3 which is not regulated by mammalian control proteins, was able to induce the lysis of sheep erythrocytes by the hemolymph of the horseshoe crab and sipunculid worm, but not that of starfish. Recent studies on invertebrate "complement" have concentrated on molecular rather than functional evidence because of the difficulty in interpretation of hemolytic assays in these animals. The thiolester-containing protease inhibitor, alpha 2 macroglobulin, has been isolated from the hemolymph of the horseshoe crab Limulus polyphemus (Quigley and Armstrong, 1985) and the american lobster Homarus americanus (Spycher et al., 1987). As considerable homology exists between alpha 2 macroglobulin and the thiolester containing proteins of mammalian complement, C3 and C4, these proteins most likely share a common evolutionary origin (Sottrup-Jensen et al., 1985). Furthermore, alpha 2 macroglobulin is considered to be the ancestral protein of this family of proteins and from which the others arose (Reid, 1986a). The presence of channel-forming proteins which are similar to the terminal complement component C9 of mammals has been established in invertebrates as well (Canicatti and Tschopp,

1989). Molecular evidence, therefore indicates that at least the potential exists for primitive complement components to be present in invertebrates.

While functional activity of the complement systems of many lower vertebrates and invertebrates has been described, the information is by no means comprehensive. It is apparent from the literature that the classical pathway of complement activation exists in representatives of most vertebrate classes; the alternative pathway though demonstrated in representatives of some vertebrate classes, has not been described in the cartilagineous fish. The question of whether or not the alternative pathway is present in these primitive vertebrates is an important one from an evolutionary point of view. The alternative pathway does not require specific antibody for activation and so may be considered an innate defense mechanism (Roitt et al., 1985). For this reason, the alternative pathway has been considered to be the phylogenetically older of the two activation pathways. Given that the classical pathway exists in the nurse shark, if the alternative pathway is truly the evolutionary predecessor of the classical then it is likely to be present in the nurse shark. Recently it has been found that a mammalian lectin, mannan binding protein (MBP), specific for mannose and N acetylglucosamine, can activate the classical pathway directly without the participation of

C1q (Ohta et al., 1990). This lectin has a gross structure homologous to C1q and has been found to replace C1q as a recognition unit and activator of C1r and C1s in lytic assays employing C1q depleted serum. The acute phase protein, C reactive protein (CRP), can also activate the classical pathway by binding C1 when it is in complex with the surface C polysaccharide of bacteria in the absence of specific antibody. These observations provide a basis on which it can be argued that the classical pathway could have evolved prior to the specific antibody response and then could be the phylogenetically older of the two pathways. A molecule similar to CRP has been detected in the coelomic fluid of the horseshoe crab which further supports this possibility (Robey and Liu, 1981). Alternatively, in the nurse shark especially, the large pool of natural antibodies may make the alternative pathway of complement activation redundant and therefore not evolutionarily advantageous. Thus the question remains does or does not alternative pathway activity exist in the nurse shark?

Functional and structural homologies between factor B and C2, the serine proteases of the alternative and classical pathway C3 convertases respectively, have been described (Bentley and Campbell, 1986). Both are unusual types of serine proteases in that their catalytic chains are over twice the size of the catalytic chains of other serine

proteases (Reid and Porter, 1981). Functionally, both enzymes, within the C3 convertase complexes, cleave the identical peptide bond between arginine 77 and serine 78 in the alpha chain of C3 to form C3b and C3a. Within the C5 convertase complexes these enzymes also cleave at the identical position in the C5 molecule to form C5b and C5a (Reid, 1986). The genes coding for factor B and C2 are closely linked (less than one kilobase apart) within the MHC complex on human chromosome 6 (Volkanis, 1986) and therefore are believed to have arisen through gene duplication, with the gene for C2 arising from the factor B gene (Reid, 1986; Farries, 1990). It would appear from this data that among the serine proteases, the alternative pathway protein (factor B) appeared before and is the ancestor of the classical pathway protein (C2). However, among the thiolester containing proteins, alpha 2 macroglobulin is considered to be the ancestral protein from which the complement proteins C3 and C4 have evolved. Therefore the presence of alpha 2 macroglobulin in invertebrate species yields no information as to which pathway may have evolved before the other. It is obvious from these observations that the phylogeny of complement evolution is no longer as clear as previously believed. Comprehensive evaluation of whether or not the alternative pathway is present in the cartilagineous fish will provide additional information on the origin of both pathways. The classical pathway has

already been defined in a representative of this class of vertebrates, the nurse shark. The alternative pathway remains to be studied.

Ginglymostoma cirratum, the nurse shark is a primitive cartilagineous fish belonging to the subclass Elasmobranchii. This particular species of shark is believed to have survived for 130 million years (Springer and Gold, 1989). The classical pathway of complement activation has been described in the nurse shark by Jensen and colleagues (Jensen et al., 1981). These studies indicate the nurse shark complement system consists of six functionally purified components C1n, C2n, C3n, C4n, C8n and C9n. C1n was shown to be functionally analogous to mammalian C1 (Ross and Jensen, 1973). The cellular intermediate EAnC1n (sheep erythrocytes optimally sensitized with nurse shark natural antibody to which C1n has been fixed) could be lysed by guinea pig complement components C4-C9 indicating C1n is capable of cleaving mammalian C4 and C2 in a manner similar to mammalian C1. Furthermore, C1n showed a single precipitin arc on immunoelectrophoresis against either anti-C1n antisera or antiserum to whole shark serum. In the presence of EDTA, a chelator of metal ions, however, three precipitin arcs could be demonstrated. These results indicated C1n to be a complex of subcomponents whose integrity depends on metal ions, a feature characteristic of

mammalian C1. One difference between mammalian C1 and nurse shark C1n was in the reversibility of the dissociation of subcomponents in the presence of EDTA by the addition of calcium, magnesium or other metal ions. Whereas the subcomponents of C1 could, after chelation, reassociate to form a functional molecule on the addition of divalent ions, the C1n complex is unable to reassociate (Ross and Jensen, 1973), possibly due to an irreversible conformational change in one or more of the subcomponents. Shark C1n however, was found to be more resistant to inactivation by EDTA than guinea pig C1 but only at 0°C. At 5°C or higher temperatures both shark and guinea pig C1 were rapidly inactivated by EDTA. These results clearly demonstrated a requirement for metal cations for the activity of C1n. Studies also indicate a functional similarity between C2n of nurse shark and mammalian C4 (Hyder-Smith and Jensen, 1986). Similarly, C8n and C9n are comparable to the terminal components of the mammalian system Jensen et al., 1973). Because functional analogies exist, it has been possible to perform hemolytic assays on nurse shark serum using mammalian erythrocyte targets and employing metal ion chelators to evaluate the complement activity of nurse shark serum. The purpose of this study was to determine whether alternative pathway activity could be demonstrated in nurse shark serum using conventional criteria previously established to successfully demonstrate and confirm

alternative pathway activity in other vertebrate classes including ectotherms.

MATERIALS AND METHODS

Nurse Shark Serum

Nurse shark serum was obtained from animals of various sizes, both male and female, held in open sea water pens at the Keys Marine Laboratory on Long Key, Florida. Sharks were anesthetized by being placed in sea water containing 1 ppm tricaine methane sulfonate and subsequently bled from the caudal vein. Once bled, sharks were revived by passing sea water over their gills until gill activity was observed and normal swimming movement could be detected. Temperature of the water in the pen was noted and recorded at the time of each collection. Whole shark blood was placed on ice and transported to the laboratory where it was allowed to clot overnight at 4^0C. Serum was then separated by centrifugation at 2000 x g and 4^0C. Serum was stored at 0^0C in a circulating water bath until assays were performed. Collection data is given in appendix 1.

Chemicals and Reagents

The sources of chemicals and reagents used in this study are indicated in appendix 2. All water used in the preparation of reagents and buffers was doubly distilled water.

Buffers and Solutions

I. Stock solutions:

5 X Veronal was prepared by dissolving 42.5g sodium chloride, 1.875g sodium Barbital and 2.875g Barbituric acid derivative in one liter of water. Barbituric acid derivative was first dissolved in about 200 ml of hot water. Sodium barbital and sodium chloride were dissolved in water at room temperature. When the Barbituric acid derivative was completely dissolved this solution was added to the barbital/sodium chloride solution at room temperature. The pH of the resulting solution was then adjusted to 7.4 with 1 N NaOH. Once the pH was adjusted, the solution was transferred to a one liter volumetric flask and the volume was adjusted with water. 5 X Veronal was stored at 4^0C until needed (Campbell et al., 1968).

5% Dextrose (w/v) was prepared by dissolving 50g of dextrose in 1000 ml of water. This solution was aliquoted into 250 ml portions and autoclaved at 121^0C, 15 pounds of pressure for 10 minutes and stored at 4^0C until required.

2% Gelatin (w/v) was prepared by dissolving 4g of gelatin in 200 ml of water with gentle heating until the gelatin was completely in solution. This solution was aliquoted into 25 ml amounts and autoclaved at 121^{0}C, 15 pounds of pressure for 15 minutes.

A 0.1 M stock solution of magnesium was prepared by dissolving 2.033g of magnesium chloride in 100 ml of water. A 0.03 M stock solution of calcium was prepared by dissolving 1.11g of calcium chloride in 100 ml of water. Stock solutions of divalent cations were not autoclaved but were stored at 4^{0}C.

0.1 M stock solutions of chelators were prepared by dissolving 39.04g ethyleneglycol-bis-(beta-amino ethyl ether) N,N,N,N-tetra acetic acid (EGTA) or 29.22g ethylenediaminetetra acetic acid (EDTA) in a final volume of one liter of water. Neither chelator will dissolve completely in the acidic conditions produced when these compounds are added to water, thus the pH had to be increased to approximately neutral by the addition of 10 N NaOH until the chelator was completely dissolved. The chelator solution was then transferred to a one liter volumetric flask and the volume adjusted with water.

All stock solutions were stored at 4^{0}C until needed.

II. Working Buffers:

Dextrose Gelatin Veronal buffer (DGVB) was prepared fresh daily in 500 ml amounts by adding 100 ml of 5 X Veronal, 25 ml of 2% gelatin and 125 ml of water to 250 ml of 5% dextrose. This solution was used for target erythrocyte preparation.

Dextrose Gelatin Veronal with calcium and magnesium (DGVB++) was prepared as above except 2.5 ml of 0.1 M $MgCl_2$ and 2.5 ml of 0.03 M $CaCl_2$ was added in place of 5 ml of water to give a final concentration of 0.15 mM Calcium and 0.5 mM Magnesium. The pH of these buffers was 7.4.

Chelating buffers were prepared in 100 ml aliquots by adding various amounts of 0.1 M stock chelator solution to 20 ml 5 X Veronal and 5 ml 2% gelatin. The pH was adjusted to 7.4 with 1 N NaOH and the solution was brought up to 100 ml with water. The exact composition of the various chelating buffers is given in appendix 3.

Chelating buffers containing magnesium were also prepared in 100 ml aliquots by adding various amounts of 0.1 M $MgCl_2$ to 20 ml 5 X Veronal, 5 ml 2% gelatin and 20 ml 0.1 M EGTA or 0.1 M EDTA. The solution was adjusted to pH 7.4 with 1 N NaOH and brought up to 100 ml with water. The exact composition of the various chelating buffers with

added magnesium is given in appendix 4.

EDTA saline was used in agglutination assays for the determination of natural antibody titre of nurse shark serum to target erythrocytes. 100 ml aliquots were prepared by adding 20 ml 0.1 M EDTA stock solution to 80 ml physiological saline (0.15 M NaCl, ACS grade).

III. Solutions for immunoblotting procedures:

Dot Blots:

A concentrated stock solution of Tris buffered saline (TBS) was provided in a commercially available immunoblot assay kit (BioRad Immun-Blot Assay kit, Richmond, CA). Once diluted to working strength this buffer contained 20 mM Tris-HCl and 500 mM NaCl in water at pH 7.5.

Tween 20 wash solution was prepared from working strength Tris buffered saline by adding Tween 20 to give a final concentration of 0.05% Tween 20 in TBS (TTBS).

Antibody buffer was prepared by dissolving enough bovine serum albumin, BSA, (Sigma, St Louis, MO) in TTBS described above to give a final concentration of 1% BSA.

Blocking solution used in this assay was prepared by dissolving enough bovine serum albumin in Tris buffered saline described above to give a 1% solution.

Western blots:

Western blots were performed using a multiscreen apparatus (BioRad, Richmond, CA) and by conventional means. Shark and human serum at 1:5 dilution were electrophoresed on 7.5% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE gels). Resolving gel for two gels was prepared by mixing 9.7 ml distilled water with 5.0 ml of 1.5 M Tris-HCl, 5.0 ml of Acrylamide/Bis (30%T, 2.67%C prepared by dissolving 29.2g Acrylamide and 0.8g Bis acrylamide in 100 ml water) and 200 ul of 10% SDS. This solution was degassed under vacuum for 15 minutes after which time the polymerizing agents, 100 ul freshly prepared 10% ammonium persulfate and 100 ul of TEMED were added. This monomer solution was then used to fill glass plate "sandwiches"(assembly is described in BioRad Mini-PROTEAN II multi-casting chamber manual and Mini-PROTEAN II dual slab cell manual for single gel casting) up to about one centimeter from the top of the small glass plate. Gel casting "sandwiches" contained 1 mm spacers between the two glass plates to produce 1 mm thick gels. Gels were then overlayed with water saturated amyl alcohol and allowed to polymerize for one hour. When the resolving gel was polymerized, the amyl alcohol was poured off and the top of the gels were rinsed with distilled water and completely drained. The stacking gel mixture described below was poured onto the top of the resolving gel. Teflon combs were placed into the glass plate "sandwich" prior to

pouring the stacking gel. For Western blots, a preparative comb (one large broad well and one small narrow standard well) was used. For other routine SDS PAGE applications, combs for 10 wells were used. The stacking gel was prepared by mixing 12.2 ml of distilled water with 5.0 ml of 0.5 M Tris-HCl 2.6 ml of Acrylamide/Bis (described above for resolving gel) and 200 ul of 10% SDS. This solution was degassed under vacuum for 15 minutes after which 100 ul of 10% ammonium persulfate and 20 ul TEMED were added. The stacking gel was allowed to polymerize for one hour at room temperature and the gels along with inserted combs were stored in sealed plastic bags with a small amount of electrode buffer at 4^0C in a glass jar until used.

A 5 X solution of electrode buffer, used to dilute samples and run electrophoresis, was prepared by dissolving 4.5g of Tris base, 21.6g of glycine and 1.5g of SDS to a final volume of 300 ml in water.

Sample buffer for electrophoresis was prepared by adding 1.0 ml 0.5 M Tris HCl, 0.8 ml glycerol, 0.2 ml 0.05% bromophenol blue and 1.6 ml 10% SDS to 4.0 ml distilled water. For reducing samples prior to electrophoresis 0.4 ml of 2-mercaptoethanol was added to the buffer and samples were heated at 95^0C for 4 minutes.

Transfer buffer, for the transfer of proteins from the electrophoresis gel to the nitrocellulose membrane, was prepared by dissolving 3.03g Tris base and 14.4g glycine in approximately 800 ml of water. 200 ml of methanol (analytical reagent grade) is then added. The pH should be approximately 8.3 and it should not need to be adjusted.

Target cells

I. Preparation of cell suspensions:

Sheep, bovine, horse and rabbit whole blood in Alsever's solution was obtained from Lampire Biological Laboratories, Pipersville, PA. An aliquot of whole blood suspension (usually 10 ml) was washed three times in 30-40 ml of chilled (4^0C) isotonic saline (0.15 M NaCl, ACS grade) in 40 or 50 ml round bottom glass centrifuge tubes. In a Beckman refrigerated Gpr centrifuge equilibrated at 4^0C the cell suspension was centrifuged at 1000 x g for 15 minutes. The buffy coat was removed from the sedimented cells using a wide tip pasteur pipet after the first wash. Following the saline washes, the cells were washed two more times in DGVB without calcium or magnesium (DGVB--) and resuspended in this buffer. Washed cells were then standardized to a concentration of 1 X 10^9 cells/ ml and stored for up to one week at 0^0C in either a circulating water bath or on ice in styrofoam coolers.

II. Standardization of target erythrocytes:

Standardized erythrocytes suspensions were prepared from washed erythrocyte stock suspensions. An aliquot of these cells was resuspended in a measured volume of DGVB--. The cells per milliliter of suspension was determined manually using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY) or by an automated method using a

Coulter counter (Coulter Corporation, Hialeah,FL). Depending on the counts obtained, the volume of DGVB-- was adjusted to yield a 1 X 10^9 cells/ml stock suspension. Stock cell suspensions were stored for up to one week at 0^0C. For use in assays, the stock cell suspensions were diluted to 1 X 10^8cells/ml in buffer. Prior to dilution, the stock cell suspensions were washed twice in DGVB--. An optional optical method that was easier to perform and more rapid was also employed to standardize cell suspensions. For each new lot number and cell type received, a manual count was performed to standardize a suspension to 1 X 10^8cells/ml. One ml of this suspension was lysed in a total volume of 15 ml of distilled water in a volumetric flask. The optical density of the solution was determined at 415 nm in a Beckman DU-68 spectrophotometer. The optical density value obtained is the OD factor in the equation: $V_f = V_i \times OD/F$, used to standardize cells to a concentration of 1 X 10^8. V_f is the final volume, V_i is the initial volume and F is the optical density factor determined for the erythrocyte suspension of a particular species and lot number (Campbell et al., 1968). The OD factor (F) was established for each erythrocyte species and each fresh erythrocyte lot number received.

III. Preparation of sensitized erythrocytes:

Nurse shark natural antibody was used to sensitize

sheep and bovine target erythrocytes. The source of nurse shark natural antibody was heat-inactivated (56^{0}C, 30 min) nurse shark serum. One volume of a 1 X 10^{9}cells/ml suspension of erythrocytes was mixed with the same volume of a subagglutinating dilution (determined by agglutination assay, see below) of heat-inactivated nurse shark serum in DGVB--. The cell suspension was incubated in a shaking water bath at 37^{0}C for 30 minutes and then at 0^{0}C for an additional 30 minutes with manual shaking at 5 minute intervals. The suspension was incubated in small flasks rather than tubes to ensure maximum exposure of the cell surfaces to antibody and to allow adequate mixing and therefore better sensitization. Following incubation cells were washed twice in DGVB-- to remove excess antibody and restandardized to 1 X 10^{9} cells/ml and stored at 0^{0}C for up to three days (Jensen, 1969).

Preparation of alternative pathway activators

Activators used in this study were zymosan, inulin, cobra venom factor (CVF) and lipopolysaccharide (LPS). Zymosan was prepared by suspending 400 mg of zymosan in 100 ml physiological saline and boiling for two hours, adding additional saline as needed (Fine et al., 1972). The zymosan particles were washed twice in saline and resuspended in 20 ml saline to a final concentration of 20 mg zymosan/ml. Lyophilized lipopolysaccharide from

Salmonella typhosa (Sigma, St Louis, MO) in 100 mg aliquots was resuspended with 5 ml of saline to a final concentration of 20 mg LPS/ml. Inulin from dahlia tubers (Sigma) was dissolved in saline to yield a stock solution of 20 mg inulin/ml. One milligram of cobra venom factor from the Indian cobra, Naja naja kaouthia, (Sigma St Louis, MO) was reconstituted with 1 ml of saline. This solution was then used in activation assays as a 1 mg/ml stock.

Agglutination assays

Agglutination assays were performed on nurse shark serum in order to determine the titre of natural antibodies to the target erythrocytes used. The assays were set up in "U" bottom polyvinyl microtitre plates (Dynatech, Chantilly, VA) using a four drop system (Ross and Jensen, 1973). Doubling dilutions of nurse shark serum were prepared by adding one drop (25 ul) of serum to one drop of EDTA-saline and serially diluting the serum with microdiluters. Two drops of EDTA-saline were then added to each well followed by one drop of 1 X 10^8cells/ml suspension of target erythrocytes. Negative controls contained three drops of EDTA-saline and one drop of target erythrocytes with no serum. Plates were incubated at 30^0C for 15 minutes with shaking on microtitre shakers and then left at room temperature for several hours to allow the cells to settle or alternatively kept overnight at 4^0C. Agglutination was

determined by comparing the behavior of the cell buttons to the control as plates are tilted at about a 45^0 angle to allow the buttons to run. Agglutination titre was recorded as the reciprocal of the highest dilution showing agglutination.

Hemolytic assays

Hemolytic assays were performed in "V" bottom polyvinyl microtitre plates (Dynatech, Chantilly, VA) using a five drop assay. Doubling dilutions of shark serum were made by adding one drop (25 ul) serum to one drop of buffer (DGVB++, EDTA/EGTA-GVB or EDTA/EGTA-GVB-Mg++) and serially diluting the serum with microdilutors. Three drops of buffer were then added to each well followed by one drop of 1 X 10^8cells/ml target erythrocytes. Spontaneous lysis controls consisted of one drop of cells in four drops of buffer. 100% lysis controls consisted of one drop of cells in four drops of distilled water. All buffers and target cell suspensions as well as serum were kept on ice while the assays were being set up so that no activation of complement occurred prior to incubation. Plates were incubated at 30^0C with shaking on microtitre shakers for one hour. After this incubation period the plates were centrifuged at 500 x g to stop the hemolytic activity and the reciprocal of the last dilution showing 50% lysis was determined as the hemolytic titre. Determination of this end point was made visually by

comparison to the spontaneous lysis control (Jensen and Ross, 1973). The plates were then stored overnight at 4^0C and read again the next day as results became clearer and easier to interpret after this time period.

Determination of the effect of chelators on the hemolytic activity of nurse shark serum

I. Incubation time of serum in the presence of chelator:

Rapid fixation of C1, C4 and C2 occurs in human serum before C1 is inactivated by removal of calcium in the presence of EGTA (Platts-Mills and Ishizaka, 1974). The fixation of shark C1n has also been shown to occur within 5 minutes even at 0^0C (Ross and Jensen, 1973); thus it is necessary to incubate shark serum in the presence of the chelator to allow complete binding and removal of calcium and or magnesium prior to performing hemolytic assays. To determine the optimum period of time necessary for removal of divalent cations from nurse shark serum by the chelators, assays were set up as described above in buffers containing chelators except for the target cells which were not added. Plates were incubated for periods of 15, 30 and 45 minutes at 30^0C without shaking before the addition of target cells. After target cells were added plates were incubated for one hour at 30^0C with shaking in microtitre shakers. The endpoint was read as described above. The effect of incubation at higher temperature (30^0C) on complement

components was evaluated by assaying the serum under identical conditions in the absence of the chelator for loss of activity.

II. Amount of chelator needed to remove calcium and or magnesium from nurse shark serum:

Normal values for serum calcium and magnesium levels have not been established in the nurse shark, however, a study conducted by Keys Marine Lab in which sharks were caught and tested for several blood chemistries revealed that calcium values for the eleven nurse sharks tested ranged from 12.1 to 16.5 mg/dL. Using 14 mg/dL as an average value mg/dL was converted to 3.5 mmol calcium. Only two of the nurse sharks in this study were tested for magnesium levels. Magnesium values were 2.6 and 3.5 meq/L which would convert to 1.3 mmol/L and 1.75 mmol/L respectively (Murru, 1984). With a limited amount of previous information available for estimating how much chelator would be required to remove these divalent cations from the serum of sharks, assays were conducted using varying amounts of chelators to establish this parameter. Hemolytic assays were set up as described above using buffers containing varying amounts of chelator. The composition of these buffers are given in appendix 3. The serum was chelated by incubating the plates without shaking for the optimal time established from the procedure outlined

above before the addition of target cells. After the addition of target cells the plates were incubated at 30^0C for one hour with shaking. The endpoint was again read as above. To determine the effect of EGTA, in concentrations ranging from 5 to 25 mM, on a larger number of individual sera, an alternative procedure was developed. In this procedure shark serum was mixed with various amounts of 0.1 M stock EGTA solution, 0.1 M stock EDTA solution or DGVB++ and incubated at 30^0 C for 30 minutes previously established as optimal time and then assayed for hemolytic activity. The various amounts of shark serum and chelators are given in appendix 4. Chelated serum was then used in hemolytic assays, as described above, using buffers containing an equivalent amount of chelator.

III. Determination of the effect of added magnesium on the hemolytic activity of chelated nurse shark serum:

Nurse shark serum was chelated as described above with 20 mM EGTA. The hemolytic activity of serum was evaluated by conducting microtitre assays in buffers containing 20 mM EGTA and increasing amounts of magnesium ions. The composition of these buffers are described in appendix 5. The addition of magnesium ions to a solution containing EGTA or EDTA results in a drop in pH of the solution (Platts-Mills and Ishizaka, 1974). Complement activity as well as stability of target cells is profoundly affected by pH

(Kabat and Mayer, 1961). Therefore, to obtain meaningful comparative data on hemolytic activity as a function of magnesium ion concentration the pH of all buffers used in these experiments was adjusted to 7.4 with 1 N NaOH before the volume was adjusted to 100 ml with distilled water.

Determination of the effect of activators of the mammalian alternative pathway on the hemolytic activity of nurse shark serum

No information is available on the amount of alternative pathway activators needed to deplete nurse shark serum of hemolytic activity. Therefore, various amounts of activators were used ranging from 1 to 10 mg/ml suspensions for zymosan, LPS and inulin and 0.05-0.25 mg/ml suspensions for cobra venom factor. Activation assays were set up in 30 ml round bottom glass centrifuge tubes.

Stock suspensions of activators were prepared as described and various quantities added to the reaction tubes. The volume in each tube was then brought up to one ml as necessary by the addition of buffer (DGVB++ or 20 mM EGTA-10 mM Mg++-GVB). Exact ratio of buffer to stock activator is given in appendix 6. One ml of shark serum was then added to each tube and incubated at 30°C in a shaking water bath. A sufficient number of tubes were set up so that one complete set (containing 0, 1, 5 and 10 mg/ml

activator) could be removed at zero time, 90 minutes, three hours, 4.5 hours and overnight as the protocol required. Once removed from incubation these tubes were centrifuged and kept at 0^0C until residual hemolytic activity was determined by microtitre assay as described previously.

Immunoblotting

The presence in nurse shark serum of complement proteins which would antigenically cross react with antisera raised against human complement components was investigated using immunoblotting techniques.

I. Dot blots:

Dot blots were performed using both the Minifold (Schleicher and Schuell, Keene, NH) and Biodot (BioRad, Richmond, CA) dot blotting manifolds. Nitrocellulose membranes were prepared for the procedure by wetting in Tris buffered saline (TBS). Wet membranes were then placed in the dot vacuum manifold and each well was wetted again with 100 ul of TBS. 100 ul of each diluted serum sample to be tested was then placed in each well of the dot blot template. Dilutions of nurse shark serum were prepared in TBS to yield 1:10, 1:100 and 1:200 dilutions. Human and guinea pig serum as well as lysozyme (65 units/ml), were prepared in a similar manner to yield dilutions of 1:100 and 1:200. Serum and control samples were allowed to flow

through the membrane by gravity. When the entire sample had passed through the membrane, it was washed with 100 ul TBS per well. Unreacted sites on the membrane were then blocked using 300 ul per well of a 3% bovine serum albumin blocking solution. The blocking solution was left on the membrane for one hour and then the membrane was washed twice with 200 ul Tris buffered saline with Tween 20 (TTBS) using a vacumn. 100 ul of diluted primary antibody (1:15 or 1:30 in antibody buffer) was then added to the appropriate wells. The primary antibody solutions were allowed to drain through the membrane by gravity. Once the primary antibody solutions had passed through, the membrane was washed three times with 300 ul TTBS per well. Following the last wash, 100 ul of diluted secondary antibody labelled with horse radish peroxidase was added to each well and allowed to drain through by gravity. After the secondary antibody had drained through, the membrane was washed twice with 300 ul TTBS per well. The membrane was then removed from the manifold and washed twice in TBS. The blot was then developed following the directions of the BioRad ImmunoBlot Assay kit until good contrast was developed between the control and samples. Color development was stopped by placing the membrane in distilled water.

II. Western Blots:

Human and shark serum were diluted 1:5 in electrode buffer and then 1:4 in sample buffer. These samples were heated at 95^{0}C for 4 minutes before loading SDS-PAGE gels (Laemmli, 1970) for electrophoresis. Electrophoresis was conducted using the BioRad Mini-Protean II dual slab cell at 200 volts. Electrophoresis was discontinued when the tracking dye reached the end of the gel which took approximately 45 minutes. Runs using gels cast with preparative combs (for use in the multiscreen apparatus) were loaded with 1500 ul of sample diluted 1:4 in sample buffer and heated as described. Five ul of a prestained low molecular weight standard mixture (BioRad, Richmond,CA) was loaded in the small well of the preparative gel directly without the dilution and preparation described above. Ten microliters of sample and 10 ul of a prestained molecular weight standard (BioRad, Richmond CA) was loaded into wells when using gels cast with ten well combs. Once electrophoresis was complete, the gels were removed and equilibrated in 500 ml of transfer buffer for 15 minutes. The BioRad Mini transblot apparatus was used to electrophoretically transfer proteins from the polyacrylamide gel to the nitrocellulose membrane for immunoblotting (Towbin et al., 1979). The membrane was first wetted in transfer buffer before assembling the gel/membrane sandwich. The electrophoretic transfer was

conducted at 70 V for one hour. Following transfer, unreacted sites on the membrane were blocked by incubation in 1% BSA for one hour at room temperature and then rinsed in TBS. At this point, if the multiscreen apparatus was to be used the membrane was placed inside the apparatus and it was assembled. Primary antibody, 600 ul, was added to a single channel of the multiscreen. Multiple antisera could be screened simultaneously against a single serum sample using the multiscreen apparatus. For Western blots using a single antisera, the membrane was flooded with 6 ml of primary antisera in a large shallow weigh boat. For both procedures, the membrane was incubated at room temperature with the primary antibody for one hour. Following this incubation period, the membrane was washed three times in TTBS and the secondary antibody was applied. Secondary antisera was applied to each individual channel of the multiscreen (600 ul) or the entire membrane was submerged in antiserum (6 ml) in a weigh boat. The secondary antibody was allowed to incubate on the membrane for one hour at room temperature. Following this incubation the membrane was washed five times with TTBS and then in TBS for 5 minutes. The blot was developed using a BioRad color development kit as described above. Description and sources of primary and secondary antibodies used in immunoblotting are given in appendix 7.

RESULTS

Agglutination assays:

Results of agglutination assays are given in table 1. These results indicate a high level of natural antibody in nurse shark serum against rabbit cells, moderate levels of antibody to sheep and horse erythrocytes and a minimal level against bovine erythrocytes. These levels of natural antibody were demonstrable consistently in all nurse shark sera tested.

Effect of chelators on the hemolytic activity of nurse shark serum

I. Incubation time of serum in the presence of chelator:

Table 2 show the results of hemolytic assays in which nurse shark serum was incubated with various concentrations of EDTA for several different periods of time, prior to assaying for residual activity. These results represent experiments on a single individual. Complete inhibition of hemolytic activity was seen against sensitized sheep and unsensitized horse erythrocyte target cells when serum was chelated with 5 mM EDTA for 30 minutes. Some reduction in the hemolytic titre of the serum control (containing no chelator) was seen against horse erythrocytes when the serum was preincubated for 45 minutes. From these experiments with sheep and horse erythrocytes it was determined that in

order to demonstrated depletion of hemolytic activity of the serum preincubation of the serum with the chelator was necessary for chelators to bind the metal cations present in nurse shark serum. The 45 minute preincubation time which resulted in a decrease in lytic activity, is most likely due to a spontaneous deterioration of one or more complement components, against at least one target cell. Therefore, time periods of 15 and 30 minutes were chosen to be near optimal for chelation of serum prior to assaying hemolytic activity against rabbit cells. Results for rabbit cell targets, given in table 3, demonstrate a preincubation period of 30 minutes, in the presence of 10 mM EDTA is necessary to completely prevent lysis of rabbit erythrocytes by nurse shark complement. Experiments were then conducted with sensitized and unsensitized bovine erythrocytes as targets, using nurse shark serum preincubated with varying amounts of EDTA for 30 minutes. Results, presented in table 4, show that there is a complete loss of hemolytic activity of nurse shark serum against bovine erythrocyte targets when the serum is preincubated for 30 minutes. From these chelation studies it was determined that serum hemolytic activity against bovine, sheep, horse and rabbit erythrocytes could be depleted by preincubation for 30 minutes in the presence of 10 mM EDTA without incurring spontaneous reduction of lytic activity from inactivation of complement components at the higher than ambient temperature

(i.e. 30^0C). Following the experiments employing EDTA, similar experiments were conducted using EGTA as the chelator. At this point in the study results suggested that a preincubation period was necessary for the chelator to be effective in removing metal ions and thereby decreasing hemolytic activity of the serum. Data also suggested that a 45 minute preincubation time at 30 ^{0}C may result in spontaneous loss of hemolytic activity not due to the chelator. Therefore, for studies investigating the effect of EGTA, serum was preincubated for periods of 15 and 30 minutes only, in the presence of various concentrations of the chelator. From the results of these experiments, presented in table 5, it was concluded that 30 minutes was sufficient preincubation time to allow EGTA to chelate nurse shark serum without spontaneous loss of hemolytic activity. The purpose of chelation experiments was to establish a general protocol for chelating nurse shark serum in order to assess the effect of chelators on the hemolytic activity of the serum. Although these experiments were limited in number of animals tested, it could be concluded that a 30 minute preincubation time in the presence of the chelator does allow maximal activity of the chelator without concomitant loss of serum hemolytic activity as a result of factors other than the effect of the chelator.

II. Concentration of chelator needed to remove cations from nurse shark serum:

Results of the above experiments demonstrate that a concentration of 10 mM EDTA, preincubated with serum for 30 minutes was sufficient to inhibit lysis of target cells by nurse shark complement. Table 6a provides data on the residual hemolytic activity of serum from several individuals in the presence of 10 mM EDTA. These data demonstrate that, for the great majority of nurse shark serum/target cell combinations, 10 mM EDTA is sufficient to completely inhibit hemolytic activity of whole nurse shark complement. With a 30 minute preincubation time established as being optimal for both EDTA and EGTA to completely chelate metal cations from nurse shark serum, it was then necessary to determine the concentration of EGTA which would maximally inhibit lysis of target cells by nurse shark complement. Table 6 presents the results of an experiment employing serum from a single individual chelated with increasing concentrations of EGTA to assess hemolytic activity of such treated serum against the mammalian erythrocyte targets. These data demonstrate no further reduction of hemolytic activity against any target cell at concentrations of EGTA greater than 10 mM. Concentrations of EGTA above 25 mM were not considered to be useful and therefore concentrations ranging from 5-25 mM were reevaluated using serum from several individual animals.

Hemolytic titres from these experiments are presented in table 7. Absolute hemolytic titres for this set of experiments were then converted to percent of the control titre (containing no chelator) in order to better illustrate and compare the phenomenon of loss of hemolytic activity due to the chelator in serum samples which had differing initial hemolytic titres. These data are presented in table 8. Figure 1 presents these data in graphic form.

Statistical analysis of the data presented in table 8 is given in table 9. From the data presented in tables 6,7 and 8, and figure 1 it can be seen that although the presence of EGTA significantly reduces the hemolytic titre, nurse shark serum retains a low level of hemolytic activity against all the mammalian target cells tested in the presence of the chelator. As EGTA selectively chelates calcium, it may be concluded that all available calcium ions had been removed from the serum, and the hemolytic activity that remained was totally independent of calcium ions. Calcium independent hemolytic activity is considered to be characteristic of the alternative complement pathway specifically.

The effect of added magnesium ions on the hemolytic activity of chelated nurse shark serum

As the alternative pathway requires magnesium ions, it was necessary to determine the effect of added magnesium on chelated serum. On the addition of magnesium ions to chelated serum an increase in hemolytic activity due to enhancement of the alternative pathway, if it operates in the nurse shark, could be expected. The effect of added magnesium, in concentrations ranging from 1-20 mM on the hemolytic activity of shark serum chelated with 20 mM EGTA is presented in table 10. In order to more clearly demonstrate the reconstitution of hemolytic activity by the addition of magnesium, the absolute hemolytic titres were converted to percent of an unchelated control titre of the same serum sample. Table 11 presents these data. The data in table 11 is presented graphically in figure 2. From the data in tables 10, 11 and 12, and figure 2 it was concluded that 10 mM Mg++ added to nurse shark serum chelated with 20 mM EGTA would yield maximum hemolytic activity in such serum. A significant level of hemolytic activity was seen against both sensitized and unsensitized sheep erythrocytes; this was unexpected as sheep erythrocytes are not lysed by the alternative pathway of mammals (Platts-Mills and Ishizaka, 1974). To confirm that the lysis of sheep erythrocytes observed in these experiments was due to the alternative pathway, nurse shark serum was chelated with 10

mM EDTA and hemolytic assays were performed using 10 mM EDTA-10 mM Mg++-GVB as the assay buffer. The chelated serum was assayed for residual hemolytic activity as described above against sensitized and unsensitized sheep erythrocytes as well as sensitized bovine erythrocytes. The results are recorded in table 12. From the data it can be concluded that a calcium-independent hemolytic system that is also magnesium dependent is responsible for the lytic activity observed in nurse shark serum. Thus, the parameters for demonstrating this alternative hemolytic system were established as serum chelated with 20 mM EGTA to which 10 mM Mg++ is added. Once it had been established that a calcium-independent pathway (i.e. the alternative pathway) existed in the nurse shark, it was of interest to determine if the 30^0C optimum temperature established by Jensen for the classical pathway in the nurse shark was also optimal for the alternative pathway as immune responses of ectotherms have been shown to be temperature-dependent (Jurd, 1983; Pettey and McKinney, 1983). Assays for total hemolytic activity (in DGVB++) and alternative pathway activity (20 mM EGTA-10 mM Mg++-GVB) were performed on nurse shark serum samples at different temperatures and the hemolytic titre recorded. These results are presented in table 13 and show that total hemolytic activity against sheep erythrocytes is best demonstrated at 30^0C similar to the optimal temperature for classical pathway as stated by Jensen (1981). The data

from these experiments though limited, did suggest, specifically when horse erythrocytes were used as targets, that incubation at 23^0C might yield higher alternative pathway hemolytic titres and therefore be the optimal temperature for demonstrating this activity. Consequently, sera from several individuals were evaluated for alternative pathway hemolytic activity at 23^0C and 30^0C using horse and rabbit erythrocytes targets. Results are presented in table 13a. No increased levels of hemolytic activity against these target cells was observed at 23^0C when compared to levels obtained at 30^0C. Additional data on a larger number of animals and a wider temperature range are needed to determine the temperature optimum for alternative pathway activity in the nurse shark. For the purposes of this study, based on the above observations, all subsequent hemolytic titrations and activation of serum were carried out at 30^0C.

The possibility was considered that the rate of the alternative pathway activity might be slower than observed for total hemolytic activity. Hemolytic assays were set up, using horse erythrocytes as targets, in which incubation time for the assays was increased from one to four hours. Results are given in table 14. For the two serum samples tested no increase in alternative pathway activity was observed at either incubation temperature (23^0C or 30^0C) as

incubation times were increased from one to four hours. In addition, spontaneous lysis of the target cells in control samples were a problem of increased incubation time.

Result from earlier experiments, employing selective chelators to remove calcium ions from nurse shark serum demonstrated a calcium-independent hemolytic pathway which was enhanced by the addition of magnesium ions, i.e. an alternative pathway of complement activation. To further study and examine alternative pathway activity in nurse shark serum it was necessary evaluate the ability of known activators of the mammalian alternative pathway to activate and in turn deplete the hemolytic activity of nurse shark serum. Table 15 gives data on the depletion of hemolytic activity, of both chelated (20 mM EGTA) and unchelated nurse shark serum following incubation with increasing concentrations of zymosan for various time periods at 30^0C. The hemolytic titres obtained were converted to percent of control samples (controls were nurse shark serum incubated for the same time period with buffer containing no activator) and presented as a bar graph in figures 3 and 4. Table 16 shows the results of a similar experiment using LPS as the activator in place of zymosan. Hemolytic titres, as before were converted to percent of controls and plotted as a bar graph in figure 5. Table 17 and 18 contain data from experiments using cobra venom factor and inulin respectively

as activators. Cobra venom factor and inulin did not produce any significant reduction of hemolytic activity of nurse shark serum under our experimental conditions. Therefore, zymosan and LPS were selected as activators for further studies using additional sera. Results of these studies are presented in tables 19, 20 and 21, and in figure 6. Serum from one animal, tag# 463 showed a depletion of hemolytic activity with increasing concentrations of zymosan and incubation times for both unchelated or chelated (20 mM EGTA) serum samples. Serum from the second animal (tag# 456), however, demonstrated a reduction of hemolytic activity on incubation with and activation by zymosan only when nonchelated serum was used. Chelated serum from this individual did not show a loss of activity. In addition, increasing the concentration of zymosan beyond 1 mg/ml, did not appreciably reduce the hemolytic titre of unchelated serum of this animal. Chelated serum of this individual did not respond to increasing concentrations of zymosan.

The above functional studies demonstrate that an alternative pathway of complement activation operates in some form in the nurse shark. Its existence is indicated by the following observations:

1. Nurse shark serum retains hemolytic activity in the presence of EGTA, a selective chelator of calcium ions.
2. This calcium-independent alternative activity is enhanced

by the addition of magnesium ions to EGTA and EDTA chelated serum and.

3. Zymosan, an activator of mammalian alternative pathway can deplete nurse shark serum of hemolytic activity in the presence of EGTA.

In addition to providing evidence for the existence of an alternative pathway of complement activation in the nurse shark these studies have helped to establish a protocol by which alternative pathway hemolytic activity can be assayed for in nurse shark serum namely, by chelation of shark serum with 20 mM EGTA for 30 minutes prior to assay employing 20 mM EGTA-10 mM Mg++-GVB as the assay buffer. Once the parameters for assaying specifically for alternative pathway activity had been established, it was then possible to compare total hemolytic activity with alternative pathway activity of several individual sharks against mammalian erythrocyte targets. Tables 22-27 present total and alternative pathway hemolytic titres of sera from several animals using the six different types of mammalian erythrocyte targets. Graphic representation of these data is presented in figures 7 and 8.

Immunoblots

Nurse shark serum was examined by immunoblotting procedures in an attempt to provide evidence of proteins antigenically similar to human complement proteins by employing antisera to human complement components. Nurse shark serum was probed with primary antibodies to human complement components C3, C4, C5, factor B and factor H, and the related thiolester-containing protein alpha 2 macroglobulin. It was hoped that nurse shark complement components contain antigenically crossreactive epitopes similar to their human counterparts which would be recognized by antibody raised to the human proteins.

Dot blots were initially attempted using the Schleicher and Schuell Minifold apparatus. Results of the initial dot blot appear in figure 8. Human serum, used as a positive control in this assay, reacted strongly with all primary antisera tested. Contrary to expectation, a reaction was also seen with normal rabbit serum, nurse shark serum and when the secondary antisera alone was applied. The reaction of human serum with normal rabbit serum and the secondary antisera alone indicated nonspecific binding of the antisera to these samples. Likewise, anti-C5 antisera as a primary antiserum gave a strong reaction with all samples, including lysozyme, used as a negative control in this assay. The nonspecific reaction of anti-human C5 antisera (rabbit anti-

human C5, Janssen Biochimica) invalidated results. Additionally, coloring of membrane areas outside of wells indicated leakage of samples from several wells. Although blots of shark serum did react with anti-C3, C4 and factor B and H antisera results were inconclusive as a reaction was noted with non-immune rabbit serum used in place of the primary antisera.

A second blot (figure 9) was prepared using a fresh sample of human serum and varying the concentration of samples and using a different source of primary antisera. Results of this blot, shown in figure 9, show an improvement in the specificity of the reaction with human serum and primary antisera preparations. Shark serum, however, demonstrated reactions with all antisera tested. The intensity of reactions were no greater than the reaction with non-immune rabbit serum. The anti-human alpha 2 macroglobulin antiserum showed uniform strong reaction with all samples and therefore was rejected. Faint reactions were seen with lysozyme.

A third blot (figure 10) was prepared using two separate preparations of lysozyme (A and B), nurse shark serum and human serum as test samples and primary antisera similar to those used in the previous blot (figure 9). The secondary antisera dilution was increased to 1:3000 from

1:1000 used in the previous two blots in an attempt to reduce the nonspecific reactions that occurred previously. Unfortunately reducing the concentration of secondary antisera produced reactions with several samples that were too weak to be reliably interpreted.

To avoid sample leakage problems that occasionally occurred with the Schleicher and Schuell Miniblot apparatus, the BioRad dot blot apparatus was used to perform a fourth blot. For the development of the fourth blot (figure 11) the secondary antisera was diluted 1:1000. The human serum sample gave the anticipated strong specific reactions with all the primary antisera tested. Guinea pig serum as antigen produced weaker but acceptable reactions with the primary antisera used. However, lysozyme reacted nonspecifically with anti-C3 and anti-C4 primary antisera. The reactions produced between nurse shark serum samples and anti-C3, C4, factor B and factor H were similar in intensity to those obtained with non-immune rabbit serum or rabbit gamma globulin fraction when used in place of the primary antisera. This nonspecific reaction with rabbit serum indicates that results with nurse shark serum are inconclusive.

Western blots, whereby serum proteins are electrophoretically separated before reaction with specific

antisera, were performed in an attempt to eliminate the apparent nonspecific reactions which had occurred in the dot blots. Initially, both human and shark serum samples were probed with several primary antisera preparations using the BioRad Multiscreen apparatus as described in Materials and Methods. In the case of human serum, blots (figure 12) showed distinct reactive bands at position corresponding to the approximate molecular weight of the human complement component against which the specific antiserum in the lane had been prepared. No analogous reactive bands appeared on the membrane which contained transferred shark serum proteins. As factor B is a complement component unique to the alternative pathway, a Western blot was prepared and screened using only anti-human factor B antisera as the primary antibody. As only a single antiserum was being used, it was not necessary to use the multiscreen apparatus. Two gels, containing identical samples, were run simultaneously. Lane 1 was loaded with a prestained low molecular weight protein standard, lanes 2 and 3 were loaded with 1:2 and 1:5 dilutions of shark serum respectively. Lane 4 was loaded with a 1:5 dilution of human serum. One gel was stained with Coomassie blue to illustrate protein separation, (figure 13), while the other was used to transfer proteins to a nitrocellulose membrane for immunoblotting. Results of this immunoblot are shown in figure 14. Strong reaction with antihuman factor B antisera

is seen in lane 1 of the Western blot, which contained human serum, however, neither dilution of nurse shark serum, lanes 2 and 3, showed a reaction with antiserum to human factor B. The antiserum also showed a weak reaction with a low molecular weight substance present in the human serum sample which may be a breakdown fragment of factor B.

TABLE 1

Agglutination of mammalian target erythrocytes by nurse shark serum.

	Target erythrocytes			
Shark Serum	Rabbit	Horse	Bovine	Sheep
010992 238*	2048	64	2	
010992 186	2048	32	2	
010992 242	2048	2048	2	
010992 241	2048	1048	2	
010992 248**	2048	512	2	
010992 244	2048	512	2	
010992 88	2048	512	0	
010992 463	2048	512	2	
040692 248**				256
040692 463				16
040692 452				64
040692 489				128
040692 238*				64
040692 477				256

Table 1: Reciprocal of highest dilution of shark serum showing agglutination of target cells. All target cells used were unsensitized erythrocytes and standardized to 1 X 10^8 cells/ml.
* and ** are the same animals bled at different times.

TABLE 2

Effect of time of preincubation of nurse shark serum in the presence of varying amounts of EDTA on the hemolytic activity of the serum

I. Horse erythrocyte targets

Concentration of EDTA (mM)

Time(min)	5mM	7.5mM	10mM	12.5mM	15mM	DGVB cont.
0	<2	<2	<2	nd	nd	>64
15	0	0	0	0	0	>64
30	0	0	0	0	0	>64
45	0	0	0	0	0	32

II. Sensitized Sheep erythrocyte targets

Concentration of EDTA (mM)

Time(min)	5mM	7.5mM	10mM	12.5mM	15mM	DGVB cont.
0	2	2	2	2	2	>64
15	<2	<2	<2	<2	<2	>64
30	0	0	0	0	0	>64
45	0	0	0	0	0	>64

Table 2: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. A value of <2 represents the presence of hemolysis at a dilution of 1:2 that is less than 50%. A value of 0 represents no hemolysis at any dilution. Serum used in these experiments was NSS 092591-2.

TABLE 3

Effect of 15 and 30 minute preincubation periods for nurse shark serum in the presence of various amounts of EDTA on the hemolytic activity of the serum against rabbit erythrocytes

	Concentration of EDTA					
Time(min)	5mM	7.5mM	10mM	12.5mM	15mM	DGVB cont.
15	<2	<2	<2	<2	0	16
30	<2	<2	0	0	0	16

TABLE 3: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. A value of <2 represents hemolysis at a dilution of 1:2 that was less than 50%. Nurse shark serum used in these experiments was NSS 092591-3.

TABLE 4

Effect of a 30 minute preincubation of nurse shark serum in the presence of various concentrations of EDTA on hemolytic activity of the serum against bovine erythrocyte targets

	Concentration of EDTA (mM)					
Target	5mM	7.5mM	10mM	12.5mM	15mM	DGVB cont.
sens.	0	0	0	0	0	>64
unsens.	0	0	0	0	0	16

Table 4: Target cells used were both sensitized (sens.) and unsensitized (unsens.) bovine erythrocytes. Values indicate the reciprocal of the last serum dilution showing 50% hemolysis of target cells. Nurse shark serum used in these experiments was NSS 092591-1.

TABLE 5

The effect of preincubation time of nurse shark serum in the presence of various concentrations of EGTA on the hemolytic activity of the serum against mammalian target erythrocytes

I.Sensitized Bovine

EGTA conc. (mM)	15 min.	30 min.
0 (DGVB cont.)	>64	>64
5	2	4
7.5	2	4
10	2	2
12.5	nd	2
15	nd	2

II.Unsensitized Bovine

EGTA conc. (mM)	15 min.	30 min.
0 (DGVB cont.)	8	16
5	4	4
7.5	8	2
10	4	2
12.5	nd	2
15	nd	2

III.Rabbit

EGTA conc. (mM)	15 min.	30 min.
0 (DGVB cont.)	8	32
5	8	4
7.5	4	4
10	4	4
12.5	nd	4
15	nd	4

IV. Horse

EGTA conc. (mM)	15 min.	30 min.
0 (DGVB cont.)	16	16
5	2	2
7.5	4	2
10	4	2
12.5	nd	nd
15	nd	nd

Table 5: Values represent reciprocal of the highest dilution showing 50 % lysis of target cells. Nurse shark serum used in these assays was NSS 092591-1. nd= not done.

TABLE 6

Effect of EGTA concentration on the hemolytic activity of nurse shark serum preincubated for 30 minutes in the presence of EGTA

	Target Erythrocytes					
mM EGTA	Sheep(S)	Sheep(U)	Bovine(S)	Bovine(U)	Rabbit	Horse
0(DGVB)	>64	>64	64	16	32	16
5	4	nd	4	2	4	2
7.5	2	nd	2	2	4	2
10	2	nd	2	2	4	2
12.5	2	nd	2	2	4	2
15	2	2	2	2	4	2
17.5	2	2	2	2	4	2
20	2	2	2	2	4	2
22.5	2	2	2	2	4	2
25	2	2	2	2	4	2
30	2	2	2	2	4	2
35	2	2	2	2	4	2
40	2	2	2	2	4	2
45	2	2	2	2	4	2
50	2	2	2	2	4	2

Table 6:Values represent reciprocal of the highest serum dilution showing 50% lysis of target cells. Sensitized cells are indicated by (S) and unsensitized cells are indicated by (U). Rabbit and horse erythrocytes were unsensitized. nd= not done.

TABLE 6a

Effect of 10mM EDTA on the hemolytic activity of nurse shark serum against mammalian erythrocyte targets

	Nurse shark serum 010992 tag#s								
Target cell	238	186	242	241	248	244	88	243	Mean
Sheep(U)	0	0	0	0	2	4	2	2	1
Sheep(S)	0	0	0	0	0	2	0	0	0
Rabbit	0	0	0	0	0	0	0	0	0
Horse	0	0	0	0	0	0	0	0	0
Bovine (U)	0	0	0	0	0	0	0	0	0

	Nurse shark serum 031892 tag#s						
Target cell	230	241	465	455	477	489	Mean
Bovine(S)	0	0	0	0	0	0	0

Table 6a: A value of zero indicates no hemolysis at any serum dilution. All sera were preincubated with 10 mM EDTA for 30 minutes.

TABLE 7

Effect of EGTA concentration on the hemolytic activity of nurse shark serum against mammalian erythrocyte targets

I. Sensitized Sheep erythrocytes

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	32	128	128	256	256	512	128	512	244	181
5	8	16	4	16	16	>32	16	32	18	10
10	8	8	4	8	16	32	16	16	14	9
15	4	8	4	8	8	32	16	16	12	9
20	4	8	4	8	8	32	8	16	11	9
25	4	4	2	16	8	16	16	16	10	6

II.Unsensitized sheep erythrocytes

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	32	32	32	128	128	1024	512	512	300	356
5	8	16	4	8	16	>32	16	>32	17	11
10	4	8	2	4	16	>32	16	32	14	12
15	4	8	2	4	16	32	8	16	11	10
20	4	4	2	4	8	32	8	16	10	10
25	2	4	2	2	8	16	8	16	7	6

III. Rabbit erythrocytes

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	64	32	32	32	64	128	128	128	72	45
5	16	16	16	16	32	>32	32	32	24	9
10	16	16	16	16	16	32	16	16	18	6
15	8	8	8	8	8	16	16	16	11	4
20	8	8	8	8	16	16	8	16	11	4
25	8	8	8	8	8	16	8	16	10	4

TABLE 7 (cont)

IV. Horse erythrocytes

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	16	64	32	32	32	256	64	32	66	79
5	4	4	4	4	4	4	8	8	5	2
10	2	4	4	2	4	4	4	8	4	2
15	2	2	4	2	2	4	4	4	3	1
20	0	2	2	2	2	2	4	4	2	1
25	0	2	2	0	2	2	2	4	2	1

V. Unsensitized bovine erythrocytes

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	16	16	32	32	32	128	32	64	44	37
5	2	2	4	4	4	8	4	4	4	2
10	2	2	4	2	2	4	4	2	3	1
15	2	0	2	2	2	4	2	2	2	1
20	0	0	2	2	2	2	2	2	2	1
25	0	0	2	2	2	2	2	2	2	1

VI. Sensitized bovine erythrocytes

Nurse shark serum 022792 tag#

mM EGTA	230	241	465	455	477	489	Mean	SD
0(DGVB)	32	64	16	32	32	32	35	16
5	8	8	4	2	8	4	6	3
10	8	4	2	2	8	2	4	3
15	8	4	2	2	4	4	4	2
20	4	4	2	0	4	2	3	2
25	4	4	2	0	4	2	3	2

Table 7: Values represent reciprocal of the highest serum dilution showing 50% lysis of target cells. All serum was preincubated for 30 minutes with the chelator.
SD=standard deviation of the mean.

TABLE 8

Effect of EGTA on the hemolytic activity of nurse shark serum against mammalian erythrocytes expressed as percent of the control value

I. Sensitized sheep erythrocytes

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	243	88	Mean	SD
0(DGVB)	100	100	100	100	100	100	100	100	100	74
5	25	12.5	3.13	6.25	6.25	6.25	12.5	6.25	9.77	6.97
10	25	6.25	3.13	3.13	6.25	6.25	12.5	3.13	8.21	7.46
15	12.5	6.25	3.13	3.13	3.13	6.25	12.5	3.13	6.25	4.09
20	12.5	6.25	3.13	3.13	3.13	6.25	6.25	3.13	5.47	3.23
25	12.5	3.13	1.56	6.25	3.13	3.13	12.5	3.13	5.67	4.41

II. Unsensitized sheep erythrocytes

Nurse shark serum 010992 tag#

mMEGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	100	100	100	100	100	100	100	100	100	117
5	25	50	12.5	6.25	12.5	3.13	3.13	6.25	14.9	15.9
10	12.5	25	6.25	3.13	12.5	3.13	3.13	6.25	9.0	7.55
15	12.5	25	6.25	3.13	12.5	3.13	1.56	3.13	8.4	7.96
20	12.5	12.5	6.25	3.13	6.25	3.13	1.56	3.13	6.06	4.29
25	6.25	12.5	6.25	1.56	6.25	1.56	1.56	3.13	4.88	3.78

III. Rabbit erythrocytes

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	100	100	100	100	100	100	100	100	100	59
5	25	50	50	50	50	25	25	25	37.5	13.4
10	25	50	50	50	25	25	25	12.5	34.9	12.9
15	12.5	25	25	25	12.5	12.5	12.5	12.5	17.2	6.5
20	12.5	25	25	25	25	12.5	6.25	12.5	18.0	7.8
25	12.5	25	25	25	12.5	12.5	6.25	12.5	16.4	7.4

TABLE 8 (cont)

IV. Horse

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	100	100	100	100	100	100	100	100	100	119
5	25	6.25	12.5	12.5	12.5	1.56	12.5	25	13.5	8.1
10	12.5	6.25	12.5	6.25	12.5	1.56	6.25	25	10.2	7.1
15	12.5	3.13	12.5	6.25	6.25	1.56	6.25	12.5	7.6	4.4
20	0	3.13	6.25	6.25	6.25	0.78	6.25	12.5	5.2	3.9
25	0	3.13	3.25	0	6.25	0.78	3.13	12.5	4.0	4.3

V. Unsensitized bovine

Nurse shark serum 010992 tag#

mM EGTA	238	186	242	241	248	244	88	243	Mean	SD
0(DGVB)	100	100	100	100	100	100	100	100	100	84
5	12.5	12.5	12.5	12.5	12.5	6.25	12.5	6.25	10.9	2.9
10	12.5	12.5	12.5	6.25	6.25	3.13	12.5	3.13	8.6	4.3
15	12.5	0	6.25	6.25	6.25	3.13	6.25	3.13	5.5	3.6
20	0	0	6.25	6.25	6.25	1.56	6.25	3.13	3.7	2.9
25	0	0	6.25	6.25	6.25	1.56	6.25	3.13	3.7	2.9

VI. Sensitized bovine erythrocytes

Nurse shark serum 022792 tag#

mM EGTA	230	241	465	455	477	489	Mean	SD
0(DGVB)	100	100	100	100	100	100	100	45
5	25	12.5	25	6.25	25	6.25	16.7	9.4
10	25	6.25	12.5	6.25	25	3.13	13.0	9.8
15	25	6.25	12.5	6.25	12.5	3.13	11.0	7.8
20	12.5	6.25	12.5	0	12.5	3.13	7.8	5.5
25	12.5	6.25	12.5	0	12.5	3.13	7.8	5.5

Table 8: Values represent hemolytic titre expressed as percent of control titre determined by hemolytic assay of nurse shark serum in DGVB++. Standard deviation (SD) of controls were determined by conversion of absolute titre to 100 and determining a factor from the ratio of the absolute titre to 100. This factor was then used to multiply the standard deviation of the absolute titre to yield the standard deviation given in the table. SD= standard deviation of the mean.

TABLE 9

Statistical analysis of the effect of EGTA on the hemolytic activity of nurse shark serum expressed as percent of control

Target erythrocytes

I. Sensitized sheep

mM EGTA	Mean	SD	t value
5	9.77	6.97	36.5
10	8.21	7.46	33.8
15	6.25	4.09	66.9
20	5.47	3.23	81.5
25	5.67	4.41	60.1

II. Unsensitized sheep

mM EGTA	Mean	SD	t value
5	14.85	15.91	15.1
10	8.99	7.55	34.1
15	8.40	7.96	32.5
20	6.06	4.29	61.9
25	4.88	3.78	71.2

III. Rabbit

mM EGTA	Mean	SD	t value
5	37.50	13.36	13.2
10	34.38	12.94	12.8
15	17.19	6.47	36.2
20	17.97	7.79	29.8
25	16.41	7.42	31.9

IV. Horse erythrocytes

mM EGTA	Mean	SD	t value
5	13.48	8.14	30.1
10	10.35	7.13	35.5
15	7.62	4.37	59.7
20	5.18	3.94	68.1
25	4.01	4.25	65.7

TABLE 9 (cont.)

V. Unsensitized bovine erythrocytes

mM EGTA	Mean	SD	t value
5	10.94	2.89	93.5
10	8.60	4.34	59.6
15	5.47	3.64	73.5
20	3.71	2.89	94.4
25	3.71	2.89	94.4

VI. Sensitized bovine erythrocytes

mM EGTA	Mean	SD	t value
5	16.67	9.41	22.2
10	13.02	9.77	22.3
15	10.97	7.81	28.4
20	7.80	5.50	41.9
25	7.80	5.50	41.9

Table 9: Paired t test was performed to compare mean hemolytic titres of treated serum with those of untreated serum for each target cell and concentration of EGTA tested. For sensitized sheep, unsensitized sheep, rabbit, horse and unsensitized bovine erythrocyte targets n=8. For sensitized bovine target cells n=6. All t values were greater than the critical value ($p<0.005$), demonstrating that the drop in titre as a result of incubation in the presence of EGTA was statistically significant for all levels of EGTA tested and against all target cells used. SD= standard deviation of the mean.

TABLE 10

The effect of added magnesium on the hemolytic activity of nurse shark serum chelated with 20 mM EGTA

I.Rabbit erythrocytes

		Mg++ added					
NSS022792tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
453	128	16	64	64	64	64	128
466	256	16	32	32	64	64	64
230	128	16	32	64	64	64	64
241	256	16	32	32	64	64	64
465	32	8	32	32	32	32	32
455	32	8	16	16	32	32	32
477	128	16	32	64	64	64	64
489	64	8	32	32	32	32	32
Mean	128	13	34	42	52	52	60
SD	89	4	13	19	17	17	31

II.Sensitized sheep erythrocytes

		mM Mg++ added					
NSS043092tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
186	1024	32	128	128	256	128	256
242	64	2	16	32	32	64	64
248	1024	32	128	128	128	128	128
489	1024	>32	256	256	512	512	256
Mean	784	25	132	134	232	208	176
SD	480	15	98	88	208	205	96

III. Unsensitized sheep erythrocytes

		mM Mg++ added					
NSS031992tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
489	128	8	32	32	64	64	64
452	256	8	8	32	64	64	64
477	32	4	16	16	16	32	32
248	128	4	16	32	32	32	32
242	32	4	16	16	16	16	16
238	64	0	16	16	32	32	32
Mean	107	5	17	24	37	40	40
SD	85	3	7.9	8.8	22	20	20

TABLE 10 (con't)

IV. Horse erythrocytes

		mM Mg++ added					
NSS022792tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
465	16	4	8	16	16	16	16
241	64	4	32	64	64	128	128
455	64	8	16	32	64	64	64
453	128	16	32	64	512	256	256
466	32	8	32	64	128	128	128
230	64	8	32	64	128	128	128
477	32	4	8	16	32	32	32
489	64	4	8	16	32	32	32
Mean	58	7	21	46	122	98	98
SD	34	4	12	23	163	80	80

V. Sensitized bovine erythrocytes

		mM M++ added					
NSS043092tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
456	512	16	64	64	128	64	64
242	32	2	16	32	64	32	16
248	256	16	64	64	64	32	64
186	512	32	128	64	64	64	64
489	1024	16	64	32	64	64	64
238	256	32	32	32	128	64	64
Mean	432	19	61	48	85	53	56
SD	342	11	38	18	33	17	20

VI. Unsensitized bovine erythrocytes

		mM Mg++ added					
NSS022792tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
453	256	8	32	64	64	64	64
477	64	4	8	16	16	16	32
455	64	2	4	8	16	16	16
466	128	16	64	64	64	64	64
230	128	16	32	64	64	64	64
241	256	4	16	32	32	32	32
465	16	2	4	8	8	16	16
489	256	8	16	16	16	16	16
Mean	201	8	22	32	35	36	38
SD	94	6	20	27	25	24	23

Table 10: Values indicate reciprocal of the highest serum dilution showing 50% lysis of target cells. All serum samples were chelated with 20 mM EGTA before addition of magnesium ions. SD= standard deviation of the mean.

TABLE 11

The effect of added magnesium ions on the hemolytic activity of nurse shark serum chelated with 20mM EGTA against mammalian erythrocytes expressed as percent of control titre

I. Rabbit erythrocytes

		mM Mg++ added					
NSS022792tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
453	100	12.5	50	50	50	50	100
466	100	6.25	12.5	12.5	25	25	25
230	100	12.5	25	50	50	50	50
241	100	6.25	12.5	12.5	25	25	25
465	100	25	100	100	100	100	100
455	100	25	50	50	100	100	100
477	100	12.5	25	50	50	50	50
489	100	12.5	50	50	50	50	50
Mean	100	14	41	47	56	56	63
SD	69	7	29	27	29	29	33

II. Sensitized sheep erythrocytes

		mM Mg++ added					
NSS043092tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
186	100	3.13	12.5	12.5	25	12.5	25
242	100	3.13	25	50	50	100	100
248	100	3.13	12.5	12.5	12.5	12.5	12.5
489	100	3.13	25	25	25	50	25
Mean	100	3.13	19	25	34	44	41
SD	61	0	7	18	19	42	40

III. Unsensitized sheep erythrocytes

		mM Mg++ added					
NSS031992tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
489	100	6.25	25	25	50	50	50
452	100	3.13	3.13	12.5	25	25	50
477	100	12.5	50	50	50	100	100
248	100	3.13	12.5	25	25	25	25
242	100	12.5	50	50	50	50	50
238	100	0	25	25	50	50	50
Mean	100	6	28	31	42	50	50
SD	79	5	19	15	13	27	27

TABLE 11 (con't)

IV. Horse erythrocytes

		mM Mg++ added					
NSS022792tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
465	100	25	50	100	100	100	100
241	100	6.25	50	100	100	200	200
455	100	12.5	25	50	100	100	100
453	100	12.5	25	50	400	200	200
466	100	25	100	200	800	200	200
230	100	12.5	50	100	200	200	200
477	100	12.5	25	50	100	100	100
489	100	6.25	12.5	25	50	50	50
Mean	100	14	42	59	231	219	219
SD	58	7	28	58	255	242	242

V. Sensitized bovine erythrocytes

		mM Mg++ added					
NSS043092tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
456	100	3.13	12.5	12.5	25	12.5	12.5
242	100	6.25	50	100	200	100	50
248	100	6.25	25	25	25	12.5	25
186	100	6.25	25	25	12.5	12.5	12.5
489	100	1.56	6.25	3.13	6.25	6.25	6.25
238	100	12.5	12.5	12.5	50	25	25
Mean	100	6	22	30	53	28	22
SD	78.7	4	16	36	74	36	16

VI. Unsensitized bovine erythrocytes

		mM Mg++ added					
NSS022792tag#	DGVB++	0mM	1mM	5mM	10mM	15mM	20mM
453	100	3.13	12.5	25	25	25	25
477	100	6.25	12.5	25	25	25	50
455	100	3.13	6.25	12.5	25	25	25
466	100	12.5	50	50	50	50	50
230	100	12.5	25	50	50	50	50
241	100	1.56	6.25	12.5	12.5	12.5	12.5
465	100	12.5	25	50	50	100	100
489	100	3.13	6.25	6.25	6.25	6.25	6.25
Mean	100	6.0	18	29	30	38	40
SD	67	4.6	15.1	18.6	17.5	30	30

Table 11: Values represent the hemolytic activity of the serum expressed as a percent of the DGVB++ control titre. SD=standard deviation of the mean.

TABLE 11a

Statistical analysis of the effect of added magnesium on the hemolytic titre or nurse shark serum chelated with 20 mM EGTA expressed as a percent of control value

Target erythrocytes

I. Rabbit erythrocytes

mM Mg++ added	Mean	SD	t value
1	41	28.9	3.19 p<0.01
5	47	27.4	4.21 p<0.005
10	56	29.0	5.40 p<0.005
15	56	29.0	5.40 p<0.005
20	63	33.0	5.00 p<0.005

II. Sensitized sheep erythrocytes

mM Mg++ added	Mean	SD	t value
1	18.8	7.2	4.339 p<0.025
5	25	17.7	2.500 p<0.05
10	34.4	18.8	3.205 p<0.025
15	43.8	41.5	1.959 p<0.10
20	40.6	40.0	1.875 p<0.10

III. Unsensitized sheep erythrocytes

mM Mg++ added	Mean	SD	t value
1	27.6	19.2	3.452 p<0.01
5	31.3	15.3	5.568 p<0.005
10	41.6	12.9	7.560 p<0.005
15	50.0	27.4	4.419 p<0.005
20	50.0	27.4	3.906 p<0.01

IV. Horse erythrocytes

mM Mg++ added	Mean	SD	t value
1	42.2	27.5	3.906 p<0.01
5	59.3	57.5	3.912 p<0.01
10	231	255	2.651 p<0.025
15	219	242	8.929 p<0.005
20	219	242	8.929 p<0.005

TABLE 11a (con't)

V. Sensitized bovine erythrocytes

mM Mg++ added	Mean	SD	t value
1	21.9	15.7	2.500 p<0.05
5	29.7	35.5	1.646 p<0.10
10	53.0	73.5	1.586 p<0.10
15	28.1	35.7	1.535 p<0.10
20	21.9	15.7	2.680 p<0.025

VI. Unsensitized bovine erythrocytes

mM Mg++ added	Mean	SD	t value
1	18	15.1	5.889 p<0.005
5	29	18.6	4.420 p<0.005
10	30	17.5	5.075 p<0.005
15	38	30	3.213 p<0.01
20	40	30	3.549 p<0.005

Table 11a: Paired t test was performed to compare hemolytic titres of chelated serum to which no magnesium was added to with those of chelated serum to which magnesium had been added for each target cell and level of magnesium added. For rabbit, horse and unsensitized bovine erythrocytes n=8; for unsensitized sheep erythrocyte targets n=6; for sensitized sheep erythrocyte targets n=4. All t values greater than the critical value at p<0.05 or less were considered statistically significant.

TABLE 12

The effect of 10mM added magnesium on the hemolytic activity of nurse shark serum chelated with 10mM EDTA against sheep and bovine erythrocytes

I.Unsensitized sheep erythrocytes

NSS031992tag#	DGVB++	10mM EDTA	10mM EDTA + 10mM Mg
489	128	0	16
452	256	0	16
477	32	0	8
248	128	0	16
242	32	0	8
238	64	0	4
Mean	107	0	11
SD	85	0	5

II. Sensitized sheep erythrocytes

NSS043092tag#	DGVB++	10mM EDTA	10mM EDTA + 10mM Mg
186	1024	2	256
242	64	0	8
248	1024	2	64
489	1024	4	128
Mean	784	2	114
SD	480	2	107

III. Sensitized bovine erythrocytes

NSS043092tag#	DGVB++	10mM EDTA	10mM EDTA + 10mM Mg
456	512	2	64
242	32	0	2
248	256	0	32
186	512	2	128
238	256	0	64
Mean	314	1	58
SD	203	1	47

Table 12: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells.
SD= standard deviation of the mean.

TABLE 13

The effect of incubation temperature on the hemolytic activity of nurse shark serum against mammalian target cells

I.Rabbit erythrocytes

Temperature	NSS101891-1 Total	NSS101891-1 ACP	NSS101891-1 Total	NSS101891-1 ACP
6°C	16	8	16	8
23°C	64	8	32	16
30°C	32	4	32	4
37°C	32	16	32	8

II. Horse erythrocytes

Temperature	NSS101891-3 Total	NSS101891-3 ACP	NSS101891-6 Total	NSS101891-6 ACP
6°C	8	16	8	16
23°C	32	128	4	128
30°C	32	64	32	64
37°C	16	32	16	16

III. Sensitized sheep erythrocytes

Temperature	NSS101891-3 Total	NSS101891-3 ACP	NSS101891-1 Total	NSS101891-1 ACP
6°C	64	8	64	16
23°C	128	64	256	64
30°C	256	16	256	8
37°C	128	32	64	16

IV. Unsensitized sheep erythrocytes

Temperature	NSS101891-3 Total	NSS101891-3 ACP	NSS101891-6 Total	NSS101891-6 ACP
6°C	64	8	16	8
23°C	64	16	32	16
30°C	128	16	32	4
37°C	nd	32	32	16

TABLE 13 (con't)

V. Sensitized bovine erythrocytes

Temperature	NSS101891-1 Total	NSS101891-1 ACP	NSS101891-6 Total	NSS101891-6 ACP
6^0C	64	8	16	8
23^0C	64	32	64	64
30^0C	16	8	16	8
37^0C	16	16	32	16

VI. Unsensitized bovine erythrocytes

Temperature	NSS101891-1 Total	NSS101891-1 ACP	NSS101891-6 Total	NSS101891-6 ACP
6^0C	8	16	16	8
23^0C	32	32	32	32
30^0C	16	8	16	8
37^0C	16	16	32	16

Table 13: Values represent the reciprocal of the highest serum dilution showing 50% lysis of the target cells. nd= not done. Total complement activity was measured in DGVB++, while alternative pathway activity was assayed in 20 mM EGTA-10 mM Mg++-GVB.

TABLE 13a

The effect of incubation temperature on the alternative pathway hemolytic activity of nurse shark serum

I. Rabbit erythrocyte targets

NSS tag #	23^0C	30^0C
463	64	64
456	32	64
238	32	64
242	16	16

II. Horse erythrocyte targets

NSS tag#	23^0C	30^0C
463	32	32
456	32	32
238	16	16
242	4	4

Table 13 a: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. All serum was chelated with 20mM EGTA prior to assay. Assay buffer was 20 mM EGTA-10 mM Mg++-GVB.

TABLE 14

The effect of increased incubation times on the hemolytic activity of nurse shark serum against horse erythrocyte targets

	NSS101891-3				NSS101891-6			
	23^0C		30^0C		23^0C		30^0C	
Time	Total	ACP	Total	ACP	Total	ACP	Total	ACP
1 HR	32	128	32	64	4	128	32	64
2 HR	32	64	32	64	32	64	32	64
4 HR	16	32	64	64	16	32	32	32

Table 14: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells.

TABLE 15

The effect of zymosan on the hemolytic activity of nurse shark serum

I. Sensitized sheep targets

Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
0 time	128	nd	nd	nd
90 min	32	16	4	4
3 hours	32	4	4	4
overnight	8	2	2	2

II. Rabbit cell targets

Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
0 time	8	nd	nd	nd
90 min	8	4	2	2
3 hours	8	4	0	0
overnight	0	0	0	0

Table 15: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. Nurse shark serum used for these assays was 111491 tag#186. Serum assayed against sensitized sheep cells was nonchelated. Serum assayed against rabbit cells was chelated with 20mM EGTA prior to incubation in the presence of zymosan. nd= not done.

TABLE 16

The effect of LPS on the hemolytic activity of nurse shark serum

I. Sensitized sheep cell targets

Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
0 time	8	nd	nd	nd
90 min	4	4	4	4
4.5 hours	nd	2	2	2

II. Rabbit cell targets

Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
0 time	4	nd	nd	nd
90 min	4	4	2	2
3 hours	4	4	2	<2
4.5 hours	4	4	2	<2
overnight	0	0	0	0

Table 16: Values indicate the reciprocal of the highest dilution showing 50% lysis of target cells. Nurse shark serum used in these assays was NSS 101891-3. nd=not done. Serum used in assays against both target cells was nonchelated.

TABLE 17

The effect of cobra venom factor on the hemolytic activity of nurse shark serum

I. Sensitized sheep cell targets

Incubation time	Concentration 0mg/ml	0.05mg/ml	0.125mg/ml	0.50mg/ml
0 time	16	nd	nd	nd
90 min	16	16	16	16
4.5 hours	16	16	16	16

II. Rabbit cell targets

Incubation time	Concentration 0mg/ml	0.05mg/ml	0.125mg/ml	0.50mg/ml
0 time	16	nd	nd	nd
90 min	16	16	16	8
3 hours	16	16	16	16
4.5 hours	8	8	8	8
overnight	8	4	4	4

Table 17: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. Nurse shark serum used in these assays was NSS 101891-1. nd= not done.

TABLE 18

The effect of inulin on the hemolytic activity of nurse shark serum on rabbit erythrocyte targets

Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
0 time	4	nd	nd	nd
90 min	4	2	2	4
3 hours	2	2	4	4
4.5 hours	2	2	2	2
overnight	0	0	0	0

Table 18: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. nd= not done. Nurse shark serum used in these assays was NSS 101891-3.

TABLE 19

The effect of zymosan on the hemolytic activity of unchelated nurse shark serum (additional individuals)

I. Sensitized sheep cell targets

NSS 043092 tag#	Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
463	0 time	512	512	256	128
	90 min	256	32	32	8
	3 hours	256	16	8	4
	4.5 hours	128	4	4	4
456	0 time	512	256	256	128
	90 min	512	64	32	32
	3 hours	128	16	16	16
	4.5 hours	128	16	16	16

II. Rabbit cell targets

NSS 043092 tag#	Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
463	0 time	64	64	32	32
	90 min	32	8	8	8
	3 hours	16	8	4	4
	4.5 hours	8	8	2	2
456	0 time	128	128	64	64
	90 min	64	16	16	16
	3 hours	32	16	16	16
	4.5 hours	32	8	8	8

Table 19: Values represent the reciprocal of the last serum dilution showing 50% lysis of target cells. All serum samples used in these experiments were unchelated. Assay buffer was DGVB++.

TABLE 20

The effect of zymosan on the alternative pathway hemolytic activity of chelated nurse shark serum against rabbit erythrocyte targets

NSS 043092 tag#	Incubation time	Concentration 0mg/ml	1mg/ml	5mg/ml	10mg/ml
463	0 time	32	32	32	32
	90 min	32	16	8	2
	3 hours	16	8	2	0
	4.5 hours	8	0	0	0
456	0 time	8	16	16	8
	90 min	16	4	4	4
	3 hours	16	8	16	16
	4.5 hours	16	8	16	16

Table 20: Values represent the reciprocal of the last serum dilution showing 50% lysis of target cells. All serum used was chelated with 20 mM EGTA prior to assay. Assay buffer was 20 mM EGTA-10 mM Mg++-GVB.

TABLE 21

The effect of LPS on the hemolytic activity of nurse shark serum NSS043092 tag#463

I. Sensitized sheep cell targets

Incubation time	Concentration			
	0mg/ml	1mg/ml	5mg/ml	10mg/ml
0 time	128	128	128	128
90 min	64	64	32	16
3 hours	32	32	16	8
4.5 hours	16	16	16	8

II. Rabbit cell targets

Incubation time	Concentration			
	0mg/ml	1mg/ml	5mg/ml	10mg/ml
0 time	32	32	32	32
90 min	8	8	16	8
3 hours	8	8	8	8
4.5 hours	4	2	8	4

Table 21: Values represent the reciprocal of the last serum dilution showing 50% lysis of target cells. Serum used in assays where sensitized sheep cells were targets was unchelated and the assay buffer for these experiments was DGVB++. Serum samples used in assays where rabbit cells were targets was chelated with 20 mM EGTA prior to assay. Assay buffer for these experiments was 20 mM EGTA-10 mM Mg++-GVB.

TABLE 22

Total and alternative pathway hemolytic titres of several nurse shark sera against rabbit erythrocyte targets

Nurse shark serum	Total activity titre	Alternative pathway titre
022792 tag 477	64	64
022792 tag 489	64	32
022792 tag 230	512	64
011992 tag 248	64	32
011992 tag 244	256	128
011992 tag 88	64	32
011992 tag 463	128	128
022792 tag 453	128	64
022792 tag 466	512	64
022792 tag 241	128	64
022792 tag 465	64	32
022792 tag 455	32	32
011992 tag 238	128	64
011992 tag 186	64	32
011992 tag 242	64	32
011992 tag 241	64	32
	Mean=146 SD=152	Mean=56 SD=32

n=16

Table 22: Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. Means of total and alternative pathway titres were compared by paired t test. The two means were found to be statistically significantly different at a confidence level of $p<0.02$. SD= standard deviation of the mean.

TABLE 23

Total and alternative pathway hemolytic titres of several nurse shark sera against horse erythrocyte targets

Nurse shark serum	Total activity titre	Alternative pathway titre
022792 tag 230	128	128
022792 tag 477	32	32
022792 tag 489	64	32
011992 tag 248	128	32
011992 tag 244	512	128
011992 tag 88	64	16
011992 tag 463	128	32
022792 tag 453	512	512
022792 tag 466	64	256
022792 tag 241	128	64
022792 tag 465	32	16
022792 tag 455	128	64
011992 tag 238	32	16
011992 tag 186	64	16
011992 tag 242	64	32
011992 tag 241	64	32
	Mean=134 SD=152	Mean=88 SD=130

n=16

Table 23: Values represent the reciprocal of the highest serum dilution showing 50% lysis of the target cells. Means of total and alternative pathway hemolytic titres were compared by the paired t test. The two means were found to be not statistically significantly different. SD=standard deviation of the mean.

TABLE 24

Total and alternative pathway hemolytic titres of several nurse shark sera against sensitized sheep erythrocyte targets

Nurse shark serum	Total activity titre	Alternative pathway titre
011992 tag 248	256	16
011992 tag 244	1024	32
011992 tag 88	512	16
011992 tag 463	512	32
043092 tag 248	1024	128
043092 tag 489	1024	512
043092 tag 456	1024	256
011992 tag 238	64	16
011992 tag 186	128	8
011992 tag 241	64	8
011992 tag 242	128	8
043092 tag 186	1024	256
043092 tag 242	64	64
043092 tag 238	1024	128
	Mean=562 SD=439	Mean=106 SD=146

n=14

Table 24: Values represent the reciprocal of the highest serum dilution showing 50% lysis of the target cells. The means of total and alternative pathway hemolytic titres was compared by the paired t test. The two means were found to be statistically significantly different at a confidence level of $p<0.001$. SD= standard deviation of the mean.

TABLE 25

Total and alternative pathway hemolytic titres of several nurse shark sera against unsensitized sheep erythrocyte targets

Nurse shark serum	Total activity titre	Alternative pathway titre
031992 tag 489	128	64
031992 tag 248	128	32
022792 tag 477	64	16
011992 tag 248	128	32
011992 tag 244	512	32
011992 tag 88	256	32
011992 tag 463	256	32
031992 tag 452	256	64
031992 tag 238	64	32
031992 tag 242	nd	16
011992 tag 238	32	16
011992 tag 186	64	16
011992 tag 242	32	16
011992 tag 241	128	16
	Mean=158 SD=134	Mean=30 SD=16

n=13

Table 25: Values represent the reciprocal of the last serum dilution showing 50% lysis of target cells. The mean of total and alternative pathway hemolytic titres were compared by paired "t" test. The two means were found to be statistically significantly different at a confidence level of $p<0.003$. SD= standard deviation of the mean.

TABLE 26

Total and alternative pathway hemolytic titres of several nurse shark sera against unsensitized bovine erythrocyte targets

Nurse shark serum	Total activity titre	Alternative pathway titre
022792 tag 230	128	64
022792 tag 477	128	16
022792 tag 489	256	16
011992 tag 248	16	8
011992 tag 244	64	32
011992 tag 88	32	8
011992 tag 463	64	8
022792 tag 453	256	64
022792 tag 466	256	64
022792 tag 241	128	32
022792 tag 465	64	8
022792 tag 455	64	16
011992 tag 238	16	8
011992 tag 186	16	4
011992 tag 241	16	16
011992 tag 242	16	16
	Mean=95 SD=89	Mean=24 SD=21

n=16

Table 26:Values represent the reciprocal of the highest serum dilution showing 50% lysis of target cells. The means of total and alternative pathway hemolytic titres were compared by paired t test. The two means were found to be statistically significantly different at a confidence level of $p<0.0001$. SD= standard deviation of the mean.

TABLE 27

Total and alternative pathway hemolytic titres of several nurse shark sera against sensitized bovine erythrocyte targets

Nurse shark serum	Total activity titre	Alternative pathway titre
043092 tag 456	512	128
043092 tag 248	512	64
043092 tag 489	1024	64
011992 tag 248	1024	8
011992 tag 244	1024	16
011992 tag 88	512	8
011992 tag 463	256	8
043092 tag 242	64	64
043092 tag 186	512	64
043092 tag 238	512	128
011992 tag 238	64	16
011992 tag 186	256	16
011992 tag 241	256	16
011992 tag 242	256	16
	Mean=485 SD=333	Mean=44 SD=43

n=14

Table 27: Values represent reciprocal of highest serum dilution showing 50% lysis of target cells. Means of total and alternative pathway hemolytic titres were compared by paired t test. The two means were found to be statistically significantly different at a confidence level of $p<0.005$. SD= standard deviation of the mean.

Figure 1: The effect of EGTA on the hemolytic activity of nurse shark serum. Shark serum was incubated for 30 minutes in the presence of the stated millimolar amount of chelator prior to assay for hemolytic activity. Hemolytic assays were done in microtitre plates using EGTA-GVB-- as the assay buffer. Each serum was assayed in buffer containing an equivalent amount of EGTA to that in which it was incubated prior to performing the assay. Each bar represents the average hemolytic titre of eight individual animals expressed as a percent of the control titre. The control titre is represented by solid bar and is determined by assay of the same serum in DGVB++, without prior incubation in the presence of EGTA under similar conditions as the treatment groups. for that particular treatment group. Error bars represent the standard deviation of each group of data. Standard deviation for the control titres was determined by calculating a factor that converts the average of the absolute titres to 100 and multiplying the standard deviation of the absolute titres by this factor.

FIGURE 1

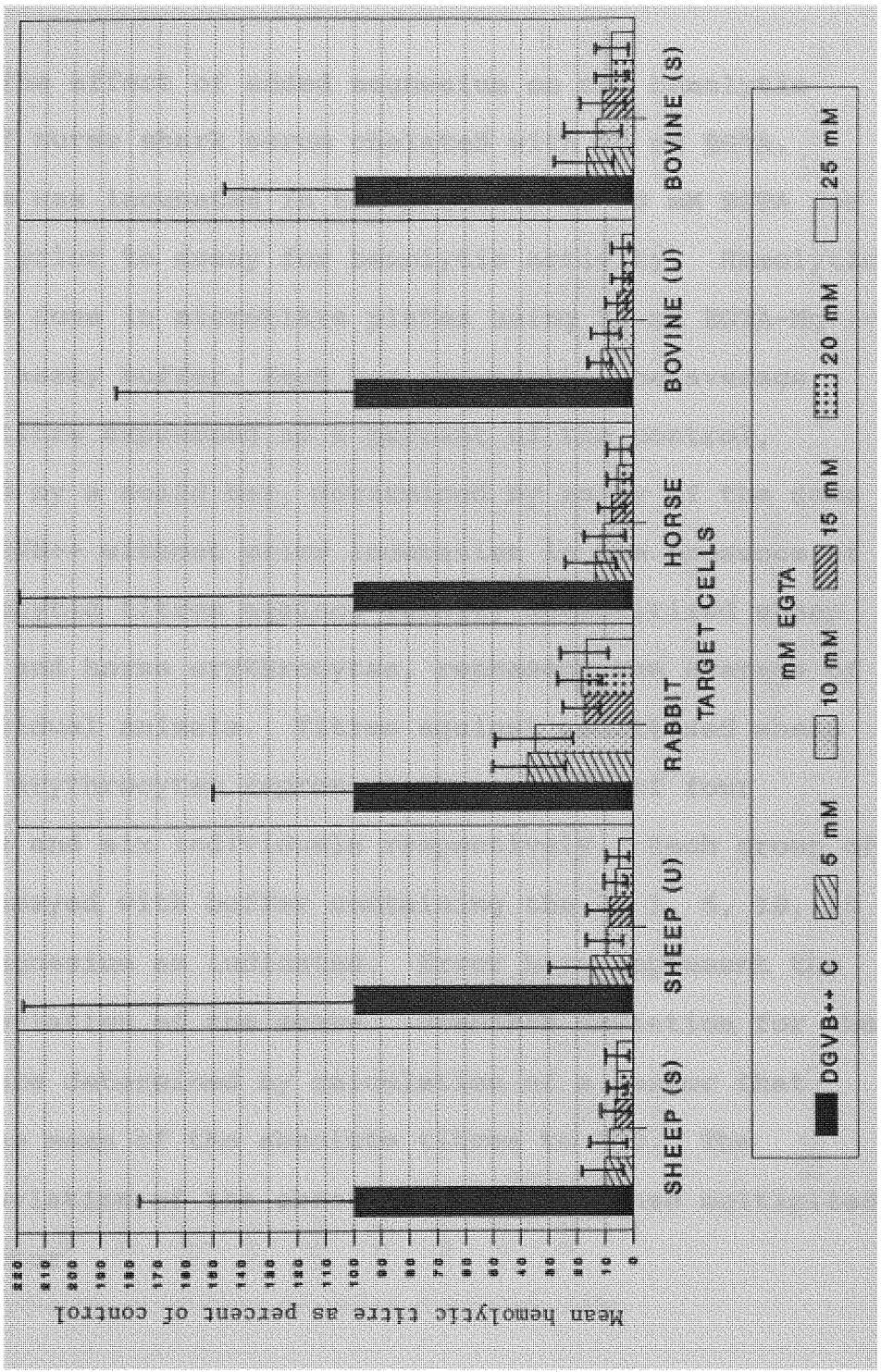

EFFECT OF EGTA ON THE HEMOLYTIC ACTIVITY
OF NURSE SHARK SERUM
Mean hemolytic titre as percent of control
SHEEP (S)
SHEEP (U)
RABBIT
HORSE
BOVINE (U)
BOVINE (S)
TARGET CELLS
mM EGTA
DGVB++ C
5 mM
10 mM
15 mM
20 mM
25 mM

Figure 2: The effect of added magnesium on the hemolytic activity of nurse shark serum chelated with 20 mM EGTA. Shark serum was incubated in the presence of 20 mM EGTA for 30 minutes prior to assay for hemolytic activity. Hemolytic assays were done in microtitre plates using 20 mM EGTA-Mg++-GVB as the assay buffer. Each bar represents the average hemolytic titre expressed as a percent of the control, represented by a solid bar, determined by assay of the same serum in DGVB++ without prior incubation in the presence of EGTA. Hemolytic titres against rabbit, unsensitized sheep and bovine and horse erythrocytes, represent the average of eight individual animals. Titres against sensitized sheep and bovine erythrocytes represent the average of four individuals and six individuals respectively. Each group of sera was assayed with buffer containing the 0, 1, 5, 10, 15 or 20 mM magnesium as indicated. Error bars represent the standard deviation of the means. Standard deviation for the controls were determined by calculation of a factor that converts the mean of the absolute titres to 100. The standard deviation of the absolute titres is then multiplied by this factor.

FIGURE 2

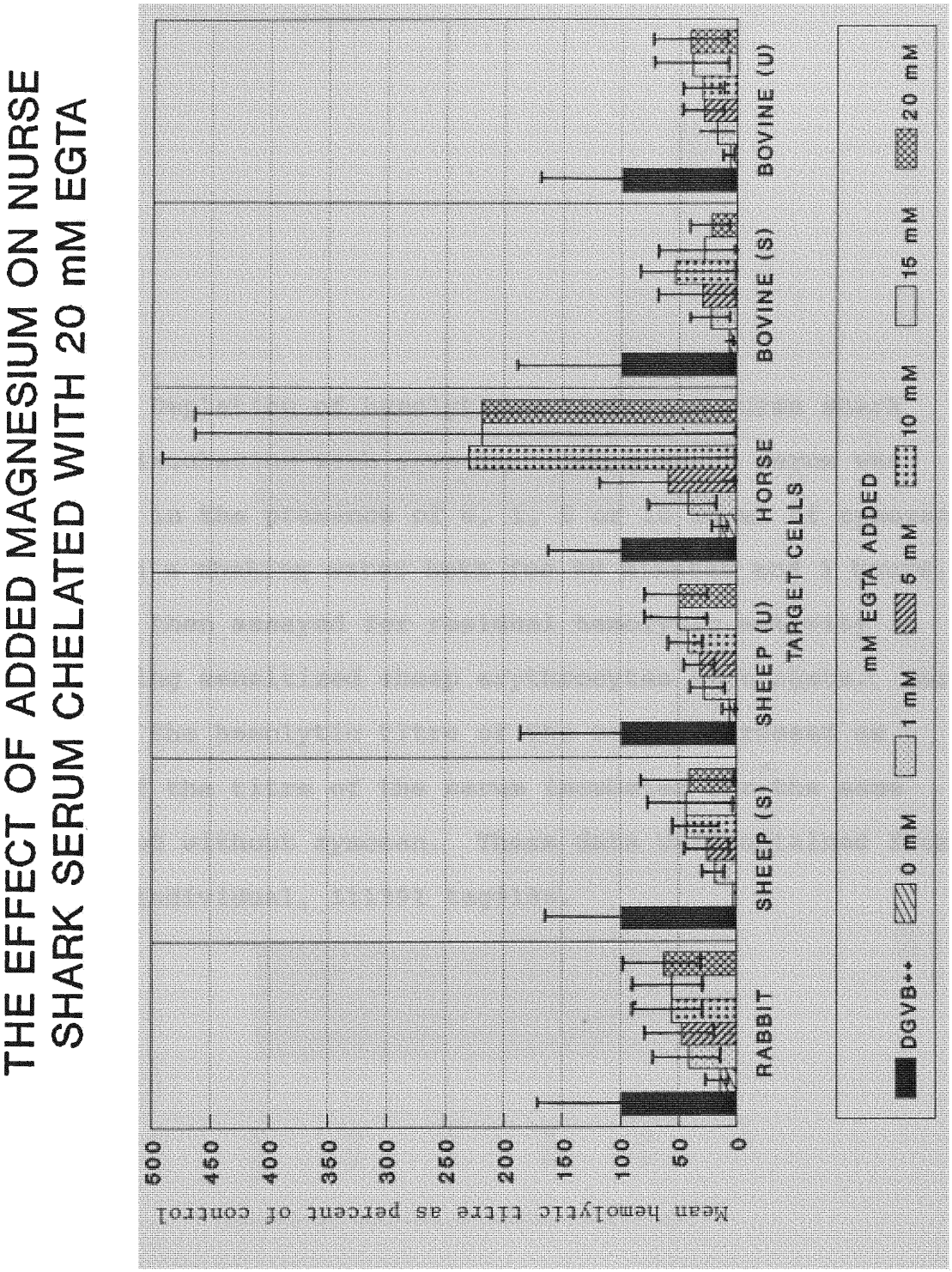

THE EFFECT OF ADDED MAGNESIUM ON NURSE SHARK SERUM CHELATED WITH 20 mM EGTA
Mean hemolytic titre as percent of control
600
450
400
350
300
250
200
150
100
50
0
RABBIT
SHEEP (S)
SHEEP (U)
HORSE
BOVINE (S)
BOVINE (U)
TARGET CELLS
mM EGTA ADDED
DGVB++
0 mM
1 mM
6 mM
10 mM
15 mM
20 mM

Figure 3: Depletion of hemolytic activity of nurse shark serum by incubation with zymosan. Nurse shark serum was incubated in the presence of 0, 1, 5 or 10 mg/ml of zymosan at 30^0C in a shaking water bath for 90 minutes and 3 hours. Serum was then assayed for residual hemolytic activity in DGVB++ using sensitized sheep erythrocytes as targets. Bars represent the hemolytic titre of the serum expressed as a percent of the titre of the serum incubated for the same time period without zymosan. These data were obtained from a single individual, 111391 tag#186.

FIGURE 3

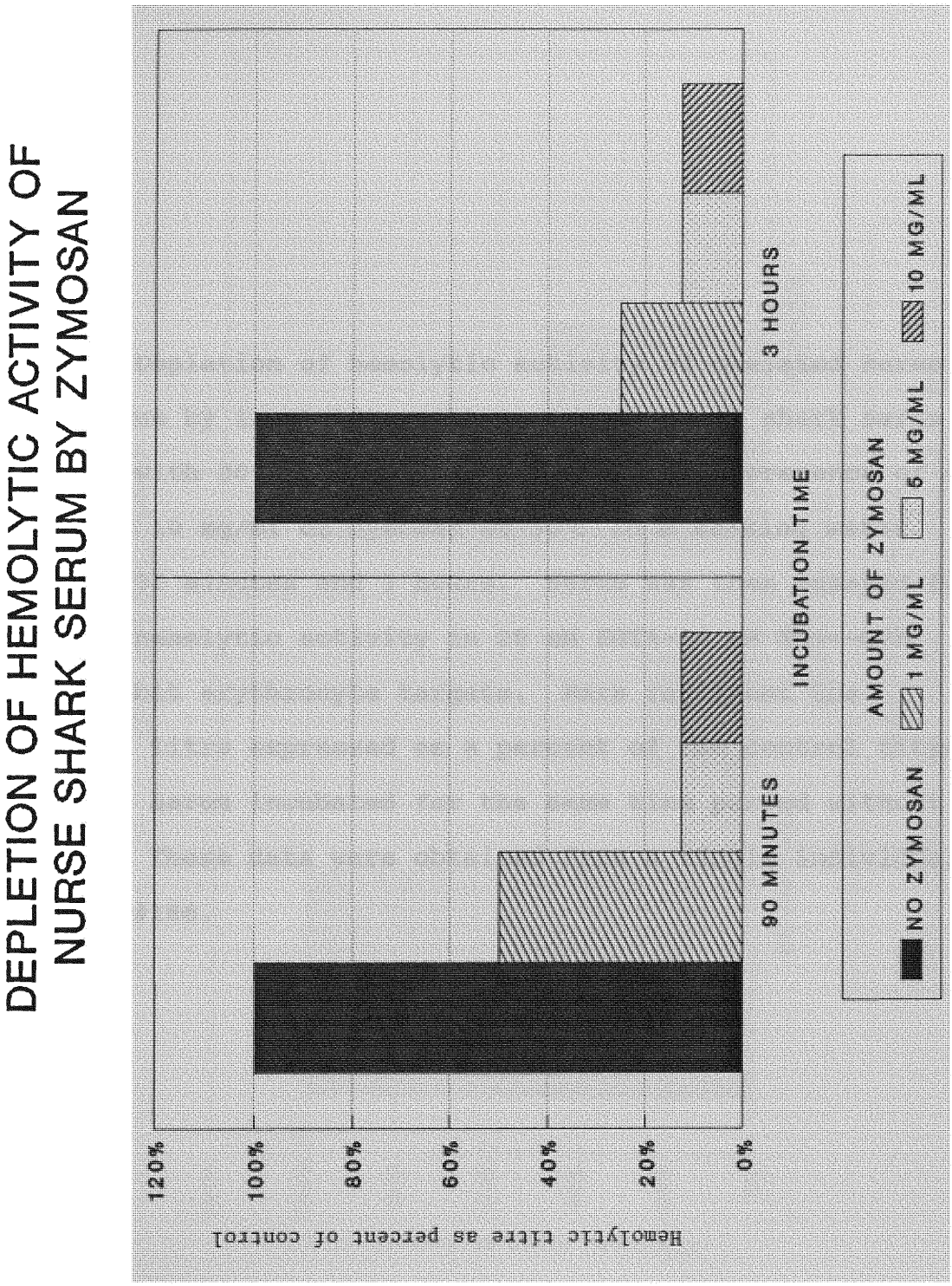

DEPLETION OF HEMOLYTIC ACTIVITY OF NURSE SHARK SERUM BY ZYMOSAN
Hemolytic titre as percent of control
120%
100%
80%
60%
40%
20%
0%
90 MINUTES
3 HOURS
INCUBATION TIME
AMOUNT OF ZYMOSAN
NO ZYMOSAN
1 MG/ML
5 MG/ML
10 MG/ML

Figure 4:Depletion of hemolytic activity of chelated nurse shark serum by incubation with zymosan. Nurse shark serum chelated with 20 mM EGTA was incubated in the presence of 0, 1, 5, and 10 mg/ml of zymosan at 30^0C in a shaking water bath for 90 minutes and 3 hours. Serum was then assayed for residual hemolytic activity in 20 mM EGTA-10 mM Mg++-GVB using rabbit erythrocyte targets. Bars represent the hemolytic titre expressed as a percent of the control titre which was serum incubated for the same time period without zymosan. These data were obtained from a single individual 11491 tag#186.

FIGURE 4

DEPLETION OF HEMOLYTIC ACTIVITY OF CHELATED NURSE SHARK SERUM BY ZYMOSAN

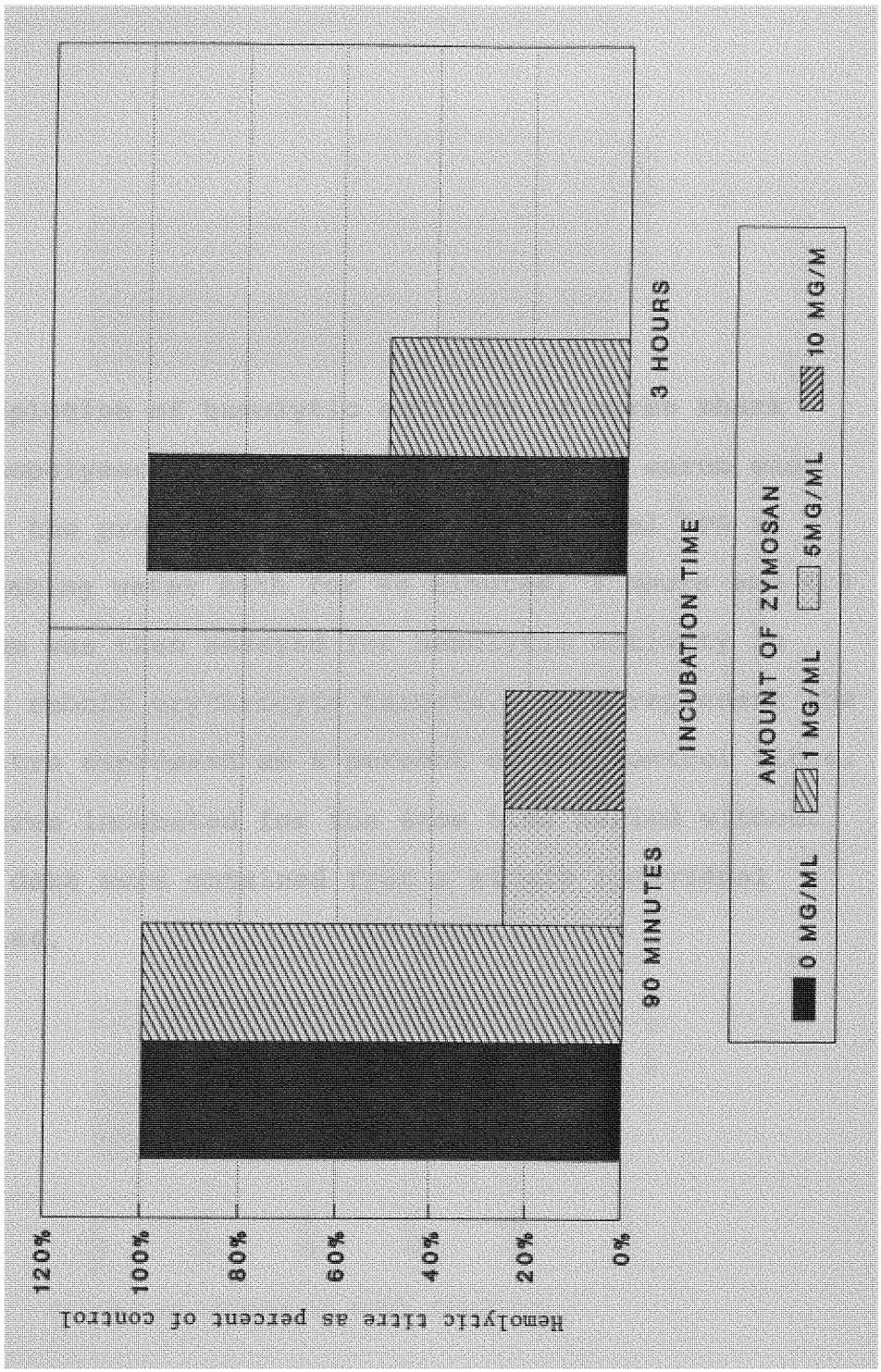


Figure 5: Depletion of hemolytic activity of nurse shark serum by lipopolysaccharide (LPS). Nurse shark serum was incubated in the presence of 0, 1, 5 and 10 mg/ml LPS at 30^0C in a shaking water bath for 90 minutes, 3 hours and 4.5 hours. Serum was then assayed for hemolytic activity in DGVB++ using rabbit erythrocyte targets. Bars represent the hemolytic titre expressed as a percent of the control titre, which was serum incubated for the same time period without LPS. These data were obtained from a single individual 111491 tag#186.

FIGURE 5

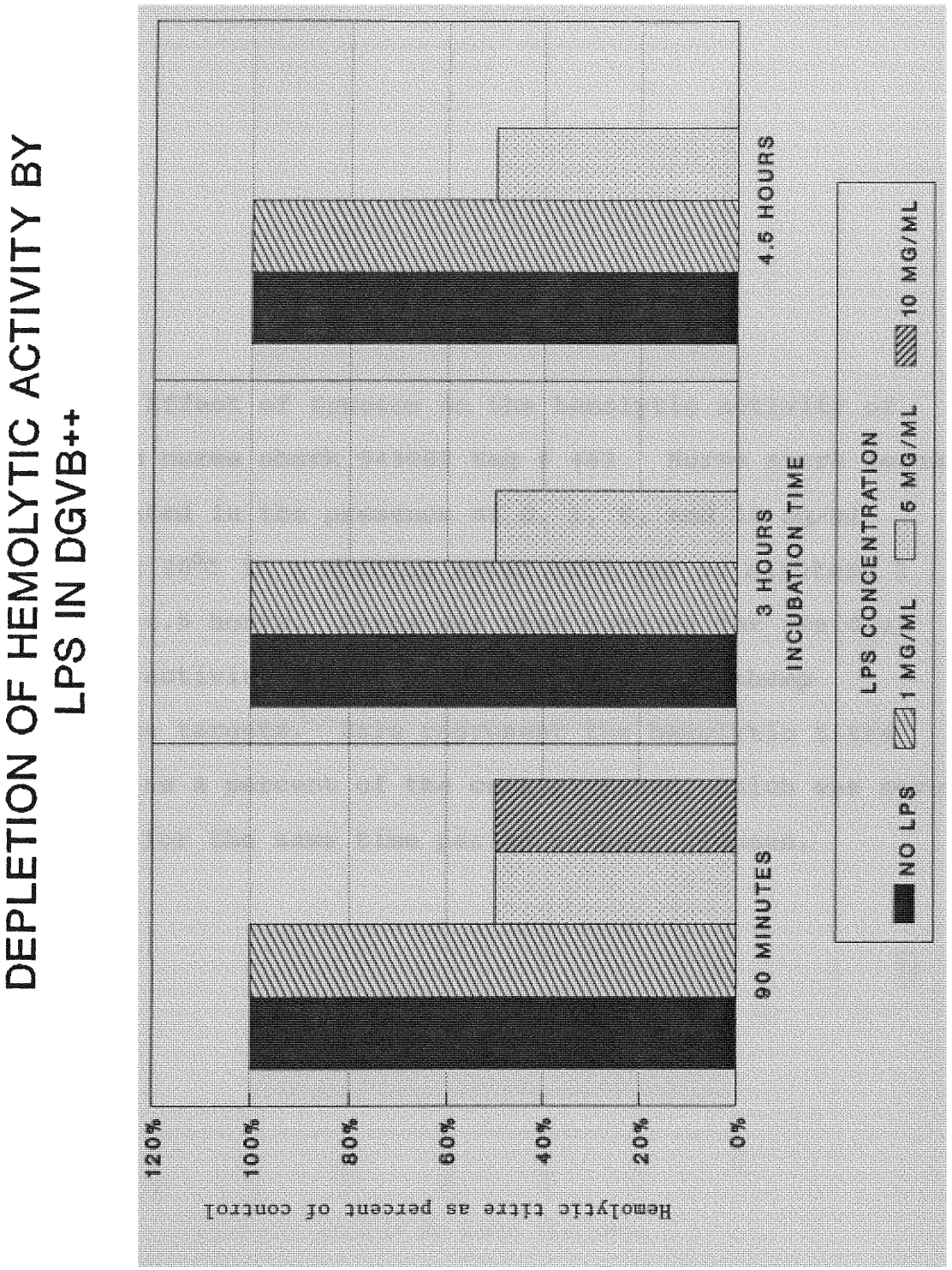

DEPLETION OF HEMOLYTIC ACTIVITY BY LPS IN DGVB++
Hemolytic titre as percent of control
120%
100%
80%
60%
40%
20%
0%
90 MINUTES
3 HOURS
4.6 HOURS
INCUBATION TIME
LPS CONCENTRATION
NO LPS
1 MG/ML
6 MG/ML
10 MG/ML

Figure 6: Effect of zymosan on the hemolytic activity of serum from nurse shark 043092 tag # 463. Nurse shark serum was incubated in the presence of 0, 1, 5, and 10 mg/ml zymosan at 30^0C in a shaking water bath for 90 minutes, 3 hours and 4.5 hours. Serum was then assayed for residual hemolytic activity in DGVB++ using sensitized sheep erythrocyte targets. Bars represent the hemolytic titre expressed as a percent of the control titre, which was serum incubated for the same time period without zymosan.

FIGURE 6

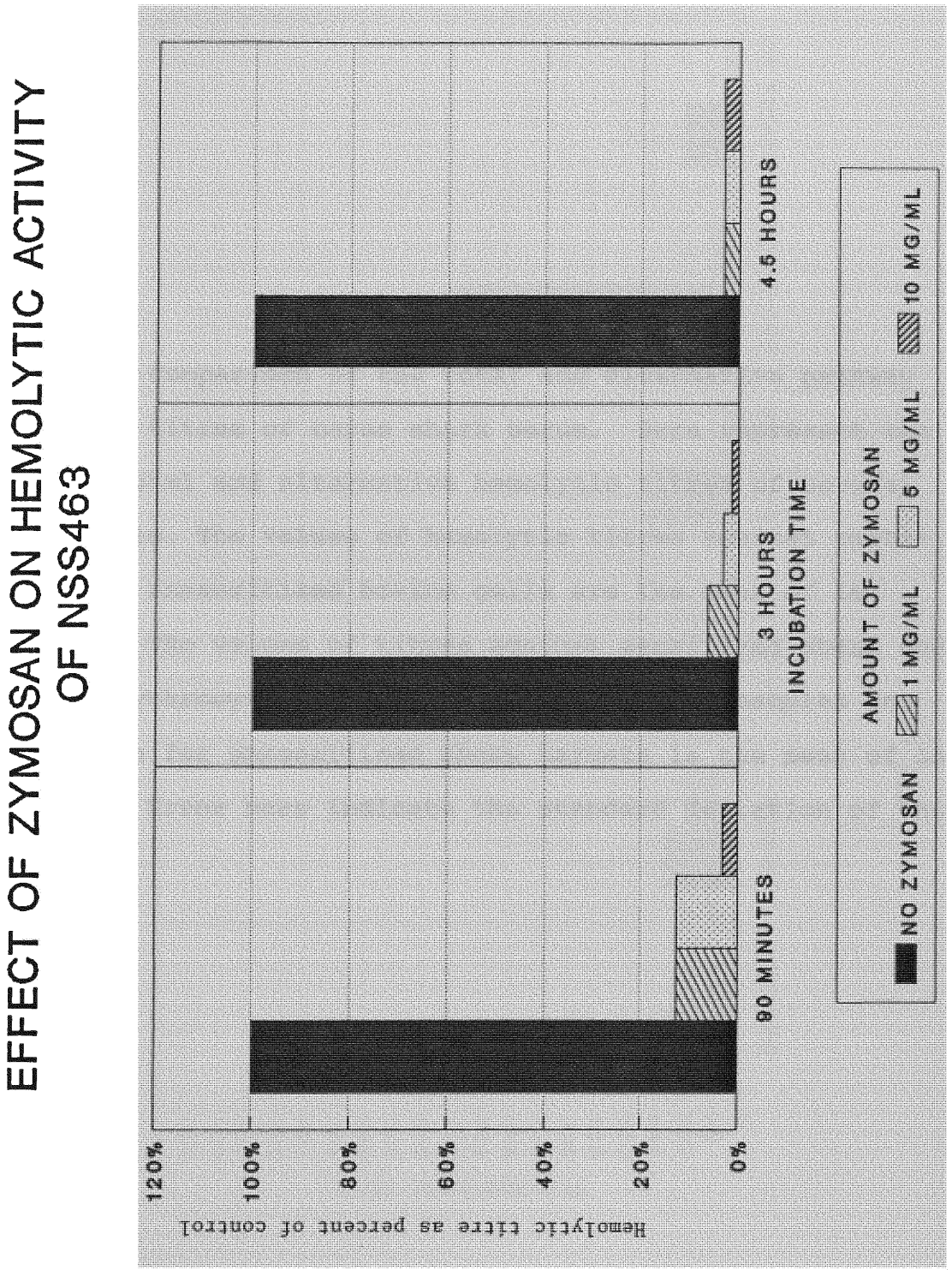

EFFECT OF ZYMOSAN ON HEMOLYTIC ACTIVITY OF NSS463
Hemolytic titre as percent of control
120%
100%
80%
60%
40%
20%
0%
90 MINUTES
3 HOURS
4.5 HOURS
INCUBATION TIME
AMOUNT OF ZYMOSAN
NO ZYMOSAN
1 MG/ML
5 MG/ML
10 MG/ML

Figure 7: Comparison of the total and alternative pathway hemolytic titres of nurse shark serum. Bars represent the average total and alternative hemolytic titres of several individuals. The values of hemolytic titres against rabbit, horse and unsensitized bovine cells are the mean of 16 samples. The values of titres against sensitized sheep and bovine erythrocyte targets are the mean of 14 samples and the values for unsensitized sheep targets is the mean of 13 samples. Error bars indicate the standard deviation of the samples.

FIGURE 7

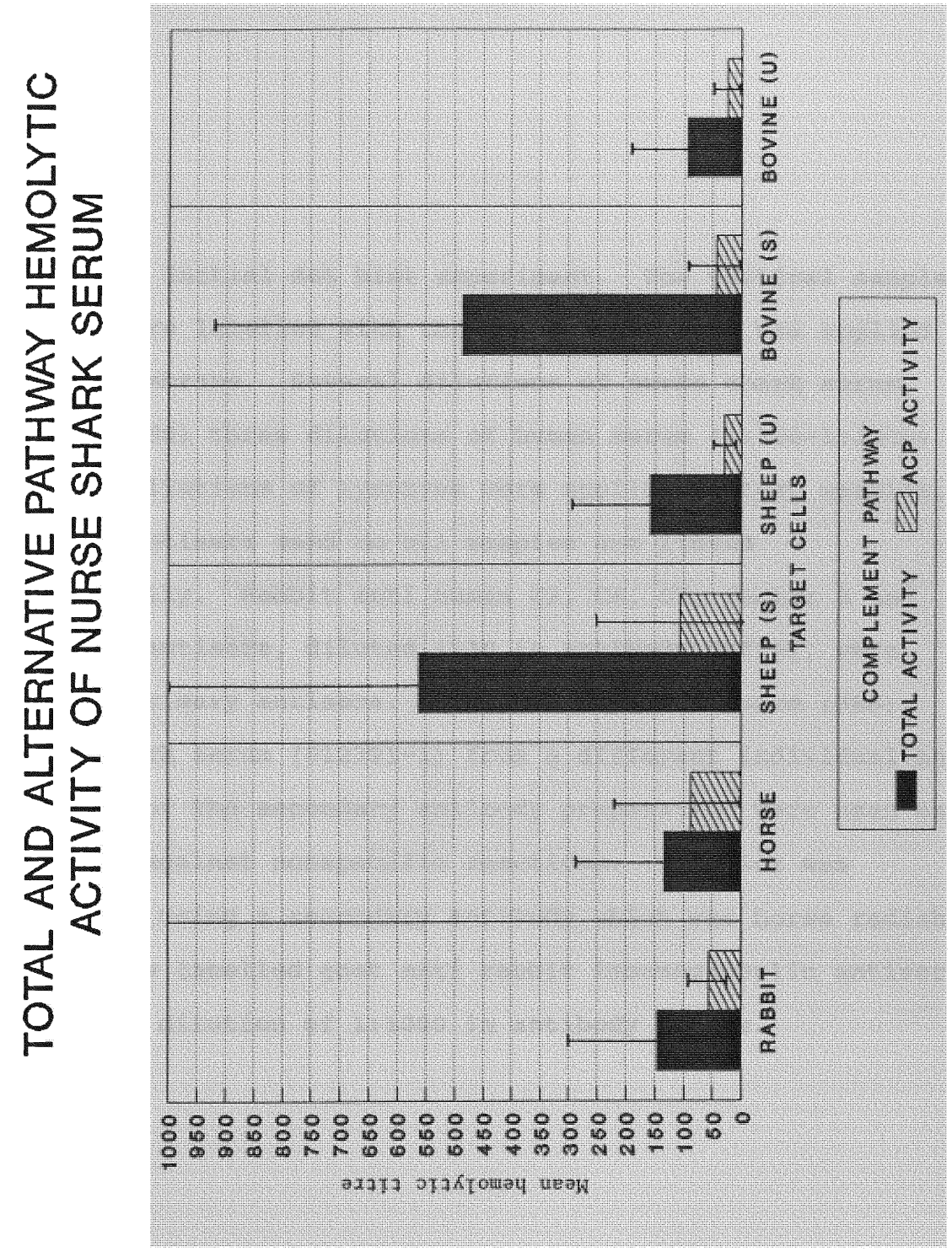

TOTAL AND ALTERNATIVE PATHWAY HEMOLYTIC ACTIVITY OF NURSE SHARK SERUM
Mean hemolytic titre
1000
950
900
850
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
RABBIT
HORSE
SHEEP (S)
SHEEP (U)
BOVINE (S)
BOVINE (U)
TARGET CELLS
COMPLEMENT PATHWAY
TOTAL ACTIVITY
ACP ACTIVITY

Figure 8: Initial dot blot experiment. The vertical sample columns are labelled with the test sample that was applied to the membrane. Several dilutions of nurse shark serum, (1:2-1:1000) three dilutions of human serum (1:100-1:1000) and two dilutions of lysozyme were applied as test samples. Primary antisera used on all samples are listed horizontally. Rabbit anti human C3, C4, C5, factor B and factor H antisera, diluted 1:30 in antibody buffer, were used as primary antisera. Non-immune rabbit serum (NRS), nurse shark serum (012391-1) from a different individual and a sample of the secondary antibody preparation were used as controls against nonspecific reactions. The blot was developed using a secondary antibody which was horse radish peroxidase labelled goat anti rabbit immunoglobulin antisera used at a dilution of 1:1000 in antibody buffer.

FIGURE 8

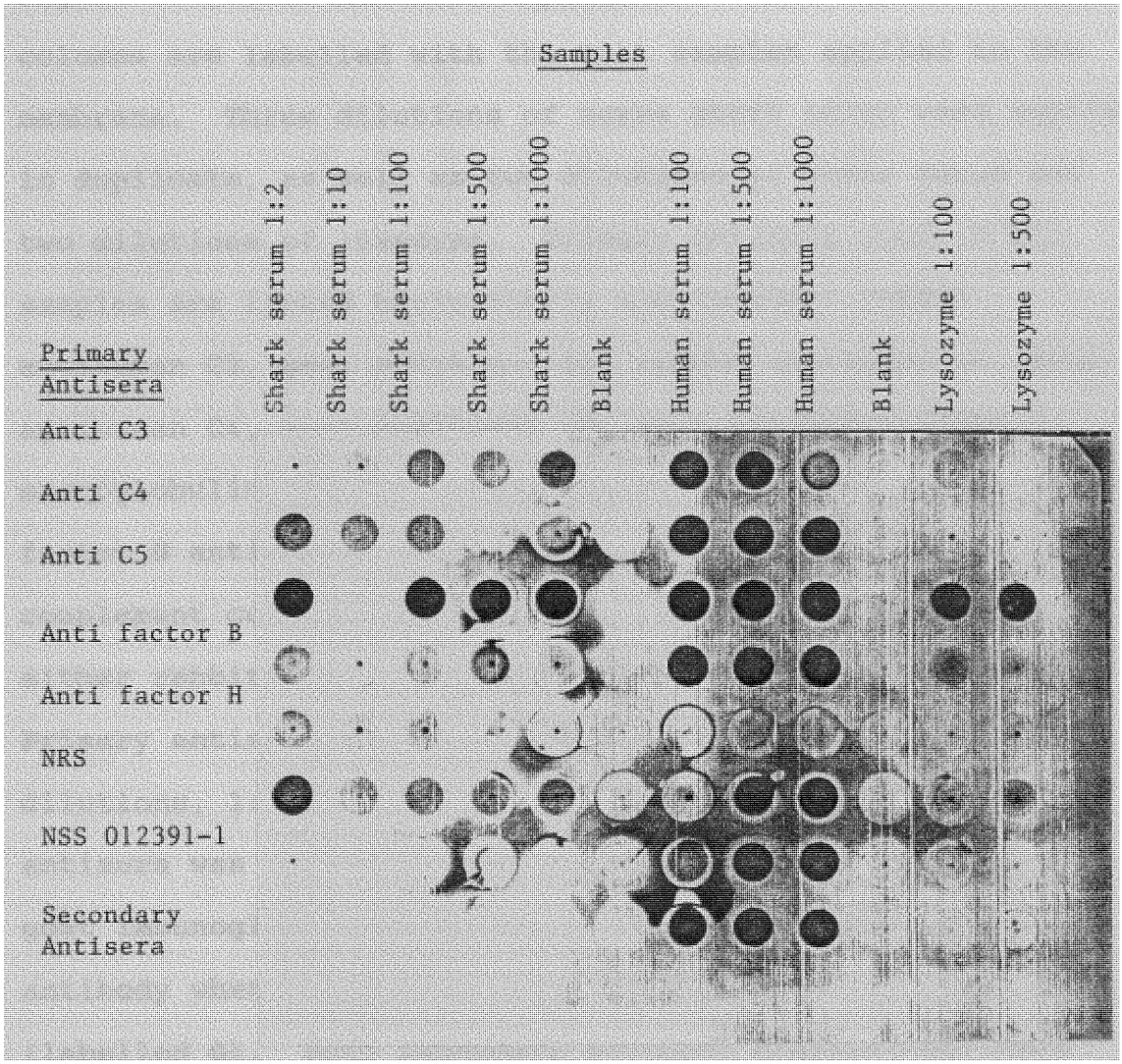

Samples
Shark serum 1:2
Shark serum 1:10
Shark serum 1:100
Shark serum 1:500
Shark serum 1:1000
Blank
Human serum 1:100
Human serum 1:500
Human serum 1:1000
Blank
Lysozyme 1:100
Lysozyme 1:500
Primary Antisera
Anti C3
Anti C4
Anti C5
Anti factor B
Anti factor H
NRS
NSS 012391-1
Secondary Antisera

Figure 9: Second dot blot experiment. The vertical sample columns are labelled with the test samples applied to the membrane. Three dilutions of nurse shark serum were applied in duplicate, as well as two dilutions of human serum and two dilutions of lysozyme. Primary antibody used in all samples are listed horizontally and include: rabbit antihuman C3, goat antihuman C3, rabbit antihuman C4, goat antihuman C4, goat antihuman C5, goat antihuman alpha 2 macroglobulin, normal rabbit serum, and rabbit anti-human factor B antisera. Primary antisera against human complement components, used in this blot, were raised in either rabbits (indicated by R) or goats (indicated by G). Primary antisera was applied to the blot at two different dilutions, 1:30 and 1:15. Goat anti-rabbit immunoglobulin antisera was raised in rabbits (labelled R). Rabbit anti-goat immunoglobulin antisera was applied as a secondary antibody when the primary antibody was of goat origin (labelled G). Both secondary antibody preparations were peroxidase labelled and applied at a dilution of 1:1000 in antibody buffer.

FIGURE 9

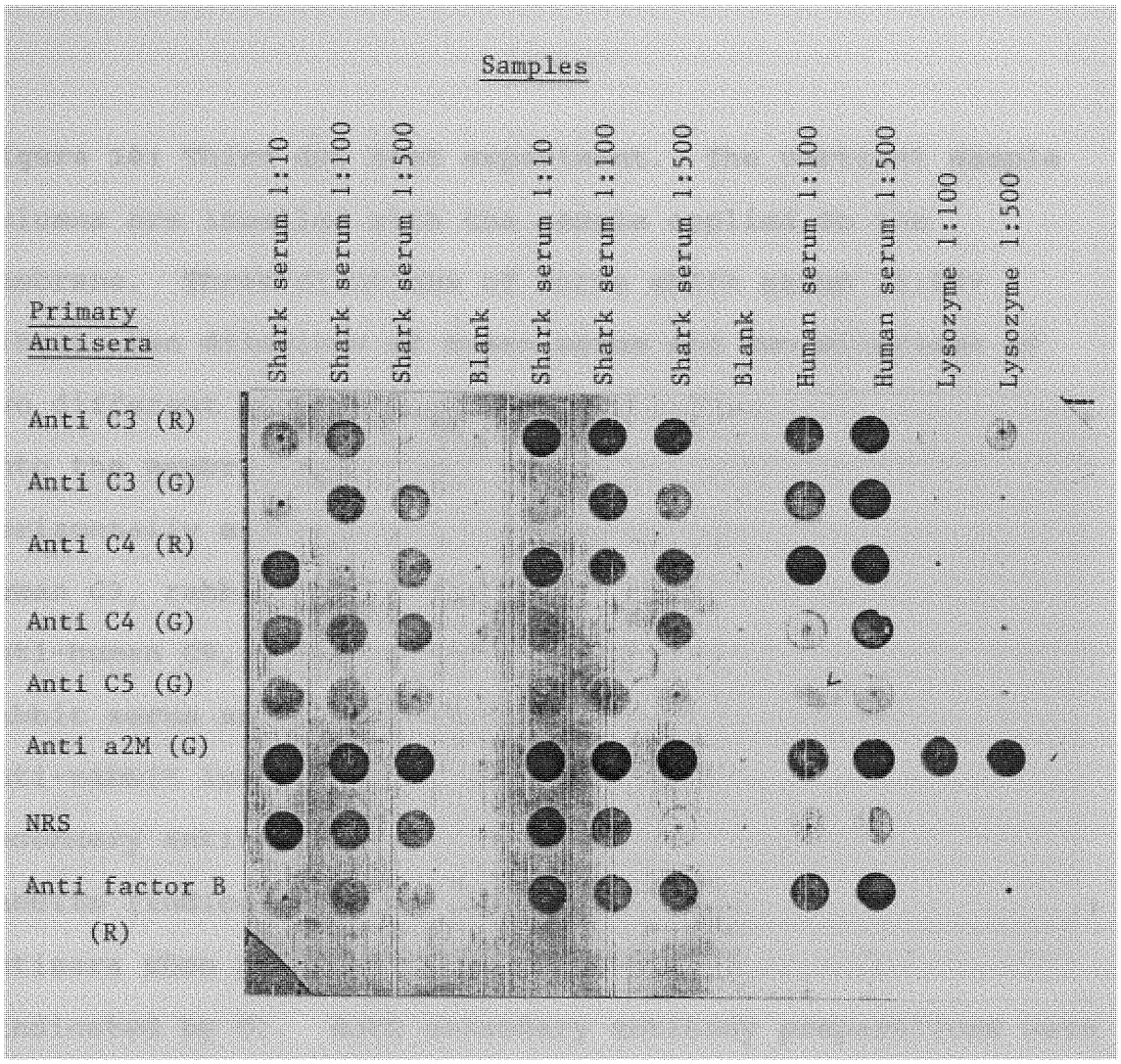

Samples
Primary Antisera
Shark serum 1:10
Shark serum 1:100
Shark serum 1:500
Blank
Shark serum 1:10
Shark serum 1:100
Shark serum 1:500
Blank
Human serum 1:100
Human serum 1:500
Lysozyme 1:100
Lysozyme 1:500
Anti C3 (R)
Anti C3 (G)
Anti C4 (R)
Anti C4 (G)
Anti C5 (G)
Anti a2M (G)
NRS
Anti factor B (R)

Figure 10: Third dot blot experiment. The vertical sample columns are labelled with the sample applied to the membrane. Three dilutions of nurse shark serum (1:10-1:500), two dilutions of human serum and two dilutions of two different samples (A and B) of lysozyme were blotted onto the membrane. Primary antibodies used are listed horizontally and include: rabbit anti-human C3, goat anti-human C3, rabbit anti-human C4, goat anti-human C4, goat anti-human C5, goat anti-human alpha 2 macroglobulin, normal rabbit serum and rabbit anti-human factor B antisera. Goat anti-rabbit immunoglobulin antisera was used as the secondary antibody when primary antisera was raised in rabbits (indicated by R) and rabbit anti-goat immunoglobulin antisera when primary antibody was raised in goats (indicated by G). Both secondary antibody preparations were peroxidase labelled and applied at a dilution of 1:3000 in antibody buffer.

FIGURE 10

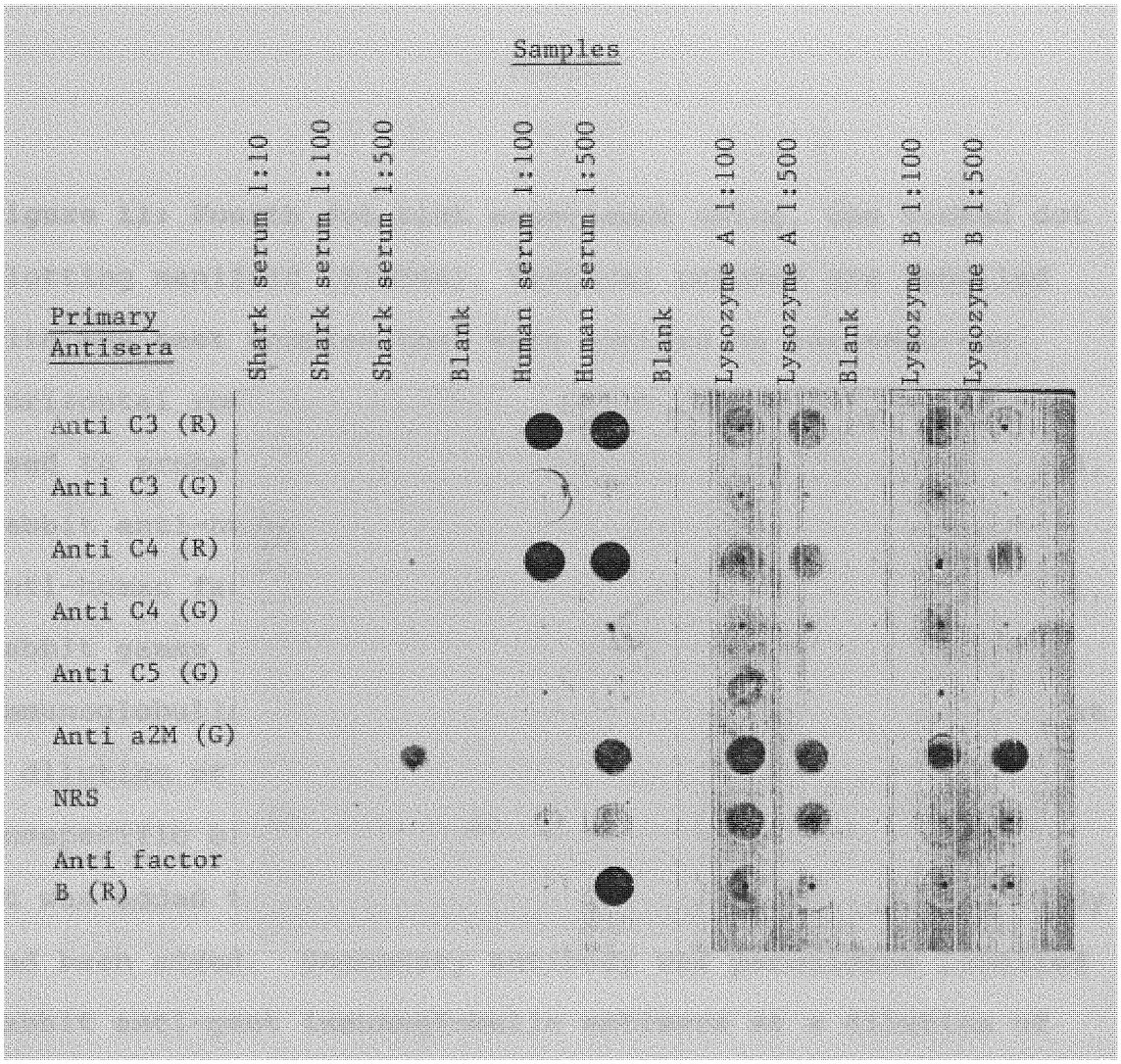

Samples
Primary Antisera
Anti C3 (R)
Anti C3 (G)
Anti C4 (R)
Anti C4 (G)
Anti C5 (G)
Anti a2M (G)
NRS
Anti factor B (R)
Shark serum 1:10
Shark serum 1:100
Shark serum 1:500
Blank
Human serum 1:100
Human serum 1:500
Blank
Lysozyme A 1:100
Lysozyme A 1:500
Blank
Lysozyme B 1:100
Lysozyme B 1:500

Figure 11: Fourth dot blot experiment using, the BioRad dot blotting manifold (Biodot). Vertical columns are labelled with the test samples that were applied to the membrane. Horizontal rows are labelled with the primary antibodies used to probe the samples and include: rabbit anti-human C3, rabbit anti-human C4, rabbit anti-human factor B, rabbit anti-human factor H antisera, non-immune rabbit serum (NRS), rabbit gamma globulin fraction, and rabbit anti-goat immunoglobulin antisera. All primary antibodies were used at a dilution of 1:15 in antibody buffer. To test for nonspecific binding of the secondary antibody to the blot, it was added to all the samples to which no primary antibody had been added. Secondary antibody was peroxidase labelled rabbit anti-goat immunoglobulin antisera at a dilution of 1:1000 in antibody buffer.

FIGURE 11

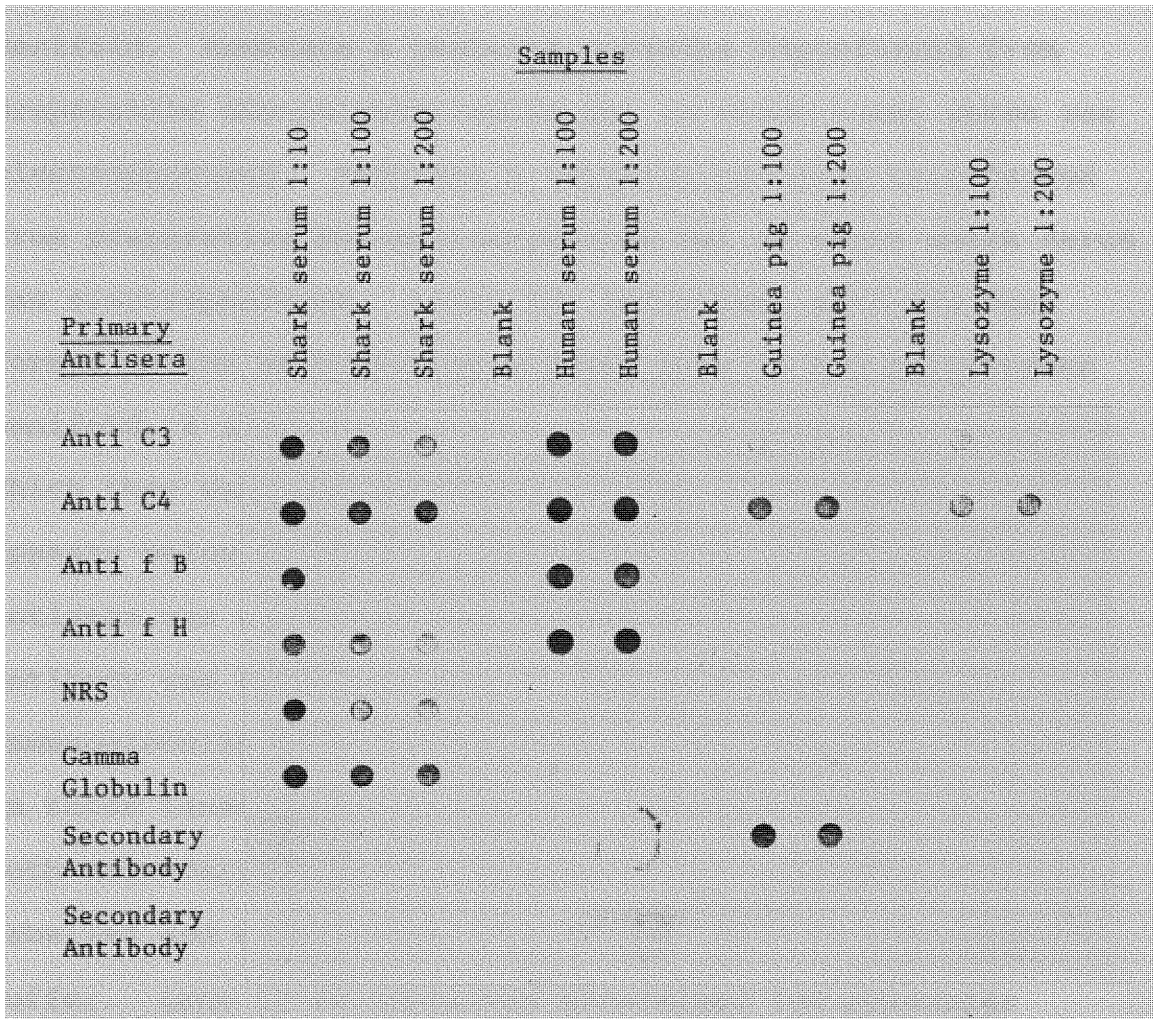

Samples
Primary Antisera
Shark serum 1:10
Shark serum 1:100
Shark serum 1:200
Blank
Human serum 1:100
Human serum 1:200
Blank
Guinea pig 1:100
Guinea pig 1:200
Blank
Lysozyme 1:100
Lysozyme 1:200
Anti C3
Anti C4
Anti f B
Anti f H
NRS
Gamma Globulin
Secondary Antibody
Secondary Antibody

Figure 12: Western blot using the BioRad multiscreen manifold. Membrane A contains proteins transferred from preparative SDS-polyacrylamide gel in which human serum was electrophoresed. Membrane B contains proteins transferred from preparative SDS-polyacrylamide gel in which shark serum was electrophoresed. Lanes for individual antisera were formed on the membranes by placing them in the multiscreen manifold. A list of primary antisera and the dilution at which they were applied and the lanes containing them appears below the blots. Goat anti-rabbit immunoglobulin antisera was used as the secondary antibody where primary antibody was of rabbit origin, and rabbit anti-goat immunoglobulin antisera was used as the secondary antibody in those lanes probed with primary antibody of goat origin. Both secondary antibody preparations were peroxidase labelled and used at a dilution of 1:1000 in antibody buffer. Lane 19-20 contained prestained low molecular weight protein standards which included: Phosphorylase B (indicated by <) 106,000 daltons; Bovine serum albumin, 80,000 daltons; Ovalbumin, 49,500 daltons; Carbonic anhydrase, 32,500 daltons; Soybean trypsin inhibitor, 27,500 daltons; and Lysozyme 18,500 daltons.

FIGURE 12

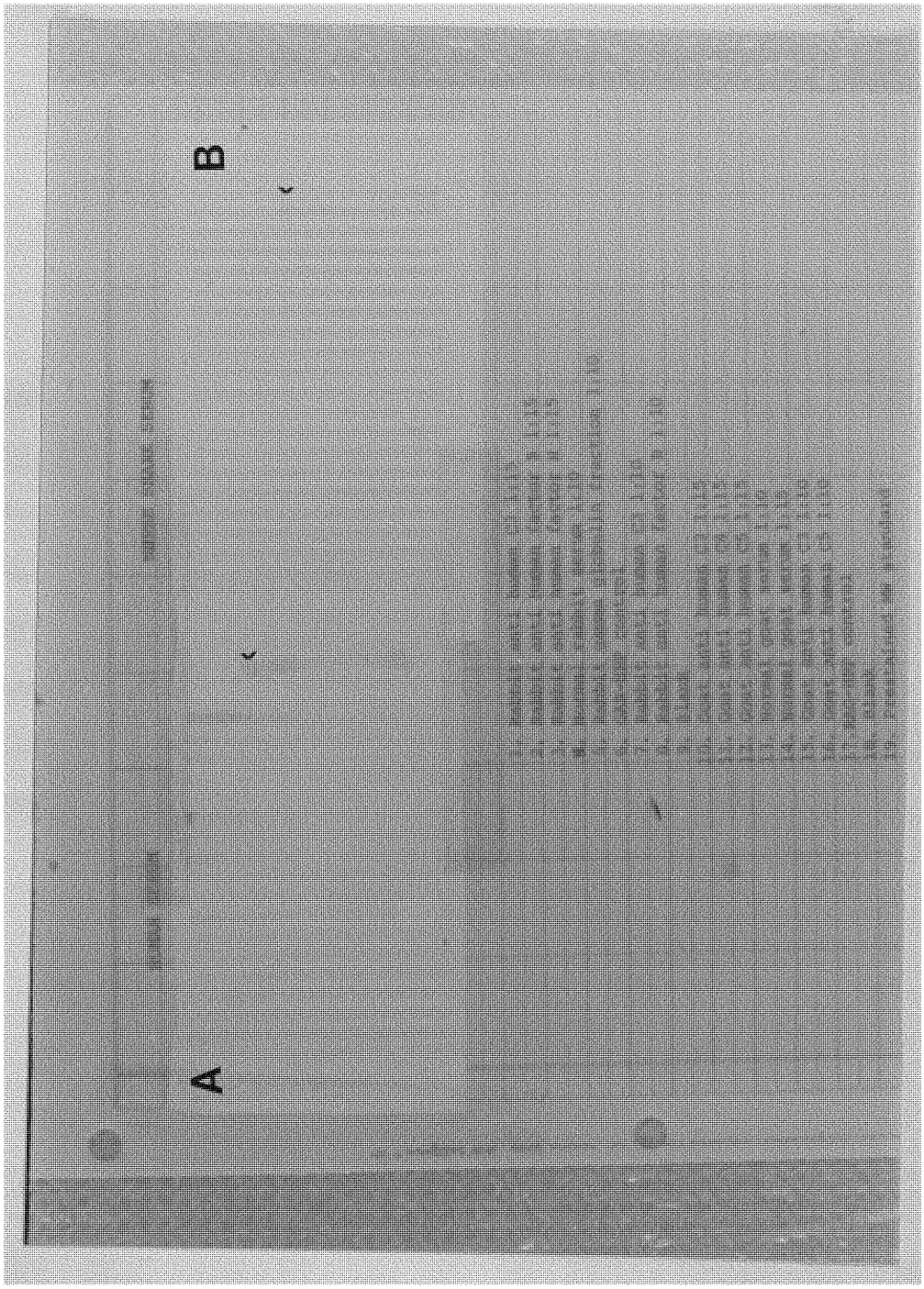


Figure 13: Coomassie Blue stained SDS Polyacrylamide gel. The gel is a duplicate run simultaneous to the gel from which the proteins were eluted and transferred to nitrocellulose for immunoblotting (figure 14). Lane 4 contains human serum diluted 1:5; lane 3 contains nurse shark serum diluted 1:5; lane 2 contains nurse shark serum diluted 1:2; lane 1 contains prestained molecular weight standards described in figure 12.

FIGURE 13

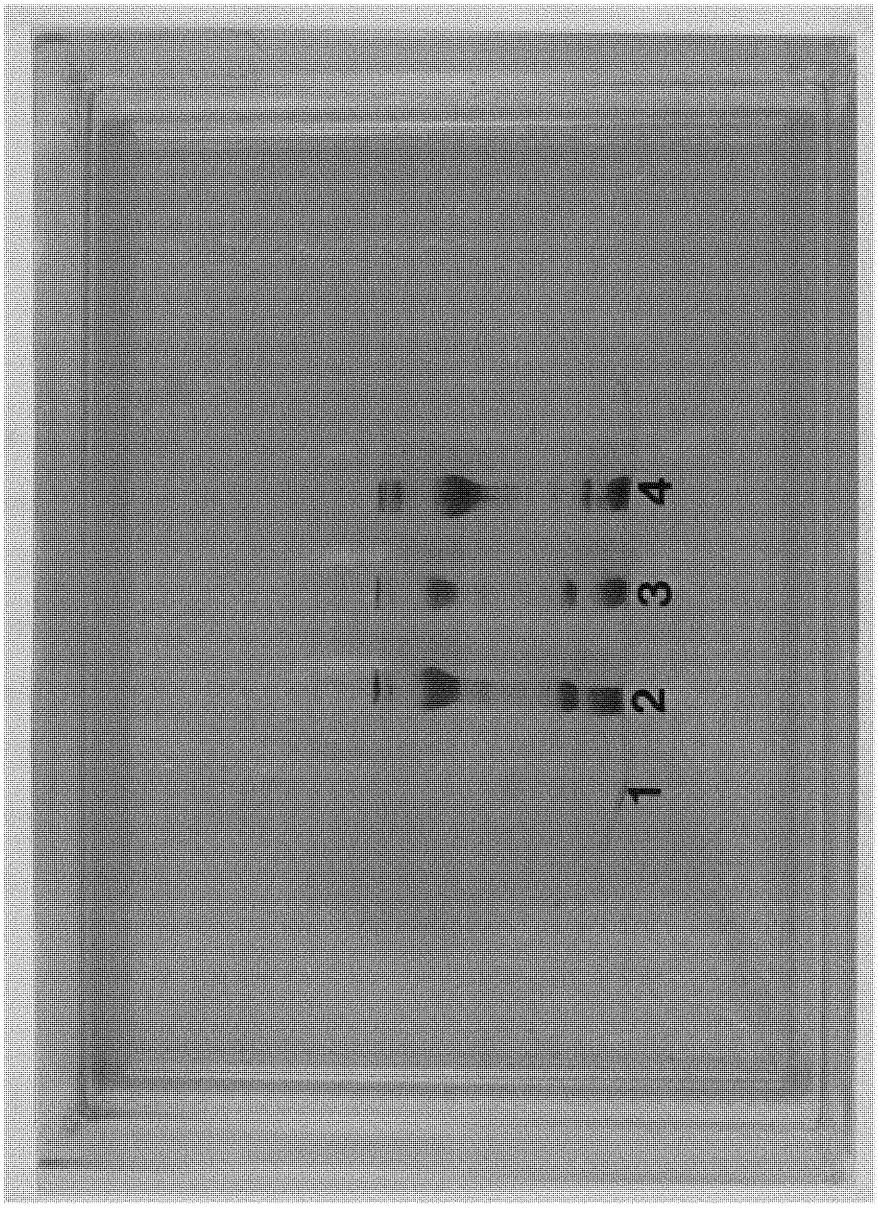
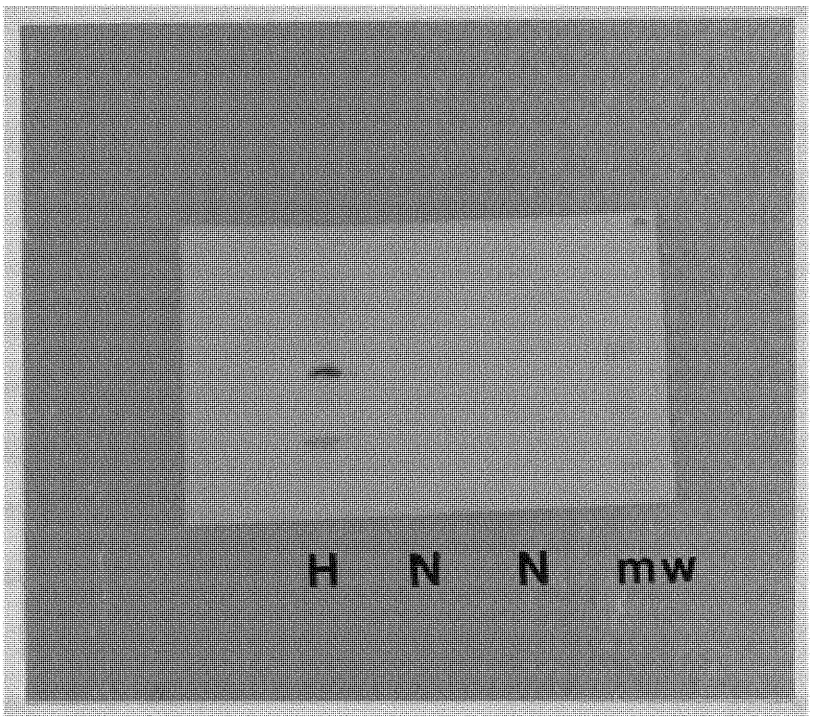


Figure 14: Western blot developed with anti-human factor B antisera. Lane 1 contains human serum diluted 1:5; lane 2 and 3 contain nurse shark serum diluted 1:5 and 1:2 respectively; lane 4 contains prestained molecular weight standards described in figure 12. Primary antibody used to probe this membrane was rabbit anti-human factor B antisera at a dilution of 1:15. Secondary antibody was goat anti-rabbit immunoglobulin antisera at a dilution of 1:1000.

FIGURE 14



CONCLUSIONS

The objective of this study was to comprehensively examine nurse shark serum for evidence of an alternative pathway of complement activation. The presence and functional characteristics of the classical pathway, in this species, was firmly established by Jensen (1981) whose studies did not indicate the presence of an alternative pathway. Whether or not the alternative pathway is present in Ginglymostoma cirratum, the nurse shark, has not been determined to date although some investigators concluded, based on the work of Jensen, that the alternative pathway does not operate in the nurse shark (Koppenheffer, 1987; Dodd, in press).

Ross and Jensen (1973) demonstrated the presence of a molecule in nurse shark serum that is analogous to mammalian C1, namely C1n. They have shown this molecule to be similar in function to mammalian C1 in that it is the first component to react in the classical complement cascade of the nurse shark which consists of six functionally isolated components (C1n, C2n, C3n, C4n, C8n, and C9n). Therefore, C1n and C1 both have recognition and activation functions in the classical pathways of shark and mammals respectively. Similarily, C1n and mammalian C1 are both complexes of subcomponents whose integrity is dependent on the presence

of divalent metal ions. In the presence of EDTA, a chelator of metal ions, C1n is completely inactivated. Once inactivated by EDTA the activity of this molecule cannot be restored by the addition of excess metal ions, unlike mammalian C1 which readily reforms under these conditions. Ross and Jensen were not able to conclusively prove that the metal cation necessary to maintain the integrity of the C1n molecule is calcium, as the molecule, once dissociated by EDTA into its subcomponents, could not be induced to reform by the addition of calcium ions under conditions demonstrated to successfully restore activity of mammalian C1 (Ross and Jensen, 1973). Experiments, in which magnesium as well as other divalent cations that are chelated by EDTA were added to dissociated-C1n did not result in reassociation of the molecule. Thus, it cannot be said that the reason EDTA-dissociated-C1n does not reform in the presence of excess calcium ions is because another metal ion is necessary for its integrity. It is more likely that the removal of calcium ions from the C1n complex results in an irreversible conformational change in one or more of its subcomponents. The requirement for calcium ions in classical complement pathway activity has been established for mammals (Sim, 1981) as well as for representatives of several other vertebrate classes (Ohta et al., 1984; Sekizawa et al.,1984; Nonaka, 1981). Therefore an absolute requirement for calcium is generally accepted as a criterion

for defining classical complement pathway activity in the serum of vertebrate species.

As EDTA also chelates magnesium ions, it predictably shuts down alternative pathway activity as well as classical complement activity as magnesium ions are required for alternative pathway activity. In the mammalian alternative pathway, magnesium ions are necessary for the binding of factor B to C3b (Reid,1986). As EGTA was originally used to titrate calcium ions in the presence of magnesium ions, (Schmid and Reilly, 1957) this chelator can be used to selectively chelate calcium ions from a solution while leaving magnesium ions available. Thus, serum chelated with EGTA would be expected to be devoid of available calcium ions while still maintaining free magnesium ions. Hemolytic activity seen in assays of EGTA treated serum can then be attributed to a calcium independent activation pathway. The alternative pathway in mammals and lower vertebrate species does not require calcium ions, indicating that a calcium-dependent recognition/activation molecule (i.e. C1) is not required to initiate this pathway. Calcium-independent activity (i.e. alternative pathway) has been demonstrated in all classes of vertebrates with the exception of the cartilaginous fish. This study has demonstrated, for the first time, that calcium-independent lytic activity does exist in nurse shark serum, as EGTA-chelated serum retained

hemolytic activity against mammalian target erythrocytes at concentrations of EGTA up to 50 mM. These results indicate that a lytic system does exist in nurse shark serum that most probably bypasses the classical complement pathway recognition/activation molecule (C1n) to produce lysis of target cells through an alternative mechanism of activation. Chelating of nurse shark serum with EGTA results in a statistically significant drop in hemolytic titre from that of unchelated serum using rabbit, horse, sheep (sensitized and unsensitized) and bovine (sensitized and unsensitized) target erythrocytes. An initial significant drop in hemolytic titre from control levels occurs when serum is chelated with 5 mM EGTA, followed by smaller decreases in titre with increasing concentration of EGTA. These observations suggest that 5 mM EGTA is sufficient to remove available calcium ions from nurse shark serum. According to a Sea World study (Murru, 1984) a calcium level of 3mM is representative of the species. A concentration of 5 mM EGTA should be sufficient to remove all the calcium ions as EGTA combines with calcium in a 1:1 ratio (Schmid and Reilly, 1957). The minimal decreases in hemolytic titre when EGTA concentration is increased may be due to a weak association of EGTA with magnesium limiting the activity. EGTA has an association constant (Ka) of 1×10^5 for magnesium versus 1×10^{11} for calcium and therefore it preferentially, but not exclusively, binds calcium. EDTA forms stable complexes

with both calcium and magnesium, with Ka values of 1 X 10^{11} and 1 X 10^{9} respectively (Fine et al. 1972). EDTA completely inhibits the lytic activity of nurse shark serum because it removes all available calcium and magnesium. In those instances where a low level of lytic activity remained in shark serum chelated with 10 mM EDTA the serum samples also demonstrated a proportionately higher level of activity against the corresponding target cell when chelated with EGTA. This most likely reflects the higher initial levels of magnesium present in these sera. It could possibly be argued that the serum calcium level (3 mM) obtained by Murru (1984) was not representative of normal levels in nurse shark serum so that even 25 mM EGTA is insufficient to remove all available calcium ions from the serum, i.e. the residual hemolytic activity would then be due to the classical pathway. If this were the case, the results would not show such an initial large drop in hemolytic titre when serum is chelated with 5 mM EGTA followed by small decreases as EGTA levels increase. Activated C1 initiates an amplification phase of the classical pathway whereby many molecules of C4 are cleaved. If sufficient calcium remains to activate C1n in chelated shark serum, the residual hemolytic activity would be expected to be at levels closer to control levels. One can conclude from these data that removal of calcium ions from nurse shark serum by EGTA is responsible for the significant decrease in hemolytic

activity noted, and that a calcium-independent lytic system, a characteristic criterion of alternative pathway activity, is responsible for the lytic activity remaining in shark serum. Furthermore, these results indicate calcium is necessary for the integrity of C1n and thus the activity of the classical pathway. If it were not, no loss of lytic activity would occur on chelating nurse shark serum with EGTA.

As lytic activity remained in serum chelated with EGTA but not in serum chelated with an equivalent concentration of EDTA, a cation that is bound by EDTA but not EGTA has to be required by the calcium-independent pathway described above. Alternative pathway activity in other species is dependent on magnesium ions. Therefore, the effect of added magnesium ions on the hemolytic activity of EGTA chelated shark serum was evaluated. The addition of magnesium ions to shark serum, chelated with 20 mM EGTA, increased hemolytic titres significantly over titres of serum chelated with 20 mM EGTA to which no magnesium had been added. This increase in activity was seen with all mammalian erythrocyte targets tested. These observations establish that the calcium-independent lytic system, is enhanced by added magnesium ions. This is in accordance with accepted criteria of alternative pathway activity namely, the ability to lyse target erythrocytes in the presence of magnesium

ions alone, i.e. in the absence of calcium ions.

The efficacy of EGTA as a chelator of calcium ions is dependent on pH (cation binding does not occur at pH values below 6.4) (Platts-Mills and Ishizaka, 1974). The addition of magnesium ions to buffers containing EGTA results in a drop in pH, thus if the pH is not adjusted when magnesium is added to EGTA-containing assay buffers bound calcium would be released by the chelator. For this study, the pH of magnesium-containing EGTA buffers was adjusted upwards with 1 N NaOH to 7.4 to eliminate such a loss of effectiveness of the chelator. Should however, a drop in pH occur inadvertantly, classical pathway activity cannot be restored by the availability of released calcium or magnesium ions as a loss of C1n activity by removal of metal cations is irreversible. Therefore the increase in hemolytic activity observed in chelated sera on the addition of magnesium ions cannot be due to the availablity of calcium ions released by the chelator. From experimental results, it is clear that a calcium-independent, magnesium-dependent hemolytic pathway operates in nurse shark serum. As these are characteristics typical of the mammalian alternative complement pathway, it would be reasonable to state that the alternative pathway of complement activation has been unequivocally demonstrated in this primitive animal.

This study has shown that some differences in the activity of the alternative pathway of mammals and sharks do exist. Whereas the lysis of rabbit and horse erythrocytes in the presence of EGTA occurs via the alternative pathway in other vertebrate species (Platts-Mills and Ishizaka, 1974; Ohta etal.,1984), the lysis of sheep erythrocytes, by human, bird and rainbow trout (Nonaka,1984) complement, is completely blocked by EGTA, indicating lysis to be exclusively via the classical pathway. In the nurse shark however, significant lysis of these target cells occured under conditions which allowed only alternative pathway activity. If the lysis of sheep cells by nurse shark complement is due to the alternative pathway then shark serum, chelated with EDTA should regain activity on the addition of magnesium ions. Experiments showed that the lytic activity of nurse shark serum, chelated with 10 mM EDTA, against unsensitized sheep and sensitized sheep and bovine erythrocytes can in fact be restored by the addition of magnesium ions. As C1n of the nurse shark classical pathway has been shown to be irreversibly inactivated by EDTA, this restoration of activity by the addition of magnesium ions has to be due to the activity of a lytic pathway that does not require C1n as the initial recognition/activation molecule.

It has been proposed (Pangburn and Muller-Eberhard,

1980) that activators of the alternative pathway provide a site in which factor B and C3b are protected from degradation by the endogenous control proteins factor H and C3b inactivator(factor I). The complement control proteins of nurse sharks may be different from those of mammals, raising the possibility of a site on the surface of sheep erythrocytes which protects C3/C5 convertase proteins from inactivation by shark control proteins. Kaidoh and Gigli (1987) have demonstrated that the serum of lower vertebrates contain factors that are able to generate cleavage fragments of C3b and C4b identical to those produced by mammalian control proteins C4bp and C3b inactivator (factor I) and factor H respectively suggesting the presence of analogous control proteins in these species. However, in this study, the serum of cartilaginous fish was not able to produce these fragments from human C3 and C4. A possible explanation for the failure of shark serum to produce the fragments could be that shark control proteins are different and do not recognize human C3 or C4, or alternatively shark proteins analogous to human C3 and /or C4 are structurally and perhaps functionally different and therefore subject to different control proteins or mechanisms. Either situation may exist in the nurse shark. These possible differences might also explain why the lysis of sheep erythrocytes by nurse shark serum occurs via the alternative pathway. It has also been shown (Kaidoh and Gigli, 1987) that the

control proteins of teleost are calcium dependent. It might be that shark complement control proteins are also calcium dependent, therefore, in the presence of EGTA these proteins are inactivated which allows sheep cells to be lysed by the alternative pathway. Similar to shark serum activity, lysis of sheep erythrocytes by cobra (Vogel and Muller-Eberhard,1984) and carp (Matsuyame,1987) serum has been shown to occur under conditions of alternative pathway activity.

Activation of nurse shark complement by known activators of the mammalian alternative pathway was attempted in this investigation. Initial experiments demonstrated that zymosan was able to deplete nurse shark serum of hemolytic activity in the presence of EGTA (which allows only alternative pathway activity) and in the absence of EGTA (both pathways operate). Nurse shark complement was found to be activated by zymosan and complete depletion of hemolytic activity occurred in serum chelated with 20 mM EGTA. However, when unchelated serum was incubated with zymosan and then assayed against sensitized sheep erythrocytes in DGVB++ a loss of hemolytic activity was observed but hemolytic activity was not totally depleted indicating that sufficient terminal components remained to affect limited lysis of the target cells via the classical pathway. It is also possible that, in the absence of the

chelator, some activation of the classical pathway occurred when serum was incubated with zymosan without chelator as it is very likely nurse shark serum contains natural antibodies to zymosan as does human serum (Nelson, 1959). The conclusions which can be drawn from this study are that zymosan can activate nurse shark alternative pathway and to a limited extent, through the interaction with shark natural antibody, the classical pathway as well. However, activation of the classical pathway could not have depleted the terminal components or early acting components of the classical pathway. In subsequent experiments using serum samples from additional individuals, reduction of hemolytic activity of unchelated nurse shark serum by incubation with zymosan was confirmed. Hemolytic activity of chelated serum from one of these individuals was also depleted completely by zymosan confirming activation of the alternative pathway. Chelated serum from the other individual showed no consistent reduction in hemolytic activity as a result of incubation with zymosan indicating no activation of the alternative pathway occurred. It is possible, since this individual was the largest animal used in this study, a difficult capture may have resulted in activation of complement <u>in vivo</u> through the release of acute phase proteins. The hemolytic titre of unchelated serum from both individuals was reduced by incubation with zymosan. These results indicate zymosan activates nurse shark complement

directly via the alternative pathway and through sensitization with natural antibody to zymosan via the classical pathway. It would appear from these results that if only the alternative pathway is activated all of the terminal components are not used up as would be expected. Rather an early acting component of the alternative pathway is the limiting factor.

LPS, an activator of the mammalian alternative pathway, reduced the hemolytic activity of shark serum, however, the decrease in activity was observed only when unchelated nurse shark serum was activated. This suggests that the classical pathway only is activated by LPS. The lipid moeity of LPS is known to activate the classical pathway of mammals directly while the polysaccharide portion activates the alternative (Klein,1990). Therefore, it is possible that the polysaccharide fails to activate shark complement whereas lipid A of LPS is an activator of the classical pathway.

Under our experimental conditions inulin and cobra venom factor failed to activate shark complement as a reduction of hemolytic activity of nurse shark serum was not observed after incubation with either inulin or cobra venom factor. In the case of inulin, it is possible that it does not activate nurse shark complement at concentrations

similar to those used for zymosan and LPS and activation may be achieved at higher concentrations. The concentration of CVF employed may also have been too low and thus the possiblity of it being an activator of nurse shark complement cannot be ruled out. It is also possible that the shark complement counterpart of factor B is not functionally compatible with CVF which in the mammalian system functions as a C3b substitute. Shark complement control proteins, as yet unidentified, could be efficient at degrading the "CVFBb" complex unlike the control proteins of mammals. Failure to produce a loss of hemolytic activity does not necessarily indicate the failure of a substance to activate nurse shark complement. Complement components may be activated without activation of the membrane attack complex. As the lysis of foreign erythrocytes is not the primary function of complement in vivo, it is possible that the alternative pathway may be activated without lysis occuring. Opsonization is considered to be a primitive function of complement (Farries et al.,1990); it may be that nurse shark serum, when activated, does not lyse targets but rather brings about the opsonization of target particles. Such activity has been demonstrated in mammalian serum when activated by cobra venom factor from the Thailand cobra (Naja haje), which interacts with factor B but does not produce a functional C5 convertase thus leaving the proteins of the membrane attack complex intact.

Although results obtained from dot and Western blots did not yield conclusive evidence for the presence, in nurse shark serum, of proteins that antigenically cross react with antisera raised to mammalian complement proteins, the possibility that antigenic relationships exist cannot be ruled out. Further refinement of these procedures may yet produce specific reactions between proteins in nurse shark serum and antibodies raised to human complement components. For example, an increase in incubation time of the primary antibody with shark serum proteins on the membrane in Western blots may facilitate the binding of the antibody even if the "fit" is not exactly perfect. Another modification to the procedure which might yield positive reactions in Western blots is to increase the concentration of primary antibody or employ primary antisera raised to complement components of lower vertebrate species. Activated nurse shark serum may give a positive reaction where unactivated serum failed to do so as "activation" may expose hidden epitopes or the configuration of epitopes may be changed. This has been demonstrated using activated cobra serum probed with antiserum to human C3 (Koppenheffer, 1987).

Nonspecific reaction may be decreased in dot blots by incubation of the primary and secondary antibodies with nurse shark serum on the membrane at 37^0C rather than at

room temperature. IgG, the primary antibody class in hyper immune mammalian antiserum reacts better at 37^{0}C than at room temperature whereas IgM, the primary antibody class of nurse shark natural antibody (Rudikoff et al., 1970) reacts best at 4-20^0. Natural antiboby is very likely to be the factor responsible for the nonspecific reactions seen in dot blots. These nonspecific reactions may also be reduced by the use of an alternate blocking agent such as BLOTTO.

This study has proven conclusively, for the first time, that the alternative pathway of complement activation is present in a representative of the cartilagenous fish. The requirements for divalent cations by the two complement pathways of this species are analogous to those found in higher vertebrates. These studies suggest that biological substances that activate the nurse shark alternative pathway and classical pathway are not as restricted as that of higher vertebrates. As the range of substances that activate the mammalian alternative pathway is extremely diverse, no common factor has been found that defines a substance as an activator or a nonactivator. One factor, sialic acid, has been shown to restrict activation of the mammalian alternative pathway (Kazatchine et al., 1979). Sialic acid is found on the surface of sheep erythrocytes and its removal has been shown to permit activation of the mammalian alternative pathway by sheep erythrocytes. As

sheep erythrocytes can activate the alternative pathway in the nurse shark, it is possible that sialic acid does not restrict activation of this pathway in nurse shark serum. Alternatively, sialic acid on the surface of sheep erythrocytes is masked by shark natural antibody. It is also possible, given the level of IgM class natural antibody to sheep erythrocytes in shark serum, that immune complexes or IgM aggregates may form during hemolytic assays providing activation of nurse shark alternative pathway and subsequent lysis of the target cells.

Given that it can now be said that both complement activation pathways exist in the primitive nurse shark, the divergence of these two pathways must have occured prior to the evolutionary divergence of the sharks from the higher vertebrates over 300 million years ago. This fact implies that both pathways are phylogenetically ancient defense mechanisms and perhaps have evolved not one from the other but parallel to one another with some "borrowing" of proteins between pathways over evolutionary time. More conclusive proof of this possibility awaits definition of alternative pathway proteins of the shark as well as those of Agnathans and invertebrates.

REFERENCES

Abbas, A.K., Lichtman, A.H. and Pober, J.S. 1991. In Cellular and Molecular Immunology. W.B. Saunders Co., Philadelphia, PA. 259-282.

Alexander, R.G. and Steiner, L.A. 1980. The first component of complement from the Bullfrog, Rana catesbeina: functional properties of C1 and isolation of subcomponent C1q. J. Immunol. **124**, 1418.

Bentley, D.R. and Campbell, R.D. 1986. C2 and factor B: Structure and genetics. Biochem. Soc. Symp. **51** 7.

Brade, E.J., Cook, C.T., Shin, H.S. and Mayer, M.M. 1972. Studies on the properdin system: Isolation of a heat-labile factor from guinea pig serum related to a human glycine-rich beta glycoprotein (GBG or factor B). J. Immunol. **109**, 1174.

Bryant, R.E. and Jenkins, D.E. 1968. Calcium requirements for complement dependent hemolytic reactions. J. Immunol. **101**, 664.

Canicatti, C. and Tschopp, J. 1989. Relationship between vertebrate channel forming proteins and invertebrate haemolysin. Dev. Comp. Immunol. **13**, 377.

Campbell, D.H., Garvey, J.S., Cremer, N.E. and Sussdorf, D.H. 1970. In Methods in Immunology. WA Benjamin Inc. New York, NY. 120.

Cowan, K.M. 1958. Phage neutralization to determine properdin levels. Science **128**, 778.

Day, N.K.B., Gerwurtz, H., Johannsen, R., Finstad, J., and Good, R.A. 1970. Complement and complement-like activity in lower vertebrates and invertebrates. J. Exp. Med. **132**, 941.

Dodds, A.W. and Day, A.J. The phylogeny and evolution of the complement system. In press.

Doolittle, R.F. 1985. The genealogy of some recently evolved vertebrate proteins. Trends Biochem. Sci. **10**, 233.

Ellis, M.G., Lamont, S.J. and Arp, L.H. 1989. Characterization of complement activity in turkeys: evidence for classical and alternative complement pathways. Poultry Science. **68**, 646

Farries, T.C., Steuer, K.L.K. and Atkinson, J.P. 1990. Evolutionary implication of a new bypass activation pathway of the complement system. Immunol. Today. **11** 78.

Fearon, D.T. and Austen, K.F. 1977. Activation of the alternative complement pathway with rabbit erythrocytes by circumvention of the regulatory action of endogenous control proteins. J. Exp. Med. **146**, 22.

Fine, D.P., Marney, S.R., Colley, D.G., Sergent, J.S. and Des Prez, R.M. 1972. C3 shunt activation in human serum chelated with EGTA. J. Immunol. **109**, 807.

Gotze, O. and Muller-Eberhard, H.J. 1971. The C3 activator system: an alterantive pathway of complement activation. J. Exp. Med. **134**, 90s.

Gotze, O. and Muller-Eberhard, H.J. 1976. The alternative pathway of complement activation. Adv. in Immunol. **24**, 1.

Hyder-Smith, S. and Jensen, J.A. 1986. The second component (C2n) of the nurse shark complement system: Purification, physicochemical characterization and functional comparison with guinea pig C4. Dev. Comp. Immunol. **10**. 191.

Jensen, J.A., Sigel, M.M., and Ross, G.D. 1968. Natural antibody (An) and compement (C′) of the nurse shark. Fed. Proc. **27**, 491.

Jensen, J.A. 1969. A specific inactivator of mammalian C4

isolated from nurse shark (Ginglymostoma cirratum) serum. J. Exp. Med. **130**, 217.

Jensen, J.A., Fuller, L., and Iglesias, E. 1973. The terminal components of the nurse shark (Ginglymostoma cirratum) complement system. J. Immunol. **111**, 306.

Jensen, J.A., Festa, E., Smith, D.S., and Cayer, M. 1981. The complement system of the nurse shark: hemolytic anbd comparative characteristics. Science **214**, 566.

Jurd, R.D. 1983. Specialization of the teleost and anuran immune response: A comparative critique. In Fish Immunology. Academic Press. 9.

Kabat, E.A. and Mayer, M. M. 1961. In Experimental Immunochemistry. Charles C Thomas Springfield, IL. 133.

Kawaguchi, S., Muramatsu, S. and Mitsuhashi, S. 1978. Natural haemolytic activity of snake serum. I. Natural antibody and complement. Dev. Comp. Immunol. **2**, 287.

Kazatchine, M.D., Fearon, D.T. and Austen, K.F. 1979. Human alternative complement pathway: Membrane associated sialic acid regulates the competition between factor B and beta 1 H for cell bound C3b. J. Immunol. **122**, 75.

Klein, J. 1990. In Immunology. Blackwell Scientific Publications Boston, MA.

Koppenheffer, T.L. 1986. Activation of alternative complement pathway by both high and low molecular weight turtle antibodies. Am. Zool. **26**, 86A.

Koppenheffer, T.L. 1987. Serum complement systems of ectothermic vertebrates. Dev. Comp. Immunol. **11**, 279.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**, 680.

Marney, S.R. and Des Prez, R.M. 1969. Comparison of the effects of divalent ion chelating agents on complement-mediated hemolysis on rabbit and human serum. J. Immunol. **103**, 1044.

Matsuyama, H., Tanaka, K., Nakao, M., and Yano, T. 1988. Characterization of the alternative complement pathway of carp. Dev. Comp. Immunol. **12**, 403.

Medicus, R.G., Gotze, O., and Muller-Eberhard, H.J. 1976a. Alternative pathway of complement: Recruitment of precursor properdin by the labile C3/C5 convertase and potentiation of

the pathway. J. Exp. Med. **144**, 1076.

Medicus, R.G., Schreiber, R.D., Gotze, O. and Muller-Eberhard, H.J. 1976b. A molecular concept of the properdin pathway. Proc. Natl. Acad. Sci. **73**, 612.

Muller-Eberhard, H.J. 1969. Complement. Ann. Rev. Biochem. **38**, 389.

Muller-Eberhard, H.J. and Schreiber, R.D. 1980. Molecular biology and chemistry of the alternative pathway of complement. Adv. in Immunol. **29**,1.

Murru, F.L. 1984. Preliminary baseline blood parameters for several sharks. Am. Assoc. Zoo. Parks and Aquariums, Ann. Conf. Proceedings. 333.

Nelson, R.A. 1958. An alternative mechanism for the properdin system. J. Exp. Med. **108**, 515.

Nelson, B. and Ruddy, S. 1979. Enhancing role of IgG in lysis of rabbit erythrocytes by the alternative pathway of human complement. J. Immunol. **122**, 1994.

Nonaka, M., Yamaguchi, N., Natsuume-Sakai, S. and Takahashi, M. 1981. The complement system of the rainbow trout (Salmo

gairdneri) I. Identification of the serum lytic system homologous to mammalian complement. J. Immunol. **126**, 1489.

Nonaka,M. Fujii, T. Kaidoh, T. and Natsuume-Sakai, S. 1984. Purification of lamprey complement protein homologous to the third component of the mammalian complement system. J. Immunol. **133**, 3242.

Ohta, M., Okada, M., Yamashina, I. and Kawasaki, T. 1990. The mechanism of carbohydrate mediated complement activation by the serum mannan binding protein. J. Biol. Chem. **256**, 1980.

Ohta, H. Yoshikawa, Y., Kai, C. Yamanouchi, K. and Okada, H. 1984. Lysis of horse red blood cells mediated by antibody-independent activation of the alternative pathway of chicken complement. Immunology. **52**, 437.

Pangburn, M.K. and Muller-Eberhard, H.J. 1980. Relation of a putative thiolester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. J. Exp. Med. **152**, 1102.

Pettey, C.L. and McKinney, E. C. 1983. Temperature and cellular regulation of spontaneous cytotoxicity in the shark. Eur. J. Immunol. **13**, 133.

Pillemer, L. Blum, L., Lepow, I.H., Ross, O.A., Todd, E.W. and Wardlaw, A.C. 1954. The properdin system and immunity: I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science. **120**, 279.

Pillemer, L., Schoenberg, M.D., Blum, L. and Wurz, L. 1955. Properdin system and immunity: II. Interaction of the properdin system with polysaccharides. Science. **122**, 545.

Platts-Mills, T.A.E. and Ishizaka, K. 1974. Activation of the alternative pathway of human complement by rabbit cells. J. Immunol. **113**, 348.

Polhill, R.B., Newman, S.L., Pruitt, K.M. and Johnson, R.B. 1978. Kinetic assessment of alternative complement pathway activity in a hemolytic system. J. Immunol. **121**, 371.

Quigley, J.P. and Armstrong, P.B. 1985. A homologue of alpha 2 macroglobulin purified from the haemolymph of the horseshoe crab Limulus polyphemus. J. Biol. Chem. **260**, 12715.

Reid, K.B.M. 1981. The proteolytic activation systems of complement. Annu. Rev. Biochem. **50**, 433.

Reid, K.B.M. 1986. Activiation and control of the complement system. Essays in Biochem. **22**, 27.

Robey, F.A. and Liu, T.Y. 1981. Limulin: a C-reactive protein from Limulus polyphemus. J. Biol. Chem. **256**, 969.

Roitt, I., Brostoff, J. and Male, D. 1985. In Immunology. C V Mosby Co. St. Louis, MO.

Ross, G.D. and Jensen, J.A. 1973. The first component (C1n) of the complement system of the nurse shark (Ginglymostoma cirratum). I. Haemolytic characteristics of partially purified C1n. J. Immunol. **110**, 175.

Ross, G.D. and Jensen, J.A. 1973. The first component (C1n) of the complement system of the nurse shark (Ginglymostoma cirratum). II. Purification of the first component by ultracentrifugation and studies of its physicochemical properties. J. Immunol. **110**, 911.

Rudikoff, S., Voss, E.W. and Sigel, M.M. 1970. Biological and chemical properties of natural antibodies in the nurse shark. J. Immunol. **105**, 1344.

Sandberg, A.L., Osler, A.G., Shin, H.S. and Oliveira, B. 1970. The biologic activityes of guinea pig antibodies. II.

Modes of complement interaction with immunoglobulins. J. Immunol. **104**, 329.

Sandberg, A.L., Oliveira, B. and Osler, A.G. 1971. Two complement interaction sites in guinea pig immunoglobulins. J. Immunol. **106**, 282.

Sandberg, A.L. and Osler, A.G. 1971. Dual pathways of complement interaction with guinea pig immunoglobulins. J. Immunol. **107**, 1268.

Schenken, H.A. and Ruddy, S. 1981. The role of immunoglobulins in alternative complement pathway activation by zymosan. I. Human IgG with specificity for zymosan enhances alternative pathway activation by zymosan. J. Immunol. **126**, 7.

Schmid, R.W. and Reilly, C.n. 1957. A new complexon for titration of calcium in the presence of magnesium. Anal. Chem. **29**, 264.

Sekizawa, A., Fujii, T. and Katagiri, C. 1984. Isolation and characterization of the third component of complement of the serum of the clawed frog Xenopus laevis. J. Immunol. **133**, 1436.

Simmons, A. 1980. In Technical Hematology. Third ed. J.B. Lippincott Co. Philadelphia PA.

Sottrup-Jensen, L., Stepnik, T.M., Kristensen, T., Lonblad, P.B., Jones, C.M., Wierzbick, D.M. Magnusson, S., Domdey, H., Wetsel, R.A., Lundwall, A., Tack, B.F. and Fey, G.H. 1985. Common evolutionary origin of alpha 2 macroglobulin and complement components C3 and C4. Proc. Natl. Acad. Sci. USA. **82**, 9.

Spycher, S.E., Arya, S., Isenman, D.E. and Painter, R.H. 1987. A functional thioester containing alpha 2 macroglobulin homologoue isolated from the haemolymph of the American lobster Homarus americanus. J. Biol. Chem. **262**, 14606.

Springer, V.G. and Gold, J.P. 1989. In Sharks in Question. Smithsonian Institution Press. Washington D.C.

Toussaint, A.J. and Muschel, L.H. 1959. Neutralization of bacteriophage by normal serum. Nature. **183**, 1825.

Towbin, H., Staelhelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. **76**, 4350.

Vogel, C.W. and Muller-Eberhard, H.J. 1985. The cobra complement system I. The alternative pathway of activation. Dev. Comp. Immunol. **9**, 311.

Volkanis, J.E. 1986. Structure and molecular genetics of complement proteins. The year in Immunol. **2**, 164.

Appendix 1

Collect Date	Animal Number	Sex of Animal	Size of Animal	Water Temp.
09/25/91	092591-1	F	2'5"	31^{0}C
09/25/91	092591-2	F	3'	31^{0}C
09/25/91	092591-3	F	2'8"	31^{0}C
10/18/91	101891-1	?	2'6"	26^{0}C
10/18/91	101891-2 TAG#186	M	2'6"	26^{0}C
10/18/91	101891-3 TAG#88	M	3'4"	26^{0}C
10/18/91	101891-5	?	2'6"	29^{0}C
10/18/91	101891-6	?	2'	26^{0}C
11/14/91	111491 TAG#186	M	2'6"	21^{0}C
01/09/92	TAG#88	M	3'4"	24^{0}C
01/09/92	TAG#186	M	2'6"	24^{0}C
01/09/92	TAG#238	F	2'2"	24^{0}C
01/09/92	TAG#241	F	2'	24^{0}C

Collect Date	Animal Number	Sex of Animal	Size of Animal	Water Temp.
01/09/92	TAG#242	M	30"	24^{0}C
01/09/92	TAG#463	M	4'4"	24^{0}C
01/09/92	TAG#244	F	3'	24^{0}C
01/09/92	TAG#248	M	4'	24^{0}C
02/27/92	TAG#453	M	27"	26^{0}C
02/27/92	TAG#466	M	31"	26^{0}C
02/27/92	TAG#230	M	53"	26^{0}C
02/27/92	TAG#241	F	30"	26^{0}C
02/27/92	TAG#465	F	28"	26^{0}C
02/27/92	TAG#455	M	28"	26^{0}C
02/27/92	TAG#477	F	40"	26^{0}C
02/27/92	TAG#489	F	36"	26^{0}C
03/19/92	TAG#246	M	4'	25^{0}C
03/19/92	TAG#463	M	4'4"	25^{0}C
03/19/92	TAG#452	F	34"	25^{0}C
03/19/92	TAG#489	F	36"	25^{0}C

Collect Date	Animal Number	Sex of Animal	Size of Animal	Water Temp.
03/19/92	TAG#238	F	34"	25^{0}C
03/19/92	TAG#242	M	30"	25^{0}C
04/30/92	TAG#456	M	6'	21^{0}C
04/30/92	TAG#248	M	3'5"	21^{0}C
04/30/92	TAG#489	F	3'5"	21^{0}C
04/30/92	TAG#463	M	4'	21^{0}C
04/30/92	TAG#457	F	3'	21^{0}C
04/30/92	TAG#186	M	2'6"	21^{0}C
04/30/92	TAG#238	F	3'	21^{0}C
04/30/92	TAG#88	M	3'4"	21^{0}C
04/30/92	TAG#242	M	2'6"	21^{0}C

Appendix 2

Sources of reagents and chemicals

Isotonic saline (0.15 M NaCl, ACS grade)	Scientific Products McGaw Park, IL
Dextrose, bacteriological grade	Difco Laboratories Detroit, MI
Barbital (Barbitone; 5,5-diethylbarbituric acid; Veronal) sodium salt	Sigma Chemical Co. St. Louis, MO
Barbital (Barbitone; 5,5-diethylbarbituric acid; Veronal)	
Magnesium chloride, hexahydrate ACS grade	
Calcium chloride, anhydrous approx. 97%	
Ethylendiaminetetra acetic acid disodium salt dihydrate	
Ethyleneglycol-bis-(beta-amino ethyl ether) N,N,N,N-tetra acetic acid approx. 97% anhydrous	

Zymosan A from *Saccharomyces cerevisiae*	
Lipopolysaccharide from *Salmonella typhosa*	
Inulin from dahlia tubers	
Cobra venom factor, *Naja naja kaouthia*	
Sodium chloride, ACS grade	Mallinckrodt Paris, KY

Appendix 3

Composition of EGTA-GVB-- and EDTA-GVB-- assay buffers

mM Chelator	ml of Stock Chelator	ml of 5 X Veronal	ml of 2% Gelatin
5	5	20	5
7.5	7.5	20	5
10	10	20	5
12.5	12.5	20	5
15	15	20	5
17.5	17.5	20	5
20	20	20	5
22.5	22.5	20	5
25	25	20	5
30	30	20	5
35	35	20	5
40	40	20	5
45	45	20	5
50	50	20	5

Appendix 4

Volume of nurse shark serum to chelator

ul of Nurse Shark Serum	**ul of Stock Chelator**	**Final Concentration of Chelator**
950	50	5
900	100	10
850	150	15
800	200	20
750	250	25

Appendix 5

Composition of 20 mM EGTA-GVB buffers used in magnesium reconstitution experiments

Final Mg++ Level	**ml of 5 X Veronal**	**ml of 2% Gelatin**	**ml of Stock $MgCl_2$**	**ml of Stock EGTA**
0 mM	20	5	0	20
1 mM	20	5	1	20
5 mM	20	5	5	20
10 mM	20	5	10	20
15 mM	20	5	15	20
20 mM	20	5	20	20

Appendix 6

Outline of protocol for activation of nurse shark serum by known activators of the mammalian alternative pathway

Final Activator Level	**ml of Shark Serum**	**ml of Stock Activator**	**ml of Buffer (DGVB++ or EGTA-Mg-GVB**
0 mg/ml	1	0	1
1 mg/ml	1	0.1	0.9
5 mg/ml	1	0.5	0.5
10 mg/ml	1	1	0

Appendix 7

Source and product description of primary and secondary antisera preparations for immunoblotting

Janssen Biochemica, distributed in US by Accurate

1. Rabbit antihuman C3b
   Protein conc. 24 mg/ml
   Antibody titre 0.15 mg/ml

2. Rabbit antihuman C4
   Protein conc. 45 mg/ml
   Antibody titre 0.46 mg/ml

3. Rabbit antihuman C5
   Protein conc. 28 mg/ml
   Antibody titre 0.18 mg/ml

4. Rabbit antihuman factor B
   Protein conc. 16 mg/ml
   Antibody titre 0.52 mg/ml

5. Rabbit antihuman factor H
   Protein conc. 23 mg/ml
   Antibody titre 1.45 mg/ml

Cedarlane, Hornby Ontario

1. Normal rabbit serum

Lampire biologicals, Pipersville, PA

1. Rabbit Gamma globulin fraction

   Protein conc. 20 mg/ml

2. Normal goat serum

Kallestad, Chaska MN

1. Goat anithuman alpha 2 macroglobulin

   Specific antibody conc. 5.22 mg/ml

2. Goat antihuman C3

   Total protein 126 mg/ml

   Specific antibody conc. 9.57 mg/ml

3. Goat antihuman C4

   Total protein 55.6 mg/ml

   Specific antibody conc. 6.5 mg/ml

Cappell, West Chester, PA

1. Goat antihuman C5

   Total protein 18 mg/ml

2. Goat antihuman C3

   Total protein 67.5 mg/ml

   Antibody protein 10 mg/ml

3. Rabbit antigoat IgG (whole molecule) peroxidase conjugated

BioRad, Richmond, CA

1. Goat antirabbit IgG (heavy and light chain specific) horse radish peroxidase conjugated