The role of humoral mediators in immunological reactions to streptokinase

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Declaration

These studies represent original work carried out by the author, and have not been submitted in any form to any other university. Where use has been made of material provided by others, due acknowledgement has been made.

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Publication and presentations

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5) Seminar, May 1995, University Department of Immunology, Glasgow, The role of humoral mediators in immunological reactions to streptokinase.

6) Immunology Colloquium, May 1995, Glasgow Western Infirmary, a) Levels of anti-streptokinase and anti-mycobacterial HSP65 (mHSP65) in patients with auto-immune diseases. b) Effects of erythrocytes on generation C3b-P and C3d, indicators of complement activation.

Abbreviations used in the text

Ab	antibody
Ag	antigen
AMI	acute myocardial infarction
ASK	neutralising anti-streptokinase antibodies
BSA	bovine serum albumin
C	Celsius
CD	cluster of differentiation
C1-INH	C1-inhibitor
CR1(CD35)	complement receptor for C3b
CV	coefficient of variation
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EGTA	1,2-di(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E	erythrocyte
E's	erythrocytes
Fc	crystallisabale fragment
FcμR	Fc receptor specific for IgG
Fab	antigen-binding site
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethaneesulfonic
HSP	Henoch-Schonlein pupura
HSPs	Heat shock proteins
IgA	immunoglobulin A
IC	immune complex
IgE	immunoglobulin E
IgG	immunoglobulin G
IEP	immunoelectrophoresis
IgM	immunoglobulin M
I.U.	international unit
mAb	monoclonal antibody
MAC	membrane attack complex (complement)

mg	milligram
mHSP65	mycobacterial heat shock protein (65kDa)
min	minutes
ml	millilitre
μg	microgram
μl	microlitre
MW	molecular weight
NIP	5-iodo-4-hydroxy 3-nitrophenacetyl
nm	nanometer
N.S.	not significant
OD	optical density
PBS	phosphate-buffered saline
PE	phycoerythrin
RA	rheumatoid arthritis
RF	rheumatoid factor
RBC	red blood cell
RIA	radioimmunoassay
RT	room temperature
r-tPA	recombinant tissue plasminogen activator
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SK	streptokinase
SLE	systemic lupus erythematosus
UV	ultraviolet

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Summary

Streptokinase is now a commonly administered thrombolytic agent in AMI patients. One of the main disadvantages of its administration is that as a foreign antigen it may provoke hypersensitivity reactions due to previous streptococcal infections, and pre-existing antibodies may reduce efficacy of treatment.

The aim of this study was to look at role of humoral immunity to streptokinase. To this end three areas were studied; 1) Specific anti-streptokinase antibodies .2) Role of complement and 3) Role of the immune complex removal.

ELISA's were developed to measure the levels of IgG, IgA, IgM, IgE and neutralising anti-streptokinase antibodies in a normal population. Only a small proportion of the normal individuals had elevated levels of anti-streptokinase antibodies (IgG 1.68%, IgA 2.7%, IgM 9.9% and neutralising anti-streptokinase antibodies 0.4%).

The levels of anti-streptokinase antibodies in 20 patients with AMI (10 patients treated with streptokinase and 10 with r-tPA) were compared to the normals. Administration of streptokinase in 10 patients studied, resulted in an immediate fall in level of anti-streptokinase antibodies and developed a specific response.

The levels of anti-streptokinase antibodies in patients treated with streptokinase and reperfused early were within the normal ranges, and levels rose in the late reperfusion or non reperfusion groups. Of the 10 patients given streptokinase, one patient with elevated levels of IgG and IgA developed serum sickness.

The levels of specific IgM anti-streptokinase antibodies in patients with rheumatoid arthritis were significantly elevated. This did not correlate with IgG anti-mycobacterium heat shock protein 65 (mHSP65). In patients with Henoch Schonlein Purpura there was a correlation between IgG anti-mHSP65 levels and IgG, IgA and neutralising anti-streptokinase antibodies levels.

The interactions of complement system and immune complexes was studied by measurement of complement activation products C1s:C1-INH, C3b-P and C5b-9

using modified techniques introduced by Auda et al 1990. An indirect ELISA for measuring serum C3d levels and a modified ELISA for detecting erythrocyte (E) with bound C3d were developed to evaluate the relationship of bound C3d and the levels of free complement components.

Patients treated with r-tPA did not generate complement activation products. All the ten patients administered streptokinase generated C1s:C1-INH and only one patient had increased levels of C3b-P and C5b-9. The extent of complement activation correlated with the pre-treatment levels of anti-streptokinase antibodies.

In vitro effects of the ICs on complement activation showed that the presence of erythrocytes did not modulate significantly C3d or C1s:C1-INH levels generated by the ICs, however, they exerted an influence on C3b-P through the actions of CR1.

The kinetics and dose response of streptokinase ICs, C3c and C3d binding to erythrocytes (E's) were studied by flow cytometry and its relationship with free complement activation was determined by measuring the free complement activation products in the accompanying supernatants. This study showed that the binding of ICs to E's depended significantly on the extent of complement activation ($p<0.001$) and the dose of ICs ($p<0.001$).

The clearance of ICs during streptokinase treatment was studied during a period of 30 minutes after streptokinase administration in 4 patients with AMI by detecting E bound ICs using FACscan analysis. Streptokinase ICs were detectable on E's in one of patients. This patient generated high levels of complement activation products which correlated with E's bound ICs ($p<0.001$), thus the clearance of streptokinase ICs depends on the extent of complement activation and the observed adverse reaction in patient CD (With elevated levels of IgA) is due to poor complement activation by the formed ICs.

Study of anti-streptokinase ICs and IgG interactions with E's revealed that they also bound to E's in absence of NHS. A haemagglutinin test among normal individuals and patient groups revealed that the of binding of rabbit IgG to human erythrocytes is not a non-specific adsorption. In addition, F(ab')2 fragment did not bind to the

E's as determined by FACScan analysis. Using flow cytometry human IgG (Fc fragment) inhibited the binding of rabbit IgG to human erythrocytes indicated that both rabbit IgG and Fc fragment of human IgG bind to the same sites on erythrocytes. Using a set of monoclonal anti-human FcγR antibodies, FcγRI (CD64) was detectable on erythrocytes.

The pattern of mouse IgG subclasses binding to human E's (IgG3 > IgG2b >IgG2a) was consistent with the pattern of CD16 (isoforms A and B) and CD64 (isoform A). These findings indicate the presence of FcγR on the erythrocytes, which may play an important role in handling of immune complexes.

Chapter 1

Introduction

1-1 Streptokinase isolation and structure. In 1933 Tillet and Garner were the first to observe that cultures of haemolytic streptococci could liquefy clotted human plasma. They separated 28 strains of haemolytic streptococci from patients who suffered from septicaemia, erysipelas, scarlet fever, acute tonsillitis, cellulitis, otitis media, empyema and 18 strains from animal sources. All human strains of haemolytic streptococci and 3 strains of animal haemolytic streptococci exhibited the biological activity of liquefying clotted plasma (Tillet & Garner 1933). This thrombolytic activity is now known to be due to the action of streptokinase.

Streptokinase is released during streptococcal infections and facilitates the dissolution of the clotted tissue in the streptococcal infected area, this in turn improves the bacteria diffusion from the site of origin (Guyton 1986).

Streptokinase was first prepared by alcoholic precipitation from extra-cellular products of haemolytic streptococci groups A and C (Garner & Tillet 1934, Weinstein 1953), and later streptokinase was purified by column chromatography, resulting in the determination of amino acid composition and molecular weight of this protein (Dillon & Wannamaker 1964, DeRenzo etal 1966). Group C haemolytic streptococci have been used as the preferred source of commercial streptokinase production since they produce the most active form of streptokinase and it also provide an easier preparation for purification (Christensen 1945).

Streptokinase has a molecular weight of 47-48 kD and consists of 415-440 amino acids (Morgan & Henschen 1969, Jackson & Tang 1982) (Table 1-1).

Unusually, in the structure of this single chain polypeptide, the amino acids cysteine and cystine are absent (DeRenzo et al 1966, Morgan & Henschen 1969, Brockway & Castellino 1974, Jackson & Tang 1982). There are however, 4

Amino acid	Number of amino acids residues
aspartic acid	41
aspargine	24
theonine	30
serine	25
glutamic acid	28
glutamine	16
proline	21
glysine	20
alanine	21
valine	23
methionine	4
isoleucine	23
leucine	40
tyrosine	22
phenylalanine	15
histidine	9
lysine	32
arginine	20
tryptophan	1
total residues	415

Table 1-1: Amino acids composition of the streptokinase molecule (Jackson & Tang 1982)

methionine residues hence cyanogen bromide cleavage, results in its breakdown into five fragments (Morgan & Henschen 1969, Bruserad et al 1992).

There are the two large regions of internal homology in streptokinase (N-terminal residues 1-173 and C-terminal residues 254-415), therefore the predicted secondary structure of streptokinase suggests that streptokinase contains two domains of similar tertiary structure (Jackson & Tang 1982).

The isoelectric point of the protein is reported to be between 4.7 and 5.2 (DeRenzo et al 1967, Einarsson et al 1979, Barth 1990) and alkaline treatment damages the biological activity of streptokinase by destruction of the amide groups of the protein (Dillon & Wannamaker 1964, Hugh et al 1965, Einarsson et al 1979).

1-2 Coagulation system. Several elements in blood and tissues contribute to the blood coagulation system. These are known as pro-coagulants and anti-coagulants. Anti-coagulants are normally dominant but damage to the endothelium of vessels or tissues initiates coagulation and results in clot formation. This can occur by two different pathways (Figure 1-1):

a) The intrinsic pathway is triggered when surfaces i.e. damaged vascular wall or foreign surface like a glass tube are exposed to factor XII (is also known as glass factor or contact factor). Healthy endothelium usually prevents the interaction between factor XII and collagen fibres in the vascular wall but damaged endothelium provokes the activation of pro-coagulants (e.g. the release of pro-coagulant cytokines, see 1-10).

b) The extrinsic pathway is triggered by cell injury and the release of a cell surface protein, tissue thromboplastin which in turn activates factor VII.

Activation of factors XII and VII (from the intrinsic and extrinsic pathways respectively) results in activation of factor X (thrombokinase). The activated form of factor X, Xa, by interacting with factor V, calcium and platelets, catalyses the

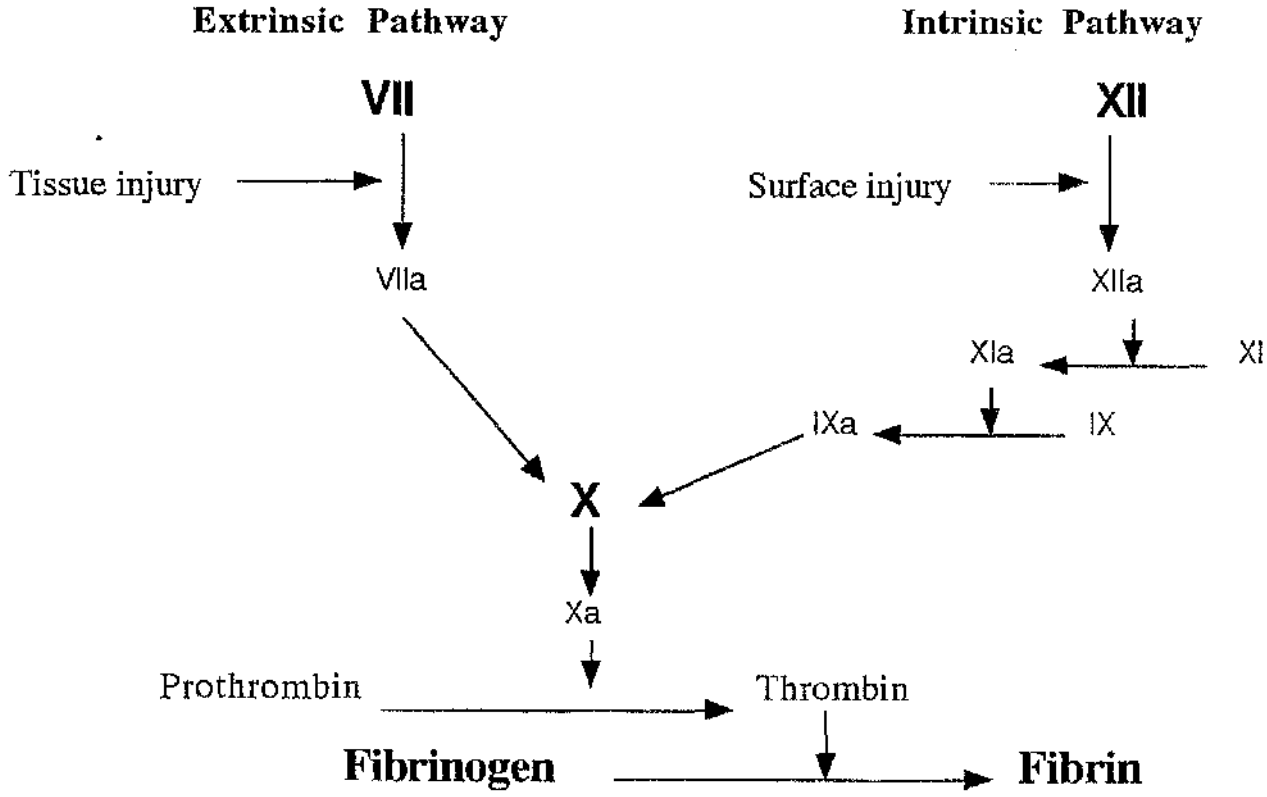


Figure 1-1:Schematic diagram of coagulation system pathways.

transformation of pro-thrombin to thrombin (Guython 1986, Ganong 1991). Calcium and phospholipids of aggregated platelets (platelet factor 3) are essential for the activation of coagulation pathways.

Thrombin is a serine protease and converts soluble fibrinogen (MW=340000 kD) to insoluble monomeric fibrin. Finally, the formation of a clot is completed when monomeric fibrin is polymerised and long fibrin threads are formed.

As mentioned before prevention of clot formation is to a large extent dependent on healthy vessels by preventing the contact of factor XII with the vascular wall and inhibition of intrinsic pathway activation. On the other hand, vascular endothelium also secretes a thrombin-binding protein (thrombomodulin) which converts thrombin to a protein C activator. Protein C is a natural anti-coagulant protein, inactivating factors V, VIII and enhancing the activation of plasminogen (Ganong 1991).

Anti-thrombin III and heparin are anti-coagulants that work in synergy. Anti-thrombin III, a protease inactivator, binds to thrombin and inactivates it. Heparin itself has little anti-coagulant effect but by combining with anti-thrombin III shows a significant thrombolytic activity. Mast cells around the connective tissues of capillary walls are the major source of heparin (Guython 1986).

Lysis of clot is initiated by the activation of the plasminogen (previously called pro-fibrinolysin) pathway. Plasminogen is a single chain protein with a molecular weight of 92000 kDa. This glycoprotein is a zymogen but when a clot is formed and a large amount of this inactive enzyme entrapped within the clot, the plasminogen activators can then initiate its activation. These activators can be, protein C (see above), activated factor XII, tissue plasminogen activator, urokinase and finally a foreign protein, streptokinase (1-3).

Plasminogen activators split the arginyl^{560}-valine^{561} bond of the plasminogen molecule resulting in formation of a two-chained plasmin molecule connected by a single disulphide bond (Bajaj & Castellino 1977, Fears & Smith 1985, Podlasek & McPherson 1989, Edelberg et al 1989). Plasmin is a potent serine protease and

digests fibrin and pro-coagulants (including fibrinogen, factor V, factor VIII, pro thrombin and factor XII) (Haire 1992).

A small amount of plasmin is produced in 24 hours in the body to remove tiny clots formed in the peripheral vessels. On the other hand, the production of plasmin is controlled by plasminogen inactivators to maintain low levels. α2 anti-plasmin is the major physiological inhibitor of plasminogen system and by binding to plasmin inactivates the enzyme (Guython 1986). Histidine-rich glycoprotein is also an inhibitor of plasminogen system, this inhibitor binds to plasminogen and protects it from the action of plasminogen activators (Mankuvad 1993).

1-3 Streptokinase and coagulation system. Since the 1930's the thrombolytic action of streptokinase has been studied (Tillet & Garner 1933). Milston in 1941 reported that human serum contains a factor (lysine-plasminogen) which is necessary for the thrombolytic activity of streptokinase. Christensen (1945) studied the effects of streptokinase on the lysis of fibrin, casein and gelatine and he showed that plasminogen, an inactive enzyme, is activated by streptokinase.

As mentioned streptokinase has a plasminogen-dependent thrombolytic activity. Native plasminogen (also known as Glu-plasminogen, with N-terminal glutamic acid) is the predominant form of plasminogen in the circulation. Streptokinase binds to a small region of the Glu-plasminogen that consists of amino acids 557 to 565 and this interaction results in formation of a one to one stoichiometric complex (Podlasek & McPherson 1989, Bajaj & Castellino 1977). The formed equimolar complex has the ability to hydrolyse the native plasminogen and to generate plasmin (Takada & Takada 1990). There is a high degree of homology between N-terminal region of streptokinase molecule (amino acids 1-253) and the serine proteases family (e.g. trypsin, Jackson &Tang 1982). Furthermore, the N-terminal region of streptokinase is an efficient binding site for the interaction of streptokinase and plasminogen (Reed et al 1993). On the other hand, C-terminal

residues of the streptokinase (amino acids 384-415) are not essential for the thrombolytic action of streptokinase (Jackson et al 1986).

The formation of clot itself enhances the thrombolytic effect of the streptokinase by two mechanisms. Firstly, the presence of fibrin is shown to increase the rate of the streptokinase-plasminogen complex formation (Fears et al 1985, Takada &Takada 1990) and secondly the complex of streptokinase-plasminogen binds to fibrin with a greater affinity than plasminogen alone (Cassel et al 1987).

The factors that prevent the thrombolytic effect of streptokinase have not been clearly identified. Lipoprotein-A has extensive homology with plasminogen but lacks the critical bond of Arg^{560}-Val^{561} that is required for the activity of plasminogen activators (see 1-2). Elevated levels of lipoprotein-a which have been shown to correlate with athrosclerotic lesions, inhibit the thrombolytic effect of streptokinase by competing with plasminogen for binding to streptokinase (Edelberg et al 1989). The presence of anti-streptokinase antibodies in circulation as a result of previous streptococcal infections or streptokinase therapy, may also prevent the therapeutic effect of the streptokinase (Massel et al 1991) (see 1-6).

1-4 Clinical indications of streptokinase administration. During the 1960's and the 1970's fibrinolytic therapy caused a 25% reduction in the mortality of patients with obstructed veins and arteries (Randomised trial of intravenous streptokinase 1988), subsequently fibrinolytic therapy became a common treatment for deep vein thrombosis (Walker et al 1973, Thayer 1981). Large-scale trials of treatment of acute myocardial infarction (AMI) in the 1980's, revealed the efficacy of thrombolytic therapy in AMI (Sharma 1982 & Sashara , GSSI-2 1990).

In the 1950's streptokinase became a known fibrinolytic agent for the treatment of a variety of diseases to remove clotted blood and purulent exudate. These included: empyema, post-operatively after pulmonary resection, constant pneumonia, hemothorax, suppurative extra-pulmonary tuberculosis, soft tissue infections, amoebic abscess, clearing the dead tissues (e.g. burns), resistant

infections of bones and joints, decubitus ulcers, pilonidal cysts (Beckman 1958, Goodman & Gilman 1975).

The results of angiographic patency studies in patients with AMI treated with streptokinase revealed a significant promotion of heart muscle reperfusion (53% Hogg et al 1990, 73% Anderson et al 1991, 77.5% Chouhan et al 1992), and the reduction of the mortality rate in these patients is now well known (White et al 1990, Munkvad 1993).

Different regimes of streptokinase administration for the treatment of AMI patients have been tested. The optimum regime is considered to be a large single bolus administered intravenously (usually 1200000 units) and this large dose is usually given during a period of one hour.

The clinical indications for streptokinase administration are summarised as followings: a) Acute myocardial infarction. b) Deep venous thrombosis. c) Pulmonary embolism. d) Occlusion of peripheral arteries. e) Central retinal venous or arterial thrombosis. f) Occlusion of shunts and intra-vascular or cavity catheters (Sharma & Sahara 1982).

1-5 Adverse reactions to streptokinase administration. Adverse reactions to streptokinase have been recorded since the advent of its use in the clinical settings. Reports come from both large-scale trials and from individual case reports. A diverse range of complications have been reported.

Many reports indicate the incidence of fever following streptokinase administration (Toty et al 1981, White et al 1990, Siebert et al 1992). Thayer (1981) reported 26.8% of a group of his patients (n=82) developed fever following streptokinase administration. Sharma & Sashara (1982) and Anderson et al (1991) reported a similar incident of fever (25%) following streptokinase administration (n=100 and 182 respectively). From these reports it can be assumed that at least one in every four patients treated with streptokinase may

experience fever, and the duration of the resulting fever can be several days (Beckman 1954).

Nausea and vomiting, headache, muscle pain, flushing and urticaria are mild, rare and usually transient complications to streptokinase administration (Thayer 1981, Sharma & Sashara 1982, Anderson et al 1991).

Hypotension and bradycardia are observed in over 7% of patients undergoing streptokinase therapy (**7.2%** GISSI-2 1990, **10%** ISSI-2 1988 and **8%** White et al 1991). Anderson et al 1991, reported a higher prevalence of hypotension and bradycardia due to streptokinase administration (46%). The discrepancy may be related to the unstable status of the AMI patients. The hypotension due to streptokinase administration usually occurs simultaneously with the peak of anaphylatoxin generation (Agostoni et all 1994a).

The possibility of bleeding as a complication of streptokinase administration has always been considered (Stehle & Schettler 1986, Chesterman 1992). Although the reports of bleeding following streptokinase administration are abundant (Rajadyalan et al 1992, Sharma & Sashara 1982, Hogg et al 1990, Califf et al 1992), results from large trials indicate that life-threatening bleeding is a rare complication (**0.9%** Thayer 1981, **0.3%** ISSI-2 1988, **0.57%** GISSI-2 1990, **0.8%** The international study group 1990).

The prevalence of stroke and cerebral haemorrhage is reported to be between 0.1% and 1% (ISSI-2 1988, GISSI-2 1990, The international study group 1990).

To date, only two reports of respiratory distress syndrome (ARDS) following streptokinase administration are documented (Kerstein & Adinolfi 1986, Siebert et al 1992). These patients developed an immediate pulmonary dysfunction after administration of streptokinase with a low arterial oxygen level and diffuse congestive atelectasis. Sallen et al (1983) have reported a case of liver function impairment following streptokinase administration. The patient showed an elevation in temperature and liver enzymes (SGOT, alkaline phosphatase) which

after discontinuing the administration of streptokinase returned to the normal levels.

Administration of streptokinase has been shown to provoke immunological reactions. Many descriptions of isolated allergic reactions have been described (Baungartner & Davis 1982, McGrath & Patterson1984, Schweitzer et al 1991, Bindels & Peters 1992). Studies of large patient groups have suggested that between 3% (Thayer 1981) and 18% (Jarvinen 7 et al 1978) of patients treated with streptokinase developed allergic reactions which necessitated discontinuation of its use. The reports describe two types of immunological reactions, immediate anaphylaxis and serum sickness.

Reports of anaphylaxis to streptokinase are rare and to date less than ten cases have been cited (Baugrtner & Davis 1982, Mc Grath & Patterson 1984, Bednayrzyk et al 1989, Callif et all 1992, Lee et al 1992, Hohage 1993). The results of large trials showed no anaphylactic reaction to streptokinase administration (Sharma & Sashara 1982, ISSI-2 1982).

The majority of reported allergic reactions to streptokinase administration were likened to serum sickness (Davies et al 1990, Siebert et al 1992, Totty et al 1981, Taylor et al 1984, Alexopoulos et al 1984, Murray et al 1986, Noel et al 1987, Dykewicz 1988, Davidson et al 1988, Schweitzer et al 1991, Siebert et al 1992, Bindles & Peters 1992, Lee et al 1992). These patients usually develop fever, arthralgia, maculopapular rash and renal function impairment (Alexopoulos et al 1984, Murray et al 1986, Schweitzer et al 1991, Bindles & Peters 1992). The nature of skin lesions has been investigated in many of the cases and reported to be like those observed in Henoch-Schonlein purpura (Siebert et al 1992, Zilliox et al 1988). The skin lesions exhibit an acute vasculitis and infiltration of neutrophils, mononuclear cells, and necrosis (Taylor et al 1984, Noel et al 1987, Ong et al 1988, Davidson et al 1988, Bindles & Peters 1992, Yvorra 1993).

A renal biopsy report from a patient who developed renal function impairment following streptokinase administration showed proliferative glomerulo-nephritis with polymorphonuclear infiltration (Murray et al 1986).

As described the current literature indicates that inflammatory reactions to immune complexes formed between streptokinase and pre-existing antibodies is more prevalent than anaphylaxis, following administration of streptokinase.

1-6 Anti-streptokinase antibodies. Tillet & Garner (1933) were the first to observe that plasma of patients who suffered from haemolytic streptococci infections when added to streptococcal cultures inhibited the lytic effect of these cultures. Since then there has been much interest in immune mediated reactions to streptokinase.

Streptokinase as described is a foreign protein released during haemolytic streptococci infections. Streptococcal infections has been shown to increase the levels of anti-streptokinase antibodies in patients sera (Anderson et al 1948, Flute 1973, Buchalter et al 1992). Furthermore, the streptokinases released from haemolytic streptococci exhibit different antigenic properties (Weinstein 1953, Dillon &Wannamaker 1965).

Elevated levels of anti-streptokinase antibodies is an important issue, as patients with high levels of anti-streptokinase antibodies could be at risk of developing hypersensitivity reactions when given streptokinase therapy (Lee & et al 1992). In spite of this fact, the role of anti-streptokinase antibodies in patients administered streptokinase is unclear (Moran et al 1984, McGrath et al 1985b, Dykewicz et al 1986, Sanjeev & Morris 1990, Lynch et al 1991, Rosenschein et al 1991).

It has been shown that elevated anti-streptokinase antibodies can prevent the thrombolytic effect of streptokinase (Grner & Tillet 1934, Lew et al 1984, Massel et al 1991, Bom et al 1993) but this elevation is not always necessarily accompanied by immunological reactions to streptokinase (Lew et al 1984), and

in contrast allergic reaction to streptokinase is not always associated with the failure of streptokinase treatment (White et al 1990).

Low levels of anti-streptokinase antibodies are presented in the normal population (Spottl & Mosumi 1974a, Moran et al 1984) and studies to date document varying levels of these pre-existing antibodies. James (1973) in an early study using a streptokinase resistance assay, showed that 15.6% of patients admitted for myocardial infarction or ischemic heart disease had a level of reactivity to streptokinase greater than the amount needed to neutralise completely 250 000 units of streptokinase. Moran et al (1984) quote a positively rate of 80% and Lynch et al (1991) 75% . However, their is no details of individuals who may be at risk when given streptokinase due to presence of high titres of specific antibodies.

During thrombolytic therapy the levels of anti-streptokinase antibodies are shown to decrease after streptokinase administration. Lynch et al (1991) showed IgG anti-streptokinase antibody levels drop at day 1 of administration and increase to pre-treatment levels by day 4 with no change in IgM levels. Fears et al (1992b) also showed that IgG anti-streptokinase levels dropped after streptokinase administration and by the day 4 of administration there was an elevation in specific antibodies to streptokinase, corresponding to an immune response to the streptokinase. Elevated levels of streptokinase antibodies persist for at least 6 month in patients treated with streptokinase (Sanjeev & Morris 1990, Lynch et al 1991, Massel et al 1991, Lee et al 1992, Buchalter et al 1992, Fears et al 1992b, Lee et al 1993, Elliot et al 1993).

There are a variety of methods for measuring anti-streptokinase antibodies: Kaplan (1944) presented the first quantitative method for measuring anti-streptokinase antibodies. This method is a neutralisation test, assessing the ability of the anti-streptokinase antibodies to prevent the lytic effect of streptokinase. In this method a constant amount of streptokinase is incubated with serial dilutions of serum and subsequently coagulation proteins (fibrinogen, plasminogen and

thrombin) are added. The last dilution of serum which prevents lysis of clot is considered as the serum titre of anti-streptokinase antibodies (Kaplan 1944, Anderson et al 1948, James 1973). This method has been commonly used to detect anti-streptokinase antibodies. Streptokinase resistance test has the same principles as neutralising anti-streptokinase assay but serial dilutions of streptokinase are added to the same sample and the lowest concentration of streptokinase that provides complete lysis is expressed as streptokinase resistance titre (Moran et al 1984).

In counter electrophoresis streptokinase is placed in the cathodic wells of an agarose plate (pH=8.6) and the serum is placed in the anodic wells and electrophoresis is performed for about 30 minutes to allow the formation of a precipitate line between antigen and antibody (Spottl & Kaiser 1974, Lew et al 1984).

Radial immunodiffusion is modified from that described by Mancini (1965), briefly agarose plates are prepared by mixing agarose and streptokinase 100 u/ml, serum to be tested for the presence of anti-streptokinase antibodies is loaded into wells and antibodies are allowed to diffuse for 24-36 hours (Spottl & Kaiser 1974).

Radio immunoassays have been used for measuring IgG and IgE anti-streptokinase antibodies using streptokinase radio-labelled with ^{125}I (Moran et al 1984, McGrath & Patterson 1984, Hoffmann et al 1988, Fear et al 1992a) and finally, enzyme linked immunoassay (ELISA) have been used. Indirect ELISA's have been employed by many investigators to measure different anti-streptokinase isotypes (McGrath & Patterson 1984, Dykewicz et al 1986, Rosenschein et al 1991, Buchalter et al 1992, Fears et al 1992b, Lee et al 1993, Elliot et al 1993 , Lynch et al 1994).

1-7 Complement system. The biological activity of the complement system in humoral immunity is now well established. These activities encompass promoting

phagocytosis, chemotaxis of neutrophils to sites of complement activation, anaphylatoxin generation, processing of immune complexes and regulation of antibody production. Some of these activities will be discussed in detail later.

The complement system consists of at least 30 serum and membrane glycoproteins which contribute about 10 % of the total serum proteins. Most of the serum proteins of this system are produced in the liver. The generation of biological activities of this system is achieved by a complex cascade reaction. The system can be divided into two early pathways (Classical and alternative pathways) and a late pathway (membrane attack complex MAC or terminal pathway).

Complement components of the classical and terminal pathways are designated with C (i.e. C1, C2 etc.). Components of alternative pathway are referred to by their individual names (i.e. Factor B, Factor P and Factor D). These are often abbreviated to letters (i.e. B, P, and D).

Cleaved fragments of complement components are showed by suffixed lower case letter (i.e. C3a, C4b). Activated C1 and its subcomponents have a bar over their name. Complement receptors are designated as CR1 to CR4 or as abbreviations of their trivial names.

The Classical pathway consists of C1, C4 and C2 (Figure 1-2). The first component, C1 is a macromolecule consisting of three proteins; one C1q molecule, two C1r and two C1s molecules. The complex of C1q:C1r:C1s is calcium dependent (Loos & Colombo 1993). C1q molecule is composed of 18 polypeptide chains (6A, 6B and 6C) each containsing a collagen-like N-terminus and a non-collagen C-terminus. The polypeptides (one A, one B and one C) are associated at their N-terminal region, the association of the 6 triple chains forms the common stem of C1q. The non-collagen region forms the six globular heads of the C1q molecule.

The globular heads of the C1q molecule impart specificity to the classical pathway by binding to the Fc regions of antibodies. The binding of at least two of

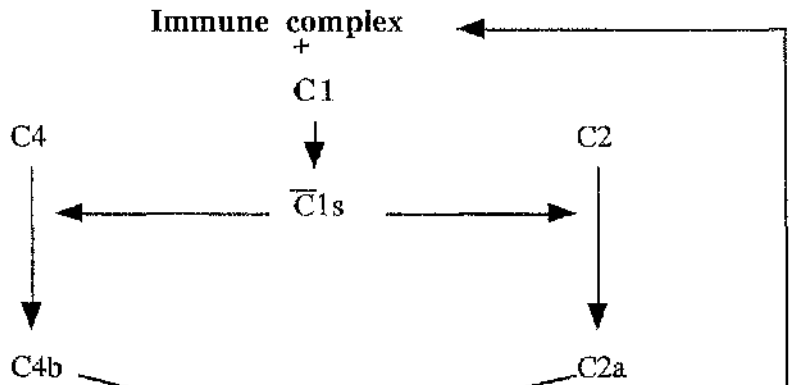


Figure 1-2: Schematic diagram of the classical pathway activation. Classical pathway activation is initiated by binding immune complexes and C1, this interaction activates C1s and activated C1s in turn cleaves C4 and C2 to form classical pathway C3 convertase (C4b 2a).

the globular heads of C1q to the Fc regions of antibodies (i.e. antibodies bound to antigens) is required for the conformational change within the C1q molecule and activation of the pro-enzyme C1r and C1s. Monomeric IgG has a weak affinity for C1q and can not activate C1.

In human the classical pathway is efficiently activated by IgG1, IgG3 and IgM. IgG2, IgG4 and IgA are poor activators of the classical pathway (Law & Reed 1988, Holme & Whaley 1989, Lucisano & Lanchmann 1991).

Activated $C^{-}1s$ has the enzymatic activity to cleave C2 and C4. The cleavage of C4 results in the formation of a small fragment C4a and a large fragment C4b. C4b does not contain an enzymatic site but through a newly exposed thiolester bond can bind to amino or hydroxyl groups on immune complexes or cell surfaces. C4b which does not bind to targets becomes inactivated through the interaction with H_20 (Holme & Whaley 1989).

If C2 binds to C4b in the presence of activated $C^{-}1s$ then C2 is split to C2a which is enzymaticaly active and C2b. The C4b2a complex is the classical pathway C3 convertase and cleaves C3 to C3a and C3b. C3 constitutes 1-2% of the total serum proteins and contains alpha (118 kD) and beta (75 kD) chains. The alpha chain contains the cleavage and binding sites for interaction of C3 with membranes and receptors. By the cleavage of C3, C3a is released into the fluid phase. C3b has a newly exposed thiolester bond (externalised) and like C4b, this allows the binding of the molecule to the cell surface or immune complexes, otherwise it becomes inactivated through the interaction with H_20. The labile thiolester bond is located in the C3d region on the alpha chain of C3.

The alternative pathway consist of Factors B, P, D and C3b (Figure 1-3). Activation of this pathway depends upon the structure of cell surfaces (i.e. micro-organisms) rather than antibodies. The pathway therefore has no immune specificity in its action. C3b is generated at a low rate in the fluid phase through nucleophilic attack and is called C3i or $C3(H_2O)$. C3b and C3i have a similar molecular conformation, when deposited on an activating surface, bind factor B in

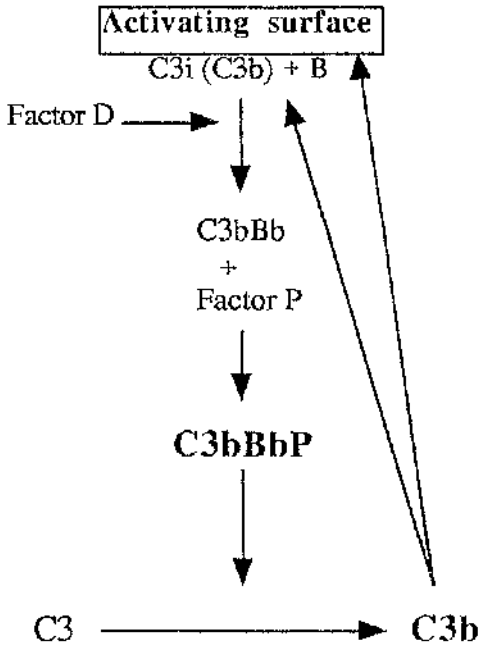


Figure 1- 3: Schematic diagram of the alternative pathway activation. Activating surfaces protect C3i (C3b) from the regulatory effects of factors I and H, and alternative pathway is initiated by theinteraction of C3i and factor B.

the presence of magnesium ions. Factor D cleaves bound factor B to enzymatically active Bb and releases Ba to the fluid phase (Holme & Whaley 1989).

The C3bBb complex which is formed in this way is very unstable and stabilised by the addition of properdin. This complex C3bBbP is the alternative pathway C3 convertase. As described the low rate production of C3i is amplified by activating surfaces forming a positive feed back cycle. The addition of an extra molecule of C3b to either C3 convertases changes their specificity from C3 to C5 convertases and allows the initiation of the terminal pathway. The cleavage of C5 molecule by C4b2a3b or C3bBbP3b results in formation C5a and C5b. C5a is released into the fluid phase and C5b remains attached to the C5 convertase via C3b. Cleavage of C5 is the last enzymatic step in the pathway.

C5b which is weakly attached to C3b in the C5 convertases, binds C6 and forms a stable C5b-6 complex. C5b-6 complex remains attached to the convertase until the binding of C7 and formation of C5b6-7 complex. The C5b6-7 complex is released into the fluid phases. If the C5b6-7 complex after release from C5 convertase is exposed to a membrane then it can bind to the membrane otherwise rapid inactivation occurs. The bound C5b6-7 complex to lipid bilayer is extremely stable and upon binding of C8, results in formation of small pores in the bilayer. Finally binding of 1 to 28 molecules of C9 to the C5b-8 complex enhances the lytic effect of the complex on the target cell (Whaley et al 1993).

The complement system has the potential to inflict cellular damage and is therefore controlled by groups of inhibitors, these are both serum and cellular in origin.

C1-inhibitor (C1-INH) is a glycoprotein and binds covalently to activated C1r and C1s, and dissociates them from the C1q. This binding results in C1r:C1-INH:C1s:C1-INH complexes and C1q which remains attached to the immunoglobulin (Loos & Colombo 1993). This controls initial activation of the classical pathway by preventing the enzymatic action of C1r and C1s.

Factor I degrades free or bound C3b to iC3b, therefore controls the amount of generated C3b, the formation of the alternative C3 convertase (C3bBbP) and C5 convertases. The further degradation of iC3b to C3c and C3dg is also through the function of Factor I and possibly plasmin (Law & Reed 1988).

Factor H binds to C3b and prevents the interaction of C3b and Factor B, accelerates the decay of the alternative pathway C3 convertase, the C5 convertase and also acts a as cofactor to Factor I. Factor H also shown to bind to C1 but the biological importance of this interaction is not clear (Holme et al 1992a).

Membrane cofactor protein (MCP) and CR1 (CD35) act as cofactors to Factor I to cleave C3b.

C4 binding protein (C4bp) and decay-accelerating factor (DAF, membrane bound) compete with C2a for binding to C4b and prevent the formation of the classical pathway C3 convertase. C4bp also act as a cofactor for Factor I to degrade C4b to C4c and C4d.

As mentioned, in the fluid phase the production of C4b and C3b which are important in the formation of C3 and C5 convertases, is controlled by the interaction of their thiolester bond with H_20.

The host cells are protected from the potential damage of MAC formation by many plasma and membrane proteins. Lipoproteins, anti-thrombin III and C8 regulate the formation of MAC. CD59 (protectin) and homologous restriction factor (HRF) are expressed on the surface of many cells, and bind to C8 and C9 preventing membrane insertion. S-protein binds to the C5b6-7 complex to prevent the binding of the complex to cell surface.

The biological activities of the complement system integrate with other aspects of the immune system to maintain homeostasis. The binding of C3b to the surface of a pathogen which initiated the activation of complement system is a major effect of complement activation and is called opsonization. Opsonised foreign particles can trigger phagocytosis. As described the lytic effect of complement is by the formation of MAC and is effective against the gram negative infections.

Anaphylatoxins C3a, C4a and C5a are released to the fluid phase after the cleavage of C5, C4 and C5 respectively and participate in the inflammation process. The anaphylatoxins trigger mast cells degranulation, increase vascular permeability and result in smooth muscle contraction. C5a is a powerful chemotactic factor and also enhances cell adherence, oxygen radical production and degranulation of neutrophils and monocytes. Anaphylatoxins are inactivated by carboxy peptidase N (Law & Reed 1988). The complement system has also a major role in prevention of immune complexes deposition in tissues.

1-8 Immune complexes and complement system. Interaction of antigen and antibody to form immune complexes is an important humoral defence mechanism, resulting in neutralising and elimination of the foreign substance. Complement plays an important role in the processing of these immune complexes.

When the reaction between antigen and antibody takes place, immune complexes of various sizes are formed and initial precipitation due to Fc-Fc reaction occurs (Shifferli & Taylor 1986). This reaction (immediate aggregation) together with F(ab')2 : antigen reaction initiates lattice formation. Large immune complexes are less soluble and can aggregate, and therefore tend to deposit in tissues. Immune complexes when deposited in tissue outside the reticuloendothelial system can provoke inflammatory reactions and damage to the tissues. The structure of immune complexes can influence their destination and this is determined by several factors. The size of immune complexes depends on the nature and amount of antigen, the antibody response, the ratio of antigen and antibody and finally the effect of complement on the processing the immune complexes.

The complement system protects the host from immune complex deposition, by preventing immune complex precipitation and promoting clearance of immune complexes.

Inhibition of immune precipitation (IIP) through binding of C1, C4b and C3b to the immune complex is an essential step for the processing the immune

complexes (ICs). The initial binding of C1, delays the immune aggregation during the initial minutes of immune complex formation. C1 reduces the immediate interaction of Fc-Fc of the immune complexes. The classical pathway activation by immune complexes results in C4b and C3b formation. C3b binds covalently to the immune complexes and inhibits their precipitation. C3b incorporation is the critical step of IIP. Activation of the alternative and terminal pathways are not vital in IIP mechanisms (Naama et al 1985, Holme et al 1992b). The isotype of antibody incorporated into the immune complex is important in initiating the activation of complement and IIP mechanisms (Johnson et al 1987). As described immune complexes formed with IgA cannot activate the complement system (Lucisano & Lanchmann 1991). IgM rheumatoid factor interferes with the IIP since it can interact with the Fc of different IgG (Whaley et al 1993).

If the IIP mechanisms fail to process the immune complexes appropriately, insoluble immune complexes are formed and precipitated. Precipitated immune aggregates can be solubilised by the complement system. Solubilization (SOL) of these complexes is by the incorporation of C3b into the preformed lattice, this reduces the forces between antigen and antibody rendering the complex more soluble. The classical pathway has importance role in accelerating deposition of C3b on ICs during the early step of solubilization (Naama et al 1985, Schifferli et al 1986c, Holme et al 1992b). Furthermore, it shown that the extent of solubilization mechanisms depend on the affinity of antibody incorporated into the immune complex (Johnson et al 1987) and even may fail due to covalent binding of antigen and antibody through the antigen binding site (Whaley et al 1989).

Immune complexes coated with C3b and its breakdown fragments have the capacity to bind to complement receptors. Predominant in the circulation are CR1 receptors on the erythrocytes. The binding of immune complexes to erythrocytes

is through C3b-CR1 interactions and influenced by the size, nature of immune complexes, and isotype of antibodies.

CR1 is a membrane glycoprotein which has 4 allotypes with molecular weights of 160kD, 190kD, 220kD and 250 kD. The number of receptor sites per erythrocyte is 200-1200, and is influenced by genetic and environment factors (Holme et al 1986, Fyfe et al 1987). The binding of the immune complexes to erythrocytes correlates with number of CR1 on the erythrocytes. The predominant ligand for CR1 is polymeric C3b, however, monomeric C3b or C4b and iC3b can also bind to CR1 with a lower affinity.

CR1 on erythrocytes is distributed in small clusters (approximately 8-20 clusters / erythrocyte), the clusters allow a better binding to multivalent opsonised immune complexes than other cells which possess CR1.

Incorporation of complement components in the immune complexes prevents the immune complexes from deposition in tissues and most of the immune complexes bound to the erythrocytes can be removed from circulation and disposed in the liver and spleen. The mechanisms involved in the removal of immune complexes from the circulation is not fully understood. In vitro studies show that by blocking Fc receptor in the reticuloendothelial cells, the immune complexes remain attached to the erythrocytes (Halma et al 1992). The presence of CR3 receptor on the Kupffer cells with a high affinity for binding to iC3b may also facilitate the release of immune complexes from the erythrocytes. Factor I accelerates the release immune complexes from erythrocytes by degradation of C3b to C3dg which has no affinity for CR1 (Yokoyama & Waxman 1994).

1-9 Fc Receptors and immune complexes. The Fc domain of antibody has an important role in the immune system either by activating classical pathway of complement system or by binding to cell surface receptors and triggering variety of cells activation (Revillard 1981). The CH2 domain near the hinge in IgG is the binding site for C1q and Fc receptors, furthermore, the differences between IgG

subclasses in triggering biological functions is related to difference of this region (Brekke & Sandile 1995).

Fc receptors are a family of glycoproteins with specificity for different immunoglobulin isotypes, they are expressed on various subsets of white blood cells and bind to native immunoglobulins or complexed immunoglobulins (immune complexes, aggregated antibodies).

Three distinct Fc gamma receptors (FcγR) with extra-cellular immunoglobulin-like domain, can interact with IgG. The affinity of IgG subclasses for binding to these receptors is not the same (Table 1-2).

Fcγ RI (CD 64) is a high affinity receptor for monomeric IgG and it is expressed on monocytes and naive macrophages. FcγRI is effective in killing the IgG-opsonised cells, it also regulates the IgG catabolism by endocytosis and degradation of the IgG molecule (Ravetch 1994).

FcγRII (CD 32) is a low affinity receptor for IgG and is expressed in two isoforms: FcγRIIA is presented on neutrophils, FcγRIIB is presented on B cells. The two isoforms of FcγRII have different cytoplasmic domains and this results in differences in signal transduction. Binding the cross-linked Fc of immune complexes or aggregated IgG to phagocytes bearing FcγRIIA results in internalisation but the binding the cross-linked Fc to B cells bearing FcγRIIB down regulates the antibodies production (Ravetch 1994).

FcγRIII (CD16) is also a low affinity receptor for IgG and is expressed on the macrophages, polymorphonuclear leucocytes (neutrophils and eosinophils) and NK cells and is responsible for mediating ADCC and the clearance of immune complexes from circulation.

Although activation of complement is essential for binding of immune complexes to the erythrocytes, there is evidence that aggregated IgG and immune complexes bind to human erythrocytes in the absence of complement mediated mechanisms (Hajos et al 1978, Virella & Sherwood 1983). The mechanism of non-complement mediated adherence of ICs to erythrocytes has not been fully investigated. Two

Receptor	Human IgG subclass	Mouse IgG subclass
FcγRI (CD64) Isoform A	IgG3>IgG1>IgG4>>>IgG2	IgG2a=IgG3>>>IgG1,IgG2b
FcγRII (CD32) isoform A	IgG3>IgG1>IgG2>>IgG4	IgG2a=IgG2b>>IgG1
FcγRII (CD32) isoform B	IgG3>IgG1>IgG4>IgG2	IgG2a=IgG2b>>IgG1
FcγRII (CD32) isoform C	ND	ND
FcγRIII (CD 16) isoform B	IgG3=IgG1>>>IgG2,IgG4	IgG2a=IgG3>>IgG2b,IgG1

Table 1-2: Affinity of human and mouse IgG subclasses for binding to human Fc gamma receptors (FcγR).

mechanisms have been suggested for this binding; Firstly, the presence of Fc receptor on erythrocytes may be responsible for the non-complement binding of ICs (Virella & Sherwood 1983) since treating the erythrocytes with Fc fragment inhibits this binding (Hajos et al 1978). Secondly, the IgG binds to erythrocytes after alterations in their membrane by oxygen radicals or proteolytic enzymes (Weiss & Mutaaugh 1992).

1-10 Streptokinase and complement system. Before the 1990's the majority of studies on immune mediated reactions to streptokinase were related to anti-streptokinase antibodies. Spotll (1974)a in an early study showed the complement system involvement during the administration of streptokinase. In the 1990's, as the administration of streptokinase has become a common clinical procedure, these studies have improved to the extent of assessing the complement system fluctuations during streptokinase treatment (Davies et al 1990, Hargreaves et al 1992, Freydottir et al 1993, Lynch et al 1993, Frangi et al 1994, Agostoni 1994).
Streptokinase activates complement through two mechanisms: a) Immune complex mediated complement activation is initiated by interaction of pre-existing antibodies and administered streptokinase which results in formation of immune complexes and classical pathway activation. By studying the extent of this activation, the highest levels of complement activation were observed in individuals who had elevated levels of anti-streptokinase antibodies (Freydottir et al 1993, Lynch et al 1993).
b) Activation of complement pathways can occur by plasmin. As described streptokinase activates plasminogen to generate plasmin which in turn dissolves fibrin (Takada &Takada 1990). On the other hand, plasmin, like trypsin, can activate the classical pathway in absence of antibodies (or independent of C1q) through the direct activation of C1s (Gewurz et al 1993, Munkvad 1993). It has also been shown that alternative pathway is activated by plasmin, since plasmin can replace Factor D to degrade Factor B and generate the alternative pathway C3

convertase (Munkvad 1993, Yamouchi et al 1994). Administration of streptokinase in patients with acute myocardial infarction may also result in formation of membrane attack complexes (Agostoni et all 1994b).

Complement system itself can have direct effects on the coagulation system. Complement activation provokes the release of pro-coagulant cytokines (interleukin-1 and tumour necrosis factor) from the leukocytes and influences the endothelial dependent coagulation (Munkvad 1993). C1 inhibitor like $\alpha 2$ anti-plasmin, by binding to plasmin can inhibit the proteolytic activity of plasmin (Levi et al 1993, Cugno et al 1993). Activated Factor B is able to convert the plasminogen to plasmin (Munkvad 1993) (Figure 1-4). C7 the component of terminal pathway, binds to plasmin and accelerates the activation of plasmingen by t-PA and also protects the plasmin from the inhibitory effect of $\alpha 2$ anti-plasmin (Reinartz et al 1995).

Streptokinase is a potent thrombolytic agent and is used in a variety of diseases in particular patients with acute myocardial infarction (AMI). The administration of streptokinase in patients suffering from AMI results in heart muscle reperfusion and reduces the mortality rate in these patients. The presence of serum anti-streptokinase antibodies may reduce the efficacy of streptokinase and provoke the immunological reactions, the mechanisms of which have not fully investigated (Lew et al 1984, Gemmil et al 1990, Hoffmann et al 1988, Fears et al 1992a). As reviewed from the literature it is difficult to predict the role of these antibodies in the clinical out-come of streptokinase administration. However, to ensure that the circulating anti-streptokinase antibodies would not prevent the effect of the administered streptokinase, a large single dose is given intravenously (usually 1200000 units) during a period of one hour (Crossland 1980).

The cost of alternative thrombolytic agents (r-tPA) and the efficacy of streptokinase have made this foreign protein a commonly used thrombolytic agent. However, there are associated problems with the administration of

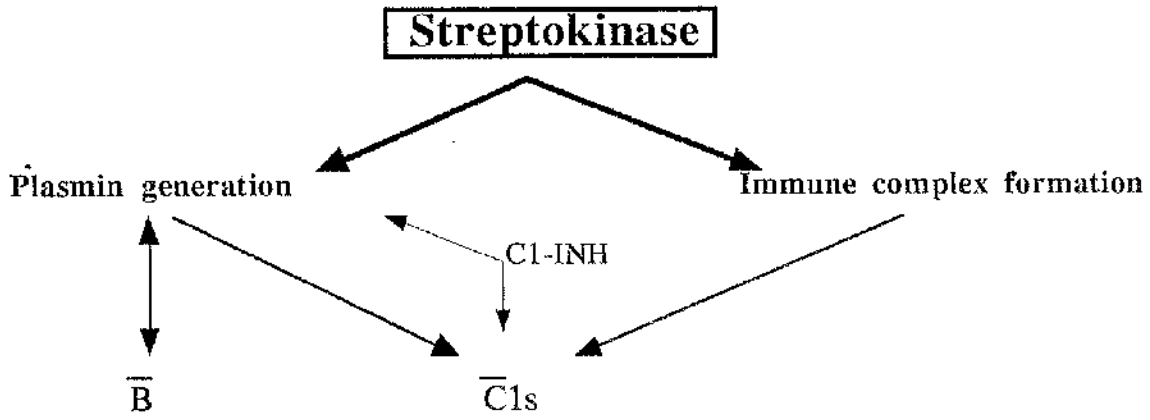


Figure 1- 4: Schematic diagram of activation of complement system by streptokinase. The activation of complement system by streptokinase is through immune complex formation and plasminogen activation.

streptokinase, firstly the factors that are responsible for the failure of streptokinase treatment are not fully understood, secondly the recognition of individuals who are at risk of developing hypersensitivity reactions when given streptokinase is difficult. Susceptibility to immunological adverse reactions to administered streptokinase is whether high streptokinase antibodies levels and other factors such as isotype specific response to streptokinase in association with appropriate complement activation and recruitment of other biological effector systems. The aim of this study was to look at role of humoral immunity to streptokinase administration. To this end three areas were studied; 1) Anti-streptokinase levels. 2) Effects of complement. 3) Role of the immune complex removal.

Chapter 2

Materials and General Methods

Introduction. This chapter details materials and general methods used. Subsequent chapters describe where appropriate more specific materials and methods.

2-1 Materials. With the exception of those chemicals listed below chemicals were of Analar quality from Merck Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leics.

Materials obtained from Sigma chemical Co. Ltd., Fancy Road, Poole, were:

Agarose (fraction V)

Ammonium per-sulphate

a-D (+) Melibiose

Biotin N-hydroxy-succinimide ester

Bovine serum albumin (type II)

Bovine thyroglobulin

Bromophenyl blue

Caprylic acid (octanoic acid)

Coomassie brilliant blue R

Horseradish peroxidase (HRP) conjugated goat anti-human IgA

HRP conjugated goat anti-human IgE

HRP conjugated goat anti-human IgG

HRP conjugated goat anti-human IgM

High molecular weight markers for gel electrophoresis (contains: carbonic anhydrase, egg albumin, bovine albumin, phosphorylase, β-galactosidase, molecular weights between 29 and 116 kD)

Human serum albumin

Human IgG subclasses (IgG1, IgG2, IgG3 and IgG4)

Isopropanol

Mouse IgG subclasses (IgG1, IgG2a, IgG2b and IgG3)

N'N-dimethyl formamide

N-Hydroxysuccinimidobiotin

N,N,N',N'-Tetramethylenediamine (TEMED)

O-phenylenediamine (OPD)

Pepsin (from procine stomach mucosa)

Plasmin (from human plasma)

Polyoxenthylen sorbitan monolaurate (Tween 20)

Protamine conjugated to Sepharose (4 % beaded agarose)

Sodium azide

Sodium dodecyl (lauryl) sulphate (SDS)

Materials obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, were:

Sheep anti-human whole serum

Sheep anti-human IgA

Sheep anti-human IgG

Sheep anti-human IgM

Mouse monoclonal anti-human AFP

Fluorescein isothiocyanate (FITC) conjugated sheep anti-human IgG

Donkey anti-rabbit IgG

FITC conjugated donkey anti-rabbit IgG

HRP conjugated donkey anti-rabbit IgG

FITC conjugated sheep anti-mouse IgG

Materials obtained from Atlantic Antibodies (INCSTAR), P.O. Box 285, 1951, North Western Avenue, Stillwater, USA, were:

Goat anti-human C3 (IgG fraction)

Goat anti-human Properdin (IgG fraction)

Goat anti-human Properdin factor B (IgG fraction)

Goat anti-human C1 (esterase) inhibitor (IgG fraction)

Goat anti-human C1s (IgG fraction)

Goat anti-human serum albumin (IgG fraction)

FITC conjugated goat anti-human properdin (IgG fraction)

FITC conjugated goat anti-human IgG (Fc fragment specific)

Materials obtained from Dako Ltd., 22 The Arcade, The Octagon, High Wycombe, Bucks, were:

Mouse monoclonal anti-human CR1 (IgG1, kappa)

Rabbit anti-human complement C3d

FITC conjugated rabbit anti-human complement C3d

FITC conjugated F(ab')2 fragment of rabbit anti-mouse immunoglobulin

Materials obtained from Cappel Laboratories, Cochramville PA 19330, USA were:

Sheep anti-rabbit IgG (Fab')2 fragment

Human IgG Fc fragment

Rabbit IgG Fc fragment

Materials obtained from Behring-Hoechst, Salisbury Road, Hounslow, were:

FITC conjugated rabbit anti-Human C3c

Streptase (Streptokinase)

Materials obtained from Difco Laboratories Ltd., P.O.Box 14B, Central Avenue, East Molesey, Surrey, were:

Freund's complete adjuvant

Freund's incomplete adjuvant

Materials obtained from Pharmacia LKB Biotechnology, Davy Avenue, Milton Keynes, were:

Protein G conjugated to Sepharose-B4 fast flow

Sepharose-4B-cyanogen bromide activated (CNBr)

Cellophane membranes

Materials obtained from The Binding Site Ltd., Birmingham Research Park, Vincent Drive, Birmingham, were:

Sheep anti-rabbit IgG (Fab')2 fragment

Materials obtained from Whatman Ltd, Maidstone, were:

Diethylaminoethyl celloluse (DE52)

pH indicator paper

Monoclonal anti-human FcγR antibodies and aggregated human IgG1 and IgG3 were kind gifts from Dr.G.Sandilands, Department of Pathology, Western Infirmary, Glasgow. The antibodies anti-human FcγR are available from Scrotec Ltd, 22 Bankside, Station Approach, Kidlington, Oxford,OX5 1JE.

Mouse monoclonal anti-human CD64 (FcγRI) (10.1)

Mouse monoclonal anti-human CD32 (FcγRII) (AT 10)

Mouse monoclonal anti-human CD16 (FcγRIII) (Leu-11a)

Sources of following materials were:

Actilyse (recombinant tissue plasminogen activator, r-tPA): Boehringer Ingelheim Ltd., Bracknell, Berkshire.

Donkey anti-rabbit IgG: Wellcome Reagents Ltd, Beckman, England.

ASK titration kit (Anti-Streptokinase): Bio-Merieux Ltd., Graften House, Graftenway, Basingstoke, Hampshire.

Rabbit anti-bovine thyroglobulin serum: was a kind gift of Dr.E.Holme, Department of Immunology, Western Infirmary, Glasgow.

Membrane peroxidase substrate tetramethylene blue (TMB), phosphatase substrate (BCIP/NBT): Kirkegaard & Perry Laboratories Inc., 2 Cessna, Gaitherburg, Maryland, USA.

Dialysis tubing: Medical International Ltd. 239 Liverpool Road, London.

Ethanol: James Burrrough, 60 Montford Place, London.

Falcon round-bottomed tubes (5ml): Becton Dickson, 2 Bridge Water lane, Lincoln Park.

Horse radish peroxidase Avidin-D, Alkaline phosphatase Avidin-D: Vector Laboratories Inc., 30 Ingold Road, Burlingame, USA.

Heparin sodium: Pharmacy, Western Infirmary, Glasgow.

Immulon type 4 ELISA plates: Dynatech Laboratories Inc. 14340, Sullyfield Circle, Chanlilly, Virginia.

Jacalin conjugated to agarose: Pierce, Rockford, Illinois, P.O.Box 117, USA.

Methanol: May and Baker Ltd., Liverpool Road, Bator Moss, Eccles, Manchester.

Nitro-cellulose transfer membrane: Amersham International Plc, Little Chalfont, Buckinghamshire.

Round-bottomed microplates: Greiner Labs Ltd., Station Road, Cambridge.

Recombinant mycobacterial mHSP$_{65}$ (mycobacterial bovis BCG): Dr.J.D.A.Van Embden, National Institute of Public Health and Environment, Biltohoven, Netherlands.

2-2 Buffers. De-ionised water was used to make up all buffers and for methods employing water.

Phosphate buffered saline (PBS) 0.15M

8g sodium chloride (NaCl), 1.2g dipotassium hydrogen orthophosphate (K_2HPO_4), 0.34g potassium dihydrogen orthophosphate (KH_2PO_4), were dissolved in 800 ml of water and made up to a total volume of one litre.

Twenty times strength phosphate buffered saline (PBS X 20)

160g NaCl, 24.2g K_2HPO_4 and 6.8g KH_2PO_4 were dissolved in 800ml water and final volume was made up to one litre.

10 % Gelatin

Ten grams of gelatin was dissolved in 80mls of boiling water and the final volume was made up to 100ml, after being autoclaved the gelatin was aliquoted and stored at 4°C.

ELISA coating buffer (pH 9.60)

1.58g sodium carbonate (Na_2CO_3) and 2.92g sodium hydrogen carbonate ($NaHCO_3$) were dissolved in 800ml water and total volume was made up to one litre.

ELISA blocking buffer

To 500ml of PBS, 5ml of 10 % gelatin was added to give a final concentration of 0.01% of gelatin.

ELISA wash buffer

1ml of Tween 20 was added to 2 litres of PBS to give a 0.05% solution.

0.2M citric acid

0.2M citric acid was made by dissolving 42g of citric acid in 800 mls of water and total volume was made up to 1 litre with water.

ELISA enzyme substrate

To 25 ml of 0.2M citric acid, concentrated KOH was added till the pH reached 4.5-5.0. The total volume was then made up to 50 ml with water, 0.025g O-phenylenediamine (OPD) and 0.025ml hydrogen peroxide (H_2O_2) were added. The substrate was used within the 15 minutes of adding the H_2O_2.

4N H_2SO_4 was made by adding 100mL of concentrated sulphuric acid gradually and cautiously to 800 mls of water and the total volume was made up to 1 litre

0.086M isotonic EDTA

197.7ml of 0.3M NaOH (12g of NaOH/1L water) was added to 500 ml of 0.12M EDTA (44.67g of EDTA/1L water) to give a final pH of between 7.2 and 7.6.

0.02M isotonic EDTA

23ml 0.086 isotonic EDTA was diluted to a final volume of 100ml.

0.1M Mg-EGTA

38.04g of EGTA was dissolved in 500ml water and concentrated NaOH was added to give a pH of 7.4. The final volume then was made up to one litre with water. To 10 ml of 0.1M EGTA, 7ml of 0.1M MgCl2 (20.33g of Magnesium chloride/1L water) was added before use.

2-3 Measurement of pH and conductivity:

pH: pH was measured using a Russell pH meter model 640 or Whatman pH indicator papers.

Conductivity: The conductivity of samples were measured at 0°C using a conductivity meter model CD M3, Radiometer, Copenhagen.

2-4 Measurement of protein concentration:

The absorbency of samples at 280 nm was measured using a Beckman spectrophotometer (model DU-64). The buffers in which the samples were suspended was used to zero the spectrophotometer.

2-5 Preparation of agarose gel:

5g agarose was added to 400ml hot barbitone buffer (2-9) and the final volume was made up to 500ml (1%). 15g polyethylene glycol (PEG, molecular weight 4000) was then added. When dissolved, the agarose was aliquoted and stored at 4°C until required.

2-6 Preparation of plates for methods employing agarose:

Gel-bond was used as a mount for agarose. Films were cut approximately to 8.5 X 8.5 cm and supported on a glass plate on a levelling table before application of agarose. Ten ml of agarose (see 2-5) was poured on the hydrophilic surface of the gel-bond and allowed to cool for half an hour before the wells or troughs were cut.

2-7 Double immunodiffusion (Ouchterlony technique, Ouchterlony 1958):

This technique is employed to identify antiserum specificity to antigen. Holes were punched in agarose gel plates (see 2-6) in a pattern consisting of central well surrounded by at least 5 equally spaced peripheral wells.

The inner well was filled with antigen and the outer wells were filled with doubling dilutions of antiserum. The plates were left a few minutes to allow the diffusion of antigen and antibody into the agarose and then they were left overnight at room temperature in a humidified chamber to allow the antibody and antigen to diffuse towards each other. When diffusion was complete the presence or absence of precipitation lines between antigen and antibody was recorded or plates were fixed and stained as described in 2-10 (Figure 2-3).

2-8 Single radial immunodiffusion (Mancini technique, Mancini 1965):

In this technique antigen is loaded in wells of an agarose gel pre-mixed with antibody. Antigen diffuses into the gel and combines with the antibody in the gel and at equivalence point an immunoprecipitin ring is formed around the well. This technique was used to measure the concentration of serum albumin. To three mls of agarose (see 2-5) at 56°C, 0.15ml of rabbit IgG anti-human albumin (47 mg/ml) was added. After mixing, the agarose was poured into 5cm petri dishes and allowed to cool. Ten wells were punched in each plate, which were filled with normal human serum (NHS) double diluted from 1/32 to 1/256 to allow the construction of a standard curve. Samples were pre-diluted 1/60, before application to individual wells of the plate. The plates were left overnight at room temperature in a humidified chamber. The diameters of precipitin rings were measured and recorded. A standard curve was constructed and from this the albumin concentrations in the unknown samples were determined (Figure 2-1).

2-9 Immunoelectrophoresis (IEP):

IEP is a combination of electrophoretic separation, diffusion and immunoprecipitation of proteins.

Barbitone buffer (for electrophoresis, pH 8.60): Barbitone buffer was made by dissolving 9.21g barbitone and 51.54g sodium barbitone separately in 500ml water then mixed in a final volume of 5 litres with water, 5g Sodium azide was added to this solution.

Agar plate:(size 8.5 X 8.5 cm, see 2-6)

Using a template a series of wells were cut in agar plate near to the anode and troughs were cut between the wells. The wells were filled with the test antigen on a levelling table and allowed to diffuse into the agarose for a few minutes. Approximately 150 mls barbitone buffer pH 8.6 was poured in each side of the electrophoresis apparatus and the plate was put on wicks (pre-soaked in barbitone buffer) and subjected to electrophoresis for 2 to 3 hours at 40 mA. The extent of

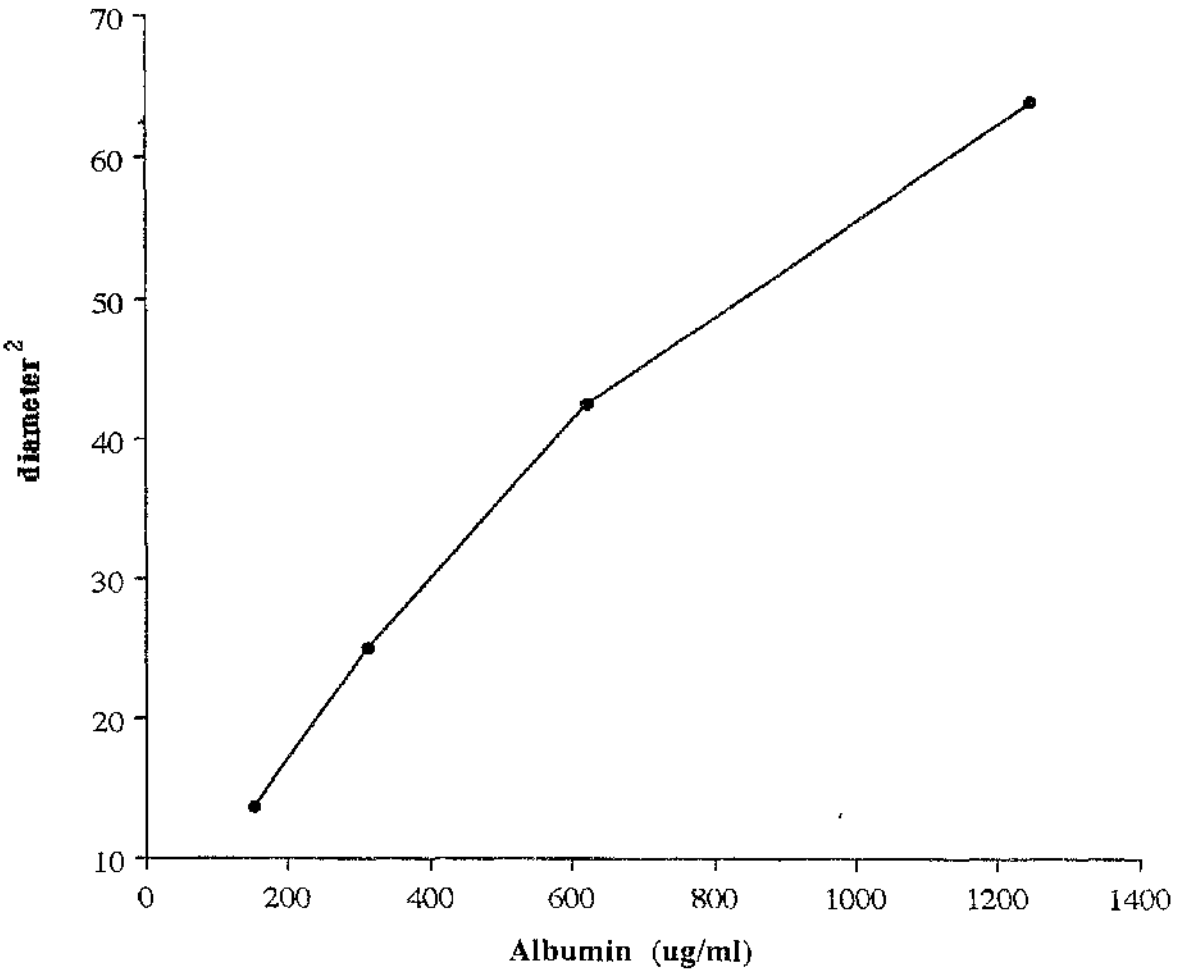


Figure 2-1: Standard curve for measuring human serum albumin by single immunodiffusion.

electrophoresis was monitored by the movement of a bromophenyl blue marker (placed in the top well) across the plate. After electrophoresis, the appropriate antisera were placed in the troughs on a levelling table. Both antigen and antibody were allowed to diffuse at room temperature in humidified chamber overnight. The resultant precipitin arcs between antigen and antibody were recorded and the gel.was stained (2-10).

2-10 Fixing and staining of agarose plates:

The agarose attached to gel-bond was soaked in PBS (diluted 1:1 with water) for two hours to remove unbound proteins. The plate was then dried at room temperature by pressing between layers of Whatman filter paper. When completely dried the gel was stained with 0.5 % coomassie brilliant blue in ethanol : water : acetic acid (50ml : 45ml : 5ml respectively) until precipitin arcs were well coloured. The plate was then destained in water : acetic acid : methanol (87ml : 8ml : 5ml respectively) and changed several times until background staining was removed. The plate was then air dried at room temperature.

2-11 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis:

This technique was employed to assess the purity and molecular weight of streptokinase used in the ELISA assays.

Buffers: 10% SDS: 10g of SDS was dissolved in 80ml water and the final volume was made up to 100 ml water.

0.75M Tris (hydroxyl methyl amino methane): 90.8g of Tris was dissolved in 800ml water and the final volume was made up to 1 litre by water.

0.75M Tris-HCl pH 6.80: was made by adding concentrated HCl to 0.75M Tris to adjust the pH 6.80 .

0.75M Tris-HCl pH 8.80: was made by adding concentrated HCl to 0.75M Tris to adjust the pH 8.80 .

0.86M Glycine: 64.4g of glycine was dissolved in 800ml water and the final volume was made up to 1 litre by water.

30 % acrylamide: 30g acrylamide and 0.8g bisacrylamide was dissolved in 80ml water and final volume was made up to 100ml. The solution then stored in dark container at 4°C.

Tank buffer: The followings were mixed together , 10ml SDS 10%, 33ml Tris 0.75 M and 220ml glycine. The final volume was then made up to 1 litre with water.

Sample buffer was made by mixing 30ml SDS 10% , 8.5ml 0.75M Tris-HCl pH 6.80, 10ml glycerol and 0.1g bromophenol blue and final volume was made up to 100ml with water. For samples using reduced condition 5ml mercaptoethanol was added to the sample buffer.

Staining buffer contained 0.1% Coomassie brilliant blue in methanol : acetic acid : water (5ml : 1ml : 4ml respectively).

Destaining buffer contained methanol: acetic acid : water (1ml : 1ml : 8ml respectively).

The following stock solutions were made up as shown for separating gel and stacking gels (10% SDS gel according to the method of Lammeli 1970) using a Raven vertical slab gel apparatus:

Stock solution	Separating gel	Stacking gel
Acrylamide, bisacrylamide	10ml	1.8ml
0.75M Tris (pH 6.8)	——	6ml
0.75M Tris (pH 8.8)	15ml	——
SDS	0.30ml	0.18ml
Ammonium per sulphate	30mg	18mg
TEMED	15µL	9µL
Water	4.7ml	13ml

The separating gel was mixed and poured immediately between two plates separated by spacers and overlaid with water saturated isopropanol until polymerised. Isopropanol then was removed and the residue was washed away with water and the stacking gel poured. A comb forming lanes was inserted and the gel allowed to set. Samples were mixed with an equal volume of sample buffer (see above). For reducing conditions samples were mixed with an equal volume of sample buffer containing mercaptoethanol (see above) and boiled for two minutes. The samples were carefully layered in the wells and electrophoresis was performed for approximately 3 hours at 40mA, until the tracking dye had reached the bottom of the gel. The gel was stained with staining buffer overnight at room temperature and destained with destain buffer (see above). The respective electrophoretic mobility (RF) for each sample was calculated by measuring the distance migrated from the bottom of the well, compared to the distance migrated by the tacking dye. The system was calibrated using reduced high molecular weight markers to form a standard curve (Figure 2-2).

Drying the gel: The gel was soaked in 5% glycerol for one hour to make it pliable and then placed between two layers of cellophane membrane (soaked in water) and dried with a gel dryer at 60° C for 3 hours.

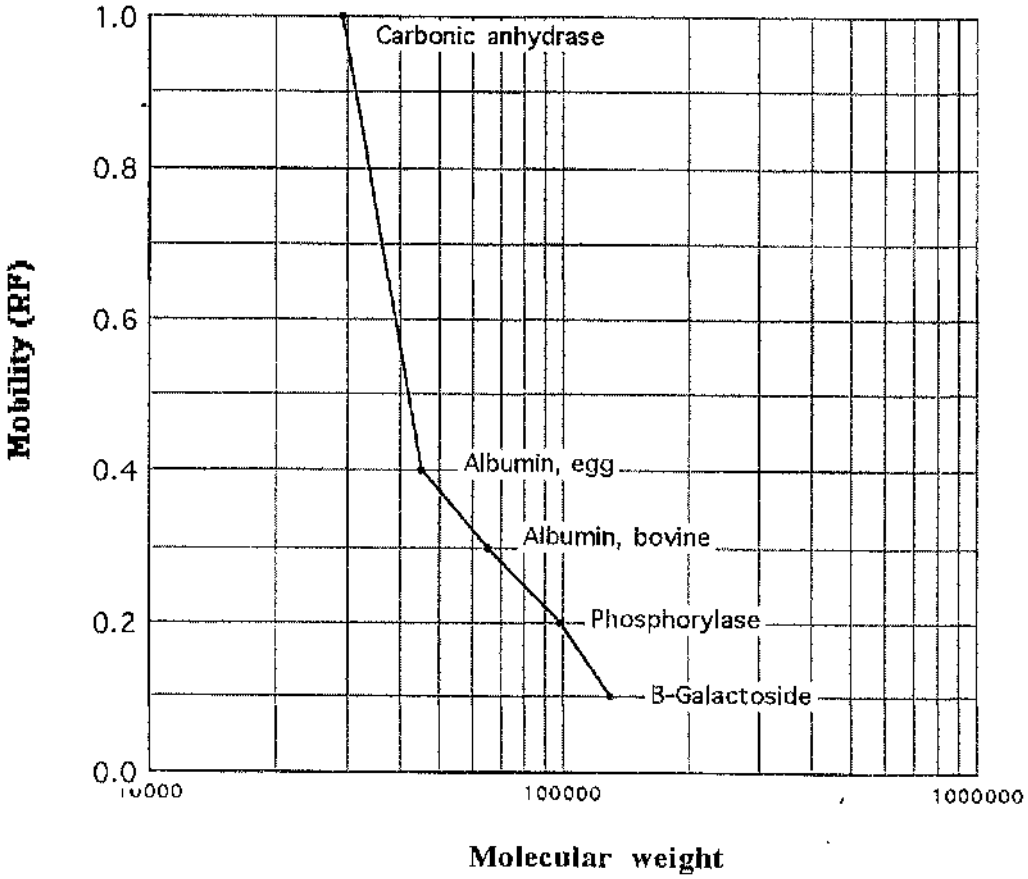


Figure 2-2: Standard curve for Sigma molecular weights markers.

2-12 Western blotting:

Buffers:

Transfer buffer: was made of 0.025M tris, 0.192M glycine and 20% methanol (12.11g of Tris, 57.67g of glycine and 800 ml of methanol was made up to a final volume of 4 litres by water).

Tris-buffered saline pH 7.50 (TBS): was made of 0.02M Tris and 0.5M NaCl (2.42g Tris and 29.22g NaCl were dissolved in 800ml of water and the final volume was made up to one litre with water).

Blocking buffer: 3g dried semi-skimmed milk was dissolved in 100ml TBS (3% Marvel).

Washing buffer: 100µl Tween 20 was added to 100ml TBS (0.1% Tween).

The specificity of antibodies to the proteins was determined by transferring the SDS PAGE gel to a nitro-cellulose membrane and performing a second electrophoresis. The electrophoresis performed at 400mA in one and half hour (Bio-Rad Trans-Blot TM cell). The membrane was then blocked with blocking buffer (see above) for one hour with constant agitation and washed with washing buffer (see above) 3 times each time for 5 minutes with constant agitation. The blot then was then incubated with anti-streptokinase antiserum (1/100 diluted with 0.15M TBS) for one hour at room temperature and constant agitation. After incubation the blot was washed and coated with anti-human IgG or IgM conjugated with HRP (diluted 1/1000 with TBS-Tween) for half an hour. The blot was then washed and developed with membrane peroxidase substrate tetramethylene blue (TMB) and hydrogen peroxide (H_2O_2), until bands were clearly seen and reaction was stopped by washing the blot in water.

2-13 Immunisation of rabbit with streptokinase:

Two New Zealand white rabbits were immunised with streptokinase (emulsified in complete Freund's adjuvant) by two intramuscular injections. After two boosts of streptokinase 1mg/1ml (emulsified in incomplete Freund's adjuvant) at days 10

and 17, the rabbits were bled at day 20 to identify the specificity of the raised antiserum to streptokinase by employing double immune diffusion technique (2-7, Figure 2-3). The rabbits were then exsanguinated and the serum separated as described in 2-14.

2-14 Separation of serum from rabbit blood:

Rabbit blood was incubated at 37°C for 30 minutes (to allow clot formation) followed by 30 minutes at 4°C (to allow clot retraction). The blood was then spun and the serum removed and heat inactivated at 56°C for half an hour to destroy complement components. Serum was then aliquoted and stored at -20°C, until required.

2-15 Separation of normal human serum (NHS):

Normal human serum was obtained from healthy volunteers. The blood was incubated in glass tubes at 37°C for 30 minutes (to allow clot formation) followed by 30 minutes incubation at 4°C (to allow clot retraction). Aliquots were stored at -70°C and used within ten days.

2-16 Preparation of human erythrocytes (RBC's):

Ten millilitres of venous human blood was mixed with preservative free sodium heparin (5 units/ml). The blood was spun at 1300 rpm, at 4°C for 7 minutes. The buffy coat layer was completely removed and the cells were washed a further three times with buffer (1% bovine serum albumin in PBS) and resuspended in buffer.

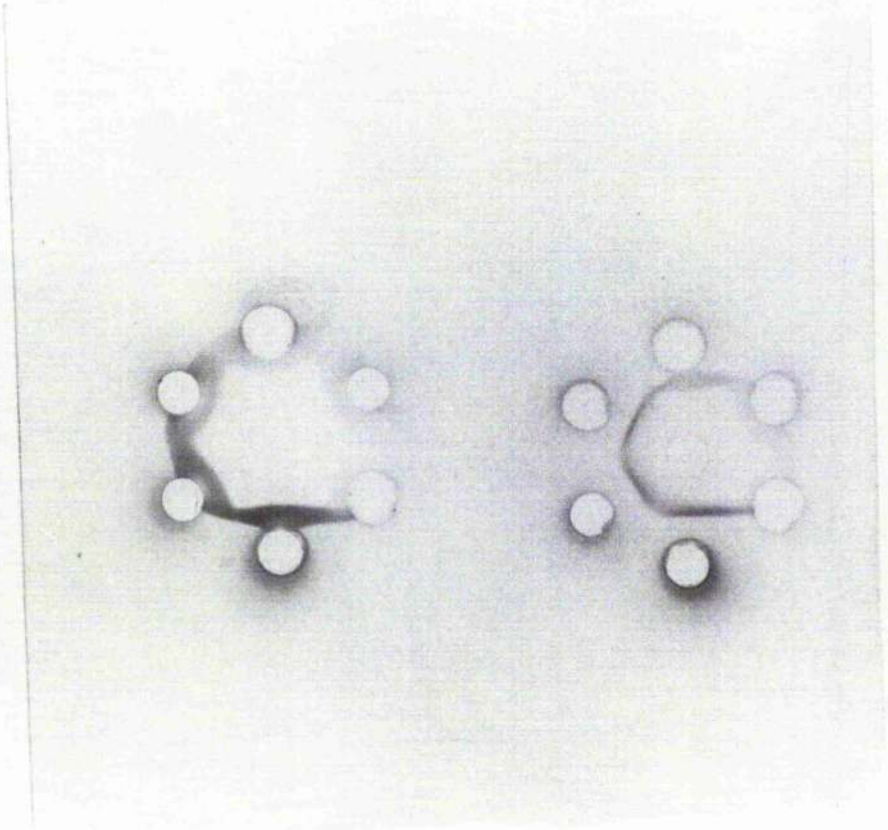


Figure 2-3: Double immunodiffusion performed on rabbit anti-streptokinase serum. The inner well filled with streptokinase and the outer wells filled with double diluted rabbit anti-streptokinase serum.

2-17 Standardisation of human erythrocytes (E's):

Washed erythrocytes were standardised using the following method:

0.1 millilitre of E's from a known volume was added to 2.9 ml of water and the optical density of lysed cells was measured at wavelength of 541nm (OD_{541}) or 414 nm (OD_{414}).

The volume of RBC suspension then adjusted to the required cell concentration by using the following formula:

Volume of E's required = (Initial volume X OD measured) / x

x was one of the following values:

OD_{414} of 0.327 = $1X10^8$ cells/ml

OD_{541} of 0.185 = $5X10^8$ cells/ml

OD_{541} of 0.37 = $1X10^9$ cells/ml

2-18 Quantitative precipitin curve:

Increasing volumes of antigen were added to a series of tubes containing a constant amount of antibody. The tubes were then incubated at 37°C for half an hour and then at 4°C overnight. The samples then spun at 3000g for 5 minutes, the supernatants removed and the precipitate washed twice with ice-cold PBS. The precipitate was re-dissolved in 0.1M sodium hydroxide. The optical densities (OD) of re-dissolved precipitates were read at 280nm and recorded. A graph was plotted of OD readings against concentration (Figure 2-4). The maximum optical density was considered as the equivalence point of the antigen and antibody immune complex (when all the antigen and antibody is complexed in the precipitate).

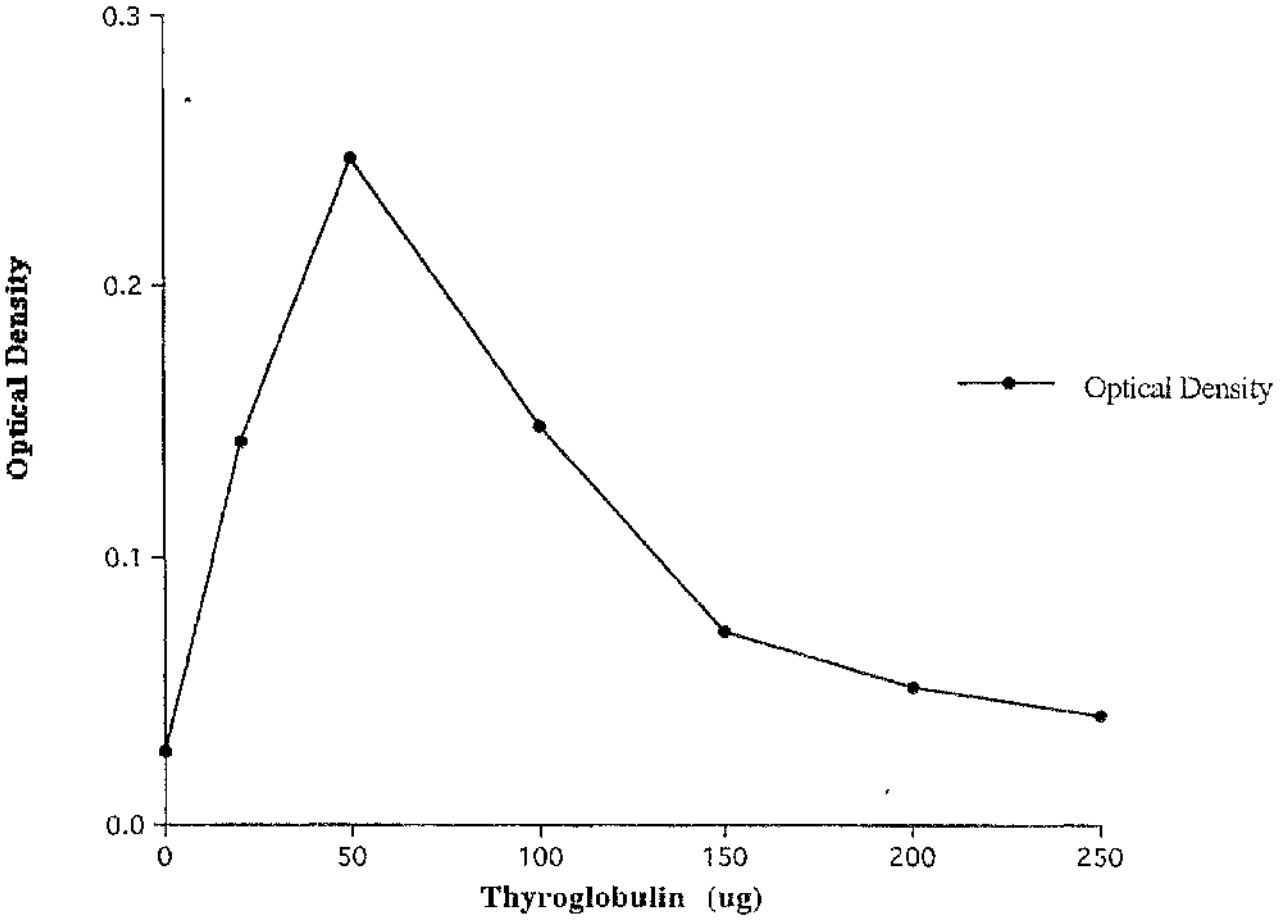


Figure 2-4: Quantitative precipitin curve of rabbit IgG anti-thyroglobulin and thyroglobulin.

2-19 Purification of IgG using Caprylic acid

Buffers: 0.1 M acetic acid: was prepared by adding 0.6ml of glacial acetic acid to 100ml water.

Saturated ammonium sulphate (SAS): 75g of ammonium sulphate was dissolved in 80ml water and final volume was made up to 100 ml with water, then stored at 4 °C to allow crystal formation. Only the supernatant was used.

The antiserum was initially treated with caprylic acid to precipitate the proteins of serum. Caprylic acid precipitates all proteins with an exception of IgG. By employing 0.1M acetic acid (see above) the pH of antiserum was reduced to 4.5-5.0. Neat caprylic acid then was added to antiserum to give a 5% suspension, mixed vigorously and spun for 5 minutes at 10000g. An equal volume of SAS was added slowly to the supernatant, antiserum and SAS were mixed for 2 hours at room temperature then spun and the supernatant discarded. The pellet was resuspended in PBS and dialysed extensively against PBS at 4°C. Absorbance of the protein at 280 nm was measured and the protein concentration was determined using an extinction coefficient of 1.42 = 1mg/ml for IgG. The purity of the prepared antibody was tested by IEP (see 2-9) and the IgG was stored at -20°C, until required.

2-20 Affinity chromatographic purification of IgM:

Buffers:

0.08M phosphate buffered saline (0.077M NaCl): 0.2g potassium dihydrogen phosphate, 1.15g disodium hydrogen phosphate, 0.2 g potassium chloride and 8.2g Sodium chloride were dissolved in water and final volume was made up to 2 litres with water.

0.8M phosphate buffered saline (1.1M NaCl): 0.2g potassium dihydrogen phosphate, 1.15g disodium hydrogen phosphate, 0.2 g potassium chloride and 120g sodium chloride were dissolved in water and final volume was made up to 2 litres with water.

Protamine binds to IgM. Sepharose coupled to protamine was spun at 10000g for 2 minutes, the supernatant discarded and the pellet was washed 3 times with 0.08M PBS containing 0.077M NaCl (see above). Serum known to contain IgM, was pre-diluted with water (1:1) and added to an equal volume of Sepharose and stirred at 4°C for 3 hours. The sample was then washed three times with PBS containing 0.077M NaCl (pre-diluted 1:1 with water). To elute the bound IgM from protamine, PBS containing 1.1M NaCl (see above) was added to the sample and stirred at room temperature for one and half hours. The sample was then spun and the supernatant was tested for purity by IEP (2-9) and was stored at -20°C, until required.

Protamine Sepharose was recycled by washing with 0.08M PBS containing 0.077M NaCl and then stored at 4°C in 0.08M PBS.

2-21 Affinity chromatographic purification of IgA:

The lectin Jacalin is obtained from the seeds of Jackfruit and when conjugated to agarose can be used to purify human IgA1.

Buffers:

0.175M Tris pH 7.50: Concentrated HCl was added to 250ml 0.75M Tris (see 2-11) till pH 7.50 was achieved, then the total volume was made up to one litre with water.

0.1M Melibiose: made by dissolving 0.34g Melibiose in 10 ml water.

Storage buffer of Jacalin agarose: was made by dissolving 0.15M sodium chloride (4.38g), 0.1M calcium chloride (5.55g), 0.02M galactose (1.8g) and 0.01M HEPES pH 7.50 (1.19g) in 400ml water and the final volume was made up to 500ml with water. Sodium azide 0.4g was added to the buffer as a preservative (0.08% W/V).

The pH of the serum known to contain IgA was adjusted to 5.50 by the dropwise addition of concentrated HCl. Solid ammonium sulphate then was added slowly to 75% saturation and the sample stirred at room temperature for one hour. The

sample was then spun, the supernatant discarded and the pellet resuspended in PBS. The resuspended pellet was then added to equal volume of Jacalin-sepharose pre-washed in 0.175M Tris pH 7.50 and mixed at room temperature for one hour. The sample was then spun and the supernatant discarded. An equal volume of 0.1M Melibiose was then added to the pellet and stirred at room temperature for half an hour. The sample was then spun and the supernatant tested for purity and the presence of IgA by IEP (2-9). The supernatant was stored at -20°C, until required.

Jacalin-agarose was recycled by washing with 0.175M Tris pH 7.5 and then resuspending in storage buffer and kept at 4°C.

2-22 Biotin labelling of IgG:

IgG was dialysed against PBS at 4 °C overnight and the concentration of dialysed sample adjusted to 5 mg/ml. Three mg of biotin N-hydroxy-succinimide ester was dissolved in 100 µL of dimethylformamide and immediately added to 1ml of IgG (5mg/ml). They were allowed to mix at room temperature for 2 hours. The sample then dialysed extensively against PBS at 4°C and stored at 4 °C until required.

2-23 Pepsin digestion of IgG:

Pepsin digestion of IgG results in a molecule which is the F $(ab')_2$ portion containing the intact antigen binding site and has a molecular weight of 100000. The amount of pepsin needed for the digestion is 1-2 milligram per 100 mg of IgG. Protein G coupled to Sepharose binds to Fc portion of IgG and can be used to separate F $(ab')_2$ and Fc fractions from pepsin digested IgG.

Buffers:

Sodium phosphate 0.1M (pH 7.0): 3.1g sodium dihydrogen phosphate 2 hydrate was dissolved in one litre water to make 0.1M NaH_2PO_4 and 2.84g disodium hydrogen phosphate anhydrous was dissolved in one litre to make 0.1M Na_2HPO_4

separately. To 50mls of the first buffer, 70 ml of latter buffer was added to give a final pH of 7.0.

0.1 M sodium acetate: made by dissolving 8.2g sodium acetate in 800 ml water and final volume was made up to 1 litre with water.

0.1M glycine: made by dissolving 7.5g glycine in 800 ml water and final volume was made up to 1 litre with water, the pH of buffer then adjusted to 2.5-3.0 with concentrated HCl.

Samples known to contain IgG were dialysed against 0.1 M sodium acetate for 3 hours at 4°C. The pH of sample was adjusted with 0.1M acetic acid (2-19) to 4.50. The required amount of pepsin was added to sample and incubated at 37°C overnight. The digested sample was then spun and any precipitate discarded and the pH was adjusted to 7.40 by addition of solid Tris. The sample was then dialysed against PBS for 3 hours at 4°C to inactivate the enzyme. Sepharose (coupled to protein G) was pre-washed with 0.02M sodium phosphate buffer (see above) and resuspended in buffer to give a ratio of 25% 0.02M sodium phosphate buffer and 75% Sepharose. The sample IgG was then added to resuspended Sepharose and mixed at room temperature for one and half hour. The sample was then spun and the supernatant containing $F(ab')_2$ was kept. The purity of $F(ab')_2$ was tested by IEP (2-9) and sample was kept at -20°C, until required.

The Sepharose was recycled by washing 3 times in 0.1M glycine and resuspended in ethanol as a preservative to give a ratio of 20% ethanol and 80% Sepharose.

Chapter 3

Measurement of isotype specific response to streptokinase in a normal population and patients with acute myocardial infarction

Introduction

The presence of anti-streptokinase antibodies in patients sera is a result of previous streptococcal infections (Anderson et al 1948, Flute 1973, Buchalter et al 1992). However, it is not clear that to what extent these antibodies exist in the population, how they influence the efficacy of streptokinase treatment for acute myocardial infarction (AMI) or provoke hypersensitivity reactions in patients given streptokinase (Moran et al 1984, Dykewicz et al 1986, Sanjeev & Morris 1990, Rosenschein et al 1991).

Development of assays to measure serum levels of anti-streptokinase antibodies, has been driven by a quest to answer these questions (James 1973, McGrath & Patterson 1985, Lynch et al 1991). Studies to date have concentrated on the measurement of IgG and neutralising antibodies to streptokinase. The aim of the present study was to determine not only the prevalence of IgG and neutralising anti-streptokinase antibodies but also the levels of the other isotypes of anti-streptokinase antibodies IgM, IgA, IgE in the normal population. The levels of anti-streptokinase antibodies in patients with acute myocardial infarction (AMI) were then compared with the normals to establish if they had elevated levels of pre-existing anti-streptokinase antibodies and whether this had any effect on clinical outcome.

Anti-streptokinase antibodies have been quantified by several methods (1-6). The classical neutralising titration assay was developed to measure anti-streptokinase antibodies which inhibit the biological activity of streptokinase on the breakdown of clots (Kaplan 1944). In this study a commercial ASK-kit was used to quantify the levels of neutralising anti-streptokinase antibodies in serum samples.

Enzyme-linked immunosorbent assay (ELISA) is a sensitive and reproducible technique for measuring the levels anti-streptokinase antibodies and is now commonly used (Lynch et al 1991, Fears et all 1992a, Elliot et al 1993). The ELISA technique is the method of choice as it is safe and cost effective compared to radioimmunoassays which are also sensitive and reproducible. In addition ELISA technique can be used successfully to measure individual antibody isotypes. In this study four enzyme-linked immunosorbent assays (ELISA's) were developed to detect IgG, IgA, IgM and IgE anti-streptokinase antibodies levels in sera of normals and patients.

Materials and methods

3-1 Quantitation of neutralising anti-streptokinase antibodies. Anti-streptokinase neutralising antibodies were quantified in serum samples by the classical neutralising titration assay, in this method the sera containing these antibodies prevents the lytic effect of streptokinase on clots.

Materials and Buffer: Sources of materials used here are described in 2-1.

ASK-kit

Round-bottomed microplates

Rabbit erythrocytes

Phosphate buffered saline (PBS) (2-2)

Method. The levels of neutralising antibodies in sera samples were measured following manufacturer's instructions. Samples were diluted 1/10 in PBS and 25 µl was applied to a round-bottomed microplate and double diluted across the plate up to 1/640 in PBS. Streptokinase (25 µl) was then added to each well and incubated at 37°C for half an hour. Fifty µl of a mixture of fibrinogen and plasminogen was then added to each well. Rabbit erythrocytes were washed in PBS and resuspended in PBS to give a 5 % suspension and then was added to dried bovine thrombin.

Twenty five µl of the rabbit erythrocytes suspension was then applied to all the wells of the plate and the plate was covered and incubated at 37°C for 2 hours. The last dilution that prevented the lytic effect of streptokinase on the formed clots and prevented the sedimentation of erythrocytes was considered as the end point titre of the sample and expressed in reciprocal titre of neutralising anti-streptokinase antibodies per ml.

A serum sample with known levels of the neutralising anti-streptokinase antibodies (80 units/ml) was used as control.

3-2 Determination of molecular weight and purity of streptokinase.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is widely used technique to determine molecular weight of proteins, the proteins bind in a constant ratio to SDS according their molecular size and allows the determination of their molecular weight. SDS-PAGE was employed to assess the purity and molecular weight of commercial streptokinase used as the antigen in the ELISA assays.

Materials: Sources of materials used here are described in 2-1.

Molecular weight markers for gel electrophoresis

Streptokinase

Buffers (2-11)

10% SDS

0.75M Tris

0.75M Tris-HCl pH 6.80

0.75M Tris-HCl pH 8.80

0.86M Glycine

30 % acrylamide

Tank buffer

Sample buffer

Method. The stock solutions were made up as described for separating gel and stacking gels (5 to 15 % SDS gel according to the method of Laemmeli 1970) (2-11).

The separating gel was mixed and poured immediately between two plates separated by spacers and overlaid with water saturated isopropanol until polymerised. Isopropanol then was removed and the residue was washed away with water and the stacking gel poured. A comb forming lanes was inserted and the gel allowed to set.

Streptokinase was mixed with an equal volume of sample buffer to give 10 to 20 μg streptokinase per track. For reducing conditions streptokinase was mixed with an equal volume of sample buffer containing mercaptoethanol and boiled for two minutes. Streptokinase mixtures were carefully layered in the wells and electrophoresis was performed for approximately 3 hours at 40mA, until the tracking dye had reached the bottom of the gel. The gel was stained with staining buffer overnight at room temperature and destained with destain buffer. The respective electrophoretic mobility (RF) for each track was calculated by measuring the distance migrated from the bottom of the track, compared to the distance migrated by the tacking dye. The system was calibrated using reduced high molecular weight markers to form a standard curve (Figure 2-2). The gel was dried as described before (2-11). Under optimum conditions of electrophoresis, streptokinase at 5-10 μg per track was run on a 10% gel, the molecular weight of precipitate bands in the gel were calculated.

3-3 Crude screening ELISA procedure for collecting positive samples with anti-streptokinase antibody activity. By neutralising titration assay the presence of anti-streptokinase in serum can be assessed. However, this technique is not specific for individual antibody isotypes and costs more than ELISA

techniques. Therefore indirect ELISA's were developed to quantify specific IgG, IgM, IgA and IgE isotypes to streptokinase. Positive serum samples with anti-streptokinase antibody activity were required to construct standard curves to measure levels of IgG, IgA, IgM and IgE streptokinase specific antibodies in unknown samples. Sera from normal individuals and patients treated with streptokinase were screened by ELISA technique as following, to select samples.

Materials: (2-1)

ELISA plates (Dynatech, Immulon 4)

Streptokinase

Horseradish peroxidase (HRP) conjugated goat anti-human IgA

HRP conjugated goat anti-human IgE

HRP conjugated goat anti-human IgG

HRP conjugated goat anti-human IgM

O-phenylenediamine (OPD)

Buffers: Prepared as described in 2-2.

Phosphate buffered saline (PBS)

10 % Gelatin

ELISA coating buffer pH 9.60

ELISA blocking buffer (0.01% gelatin)

ELISA wash buffer (0.05% Tween in PBS)

0.2M citric acid

ELISA enzyme substrate

4N H_2SO_4

Method. For detecting antibodies ELISA plates were coated with streptokinase (5 µg/ml in ELISA coating buffer pH 9.60) overnight at 4°C. After extensive washing in PBS-Tween the plates were blocked for one hour at room temperature with 200

µl per well of 0.01% gelatin in PBS. The plates were washed and samples were applied to the plates. The plates were incubated for a period of 2 hours at room temperature. After washing horseradish peroxidase conjugated anti-human IgG, IgA, IgM or IgE (diluted 1/1000 in 0.01% gelatin in PBS, 100 µl per well) were added and after a further one hour incubation the plates were washed and developed using o-phenylenediamine (OPD) and the reaction stopped with 4N H_2SO_4. Colour reaction was read at 490 nm on a Dynatech ELISA reader.
Samples which gave a strong positive signal were chosen for construction of standard curves.

3-4 Purification of human anti-streptokinase antibodies. Following sources of antisera were used to construct standard curves of isotype specific anti-streptokinase antibodies in ELISA's to measure antibody concentrations in unknown serum samples. Sources of materials used here are described in 2-1.
The commercial ASK-kit purchased for measurement of neutralising anti-streptokinase antibodies (3-1) contained a positive anti-streptokinase antibody control serum. The specificity of the positive serum for streptokinase was determined by the following procedure. SDS-PAGE electrophoresis was performed on streptokinase (10 to 20 µg streptokinase per track, 3-2) and the gel transferred to a nitro-cellulose membrane (2-12). The membrane was then blocked with 3% Marvel in TBS for one hour and washed (2-12). The blot was then incubated with the positive control sample diluted 1/100 in 0.15M TBS for one hour at room temperature with constant agitation (2-12). After incubation the blot was washed and incubated with HRP conjugated goat anti-human IgG or IgM, diluted 1/1000 in TBS-Tween, for half an hour (2-12). The blot was then washed and developed with the peroxidase substrate tetramethylene blue (TMB) and hydrogen peroxide (H_2O_2), until bands were clearly seen, the reaction was then stopped by washing the blot in water.

Human anti-streptokinase IgG was purified from this positive serum using the caprylic acid precipitation method (2-19). Briefly the positive sample contained 50.8 mg/ml protein was treated with 5% caprylic acid to precipitate the majority of serum proteins except IgG (2-19). The supernatant was treated with an equal volume of SAS to precipitate IgG and the pellet resuspended with PBS and dialysed against PBS (2-19). The final IgG concentration was 33.4 mg/ml (46% protein recovery). The purified IgG was then used to construct a standard curve for measuring IgG anti-streptokinase antibodies in unknown samples.

To find an IgM anti-streptokinase antibody positive serum, 94 samples from normal individuals and patients treated with streptokinase were screened by the crude ELISA (3-3). IgM was initially purified from normal human serum (NHS) (Contained 23 mg/ml total protein) by affinity chromatography using protamine coupled to Sepharose (2-20). As protamine binds to IgM, Sepharose-protamine was added to an equal volume of NHS and stirred at 4°C for 3 hours. The Sepharose suspension was then washed with PBS and IgM was eluted using PBS containing 1.1M NaCl (2-20). Absorbency of the eluted protein at 280 nm was 5.2 mg/ml (22.6% protein recovery). However, the availability of the serum positive for IgM anti-streptokinase activity (3-3) was low, therefore to construct standard curve for measuring IgM anti-streptokinase antibodies in unknown samples the positive serum was used directly and not the IgM fraction.

IgA was purified from a known positive sample (Contained 53 mg/ml total protein) with anti-streptokinase IgA activity (a kind gift from Dr. E. Holme) by affinity chromatography purification using the lectin Jacalin derived from Jackafruit conjugated to agarose (2-21). Solid ammonium sulphate was added slowly to 75% saturation to the serum known to contain IgA (at pH 5.50) and stirred at room temperature for one hour. The mixture was then spun, the supernatant discarded and the pellet resuspended in PBS, added to equal volume of Jacalin-agarose and mixed at room temperature for one hour (2-21). The agarose suspension was then

spun and the supernatant removed. To elute bound IgA an equal volume of 0.1M melibiose was added to the pellet and stirred at room temperature for half an hour. The suspension was again spun and the supernatant removed and retained. Absorbency of the supernatant at 280 nm was 8.4 mg/ml (15.8 % protein recovery). The purified IgA was contaminated with IgG (see results) therefore for constructing standard curve for measuring IgA anti-streptokinase antibodies in unknown samples the positive serum was used directly and IgA was not puried from the positive sample.

An IgE response was not detectable in any of the normal controls, although for capturing IgE anti-streptokinase antibody, the concentration of streptokinase was increased from 5μg/ml to 250 μg/ml (3-3).

The purity of the antibodies was checked by immunoelectrophoresis (IEP, 2-9) using sheep anti-human IgG, IgA, IgM and anti-human whole antiserum (diluted 1/5) and 8% SDS-PAGE electrophoresis, both in reduced and non-reduced conditions (2-11). The purified antibodies were stored at - 20°C until required.

3-5 Development of ELISA's for measuring isotype specific anti-streptokinase antibodies. Indirect ELISA's were developed to measure the levels of specific IgG, IgM, IgA and IgE isotypes to streptokinase in the population and study their effect in patients treated with streptokinase.

Materials: (described in 3-3)

Buffers: (described in 3-3)

Method. For detecting antibodies different ELISA conditions were examined to find the optimum conditions for each assay. ELISA plates were coated with streptokinase solution overnight at 4°C. As alkaline buffers may damage the biological activity of streptokinase by destruction of the amide groups of the protein (1-1), ELISA plate were coated with 5 μg/ml and 10 μg/ml streptokinase diluted in PBS pH 7.0 or ELISA coating buffer pH 9.60 to examine the effect of the

concentration of streptokinase and the pH of coating buffer. After extensive washing in PBS-Tween the plates were blocked for one hour at room temperature with 200 µl per well of 0.01% gelatin in PBS.

The plates were washed and standard curves for each antibody isotype (IgG, IgA and IgM) were constructed and run on the plates to allow the determination of the concentration of the unknown sample as follows.

For the IgG, purified IgG anti-streptokinase (3-4) was double diluted from 1/100 and applied to the plates, the first dilution was assigned an arbitrary value of 225 units/ml.

IgA and IgM anti-streptokinase positive sera (3-4) were double diluted from 1/100 and 1/10 respectively and applied to the plates, the first dilutions being assigned arbitrary values of 1000 units/ml and 100 units/ml respectively. For IgE no positive sample with IgE anti-streptokinase was available and no standard curve was run, samples were expressed as positive or negative based on the OD of the blank. Values were accepted as positive if the OD was 3 times the value of the blank.

To establish the dilutions of samples which corresponded to the linear part of standard curves, different dilutions of samples were examined and the optimum dilutions were 1/200 for IgG and IgA, 1/30 for IgM. For detecting IgE samples were diluted 1/5. After applying standard curves and samples on the plates, the plates were incubated for a period of 2 hours at room temperature.

After washing horseradish peroxide conjugated to human IgG(diluted from 1/1000 to 1/8000), IgA (diluted 1/1000), IgM (diluted 1/1000) and IgE (diluted 1/500) were added and after a further one hour incubation the plates were washed and developed using o-phenylenediamine (OPD) and the reaction stopped with 4N H_2SO_4. The colour reaction was read at 490 nm on a Dynatech ELISA reader. The antibody concentrations in unknown serum samples were obtained by interpolation from standard curves and the values expressed in the arbitrary units/ml.

3-6 Intra-assay and inter-assay variations of techniques used for measuring anti-streptokinase antibodies. Intra-assay and inter-assay variations of the assays used was determined by following procedure.

Intra-assay variations: Known positive samples which contained 10 units/ml IgG, 330 units/ml IgA, 16 units/ml IgM and 80 units /ml neutralising anti-streptokinase antibodies were studied for intra-assay and inter-assay variations. The levels of isotype specific anti-streptokinase antibodies, IgG, IgA and IgM, were measured by indirect ELISA and the levels of neutralising anti-streptokinase antibodies were measured by ASK-kit 12 times within the same assay as described before (3-1 and 3-5). The coefficient variations (C.V.) of the samples for each assay was then determined by the following formula: C.V. = Standard Deviation / Mean X 100.

Inter-assay variation: The levels of specific isotype response to streptokinase (IgG, IgA and IgM) and neutralising anti-streptokinase antibodies were determined in samples with known levels of anti-streptokinase antibodies (IgG 2 units/ml, IgA 330 units/ml, IgM 22 units/ml and ASK 80 units/ml) on 7 different days by ELISA's and ASK-kit respectively (3-1 and 3-5). The C.V. of them was calculated as described.

3-7 Normal control group. Two hundred and forty six normal controls were studied for the presence of isotype specific anti-streptokinase antibodies. These samples were kindly provided by Dr. Elizabeth Holme, Department of Immunology Western Infirmary, Glasgow and the details of these individuals have been documented else where (Fyfe et al 1987). Their ages ranged between 14 and 79 years and at the time of venesection they had no known current or recent illness. The sample were stored at -70°C.

3-8 Acute myocardial infarction (AMI) patients treated with thrombolytic agents. This study was performed in collaboration with Dr Elizabeth Holme (Department of Immunology, Western Infirmary, Glasgow) and

Dr.Stewart Hillis (Department of Cardiology, Western Infirmary, Glasgow). Twenty patients with AMI (13 males, 7 females, mean age 57.6, range 37-70) were studied. They all presented with chest pain of duration greater than 30 minutes and less than 6 hours. AMI was confirmed by standard ECG criteria of ST segment elevation >1mm in at least two limbs leads or >2mm in at least two chest leads. Fourteen patients had inferior and six patients had anterior infarcts.

Ten patients received intravenous streptokinase (1.5 million units over 1 hour) and ten patients received r-tPA (70-100 mg over 1 hour). Blood samples were taken prior to administration of thrombolytic agents and at timed intervals after treatment. Reperfusion in the twenty patients treated for AMI was monitored by the ECG (changes of the ST segment) and was observed from 2 hours to 24 hours after thrombolytic administration. The patients were therefore categorised into 3 groups according to their response to thrombolytic therapy, early reperfusion group responded within 2 hours, late reperfusion group responded within 24 hours and non-reperfusion group.

3-9 Measuring serum albumin levels by single radial immunodiffusion. Serum albumin levels were measured in serum samples prior to administration of thrombolytic agents and at timed intervals after treatment by single radial immunodiffusion using rabbit IgG anti-human albumin (2-8).

0.15ml of rabbit IgG anti-human albumin (47 mg/ml) was added to three mls of agarose (2-5) and mixed at 56°C. The agarose was poured into petri dishes and allowed to cool. Wells were punched in plates and were filled with normal human serum (NHS) double diluted from 1/32 to 1/256 to construct standard curves. Samples diluted 1/60 were applied to the remaining wells and the plates were left overnight at room temperature in a humidified chamber. The diameters of precipitin rings were measured and recorded. A standard curve was constructed by plotting the d2 (diameter) of the precipitin rings of controls versus their concentration, and from this the albumin concentrations in the unknown samples were determined.

3-10 In vitro effect of recombinant tissue plasminogen activator on quantitation of neutralising anti-streptokinase antibodies. The presence of recombinant tissue plasminogen activator (r-tPA) in the sera of patients treated with this thrombolytic agent may interfere with neutralising anti-streptokinase assay (3-1). Therefore this effect was studied in vitro by following procedure.

Materials and Buffer: Sources of materials used here is described in 2-1.

ASK-kit

Positive serum with anti-streptokinase activity

r-tPA

Round-bottomed microplates

Rabbit erythrocytes

PBS (2-1)

Method. The levels of neutralising anti-streptokinase antibodies in a serum sample with known levels of the antibodies (80 units/ml) were measured in the presence and absence of r-tPA. The serum sample was diluted 1/10 in PBS (Total volume 500µl) or was diluted 1/10 in PBS containing increasing amount of r-tPA from 0.3 µg/ml to 5000 µg/ml (Total volume 500µL) (Therapeutic dose of r-tPA = 1000 µg/ml). The titre of neutralising anti-streptokinase antibodies in the diluted serum samples then were measured as described in 3-1.

3-11 Statistical analysis. Statistical analysis of data was performed using Spearman rank correlation test, Wilcoxon signed rank test and Mann-Whitney test.

Results

Determination of molecular weight and purity of streptokinase. Molecular weight and purity of the commercial streptokinase used as antigen in the ELISA assays was first determined by SDS-PAGE (3-1). The mobility of

streptokinase as observed by this technique showed no difference under non-reduced and reduced conditions (Figure 3-1). Streptokinase run on a SDS-PAGE revealed two close bands with molecular weights of 47 and 50 kD (Figure 3-1). Immunoblots performed with known positive serum containing anti-streptokinase antibodies (3-4) confirmed that the two bands correspond to the streptokinase molecule at 47 and 50 kD (Figure 3-2).

Isolation of positive samples for construction of standard curves. A crude ELISA procedure was used to select serum samples which were positive for IgG, IgA, IgM or IgE anti-streptokinase antibodies. Attempts were made to purify the specific isotypes of antibody from the positive serum samples. IgG was purified by caprylic acid and IgM and IgA by affinity chromatography.

IgG was purified from the positive kit control of the ASK kit. This control was IgG as confirmed by western blotting (3-4, Figure 3-2). After caprylic acid purification SDS-PAGE electrophoresis (3-4) under non-reducing conditions showed a band with molecular weight of 150000 corresponding to the molecular weight of IgG and in reduced condition a band with molecular weight of approximately 55000 corresponding to molecular weight of the heavy chain of IgG.

When IEP was performed to examine the purity of the IgG preparation a thick precipitation arc was observed with anti-human IgG and no precipitation arc with anti-human IgM (Figure 3-3). However, the IEP showed that the purified IgG was contaminated with IgA (Figure 3-3). To assess whether this IgA contamination would influence the use of the IgG in the ELISA assay system, the levels of IgA were determined by ELISA (3-3), using HRP conjugated anti-human IgA utilising the IgG ELISA protocol. The optical density of the purified sample for IgA (OD_{490nm}=0.08) was close to the optical density of blank (OD_{490nm}=0.05) thus the presence of IgA did not appear to interfere in the IgG ELISA.

Five of the samples screened for IgM anti-streptokinase antibodies (n=94) showed elevated levels of IgM when the optical densities were compared with the optical

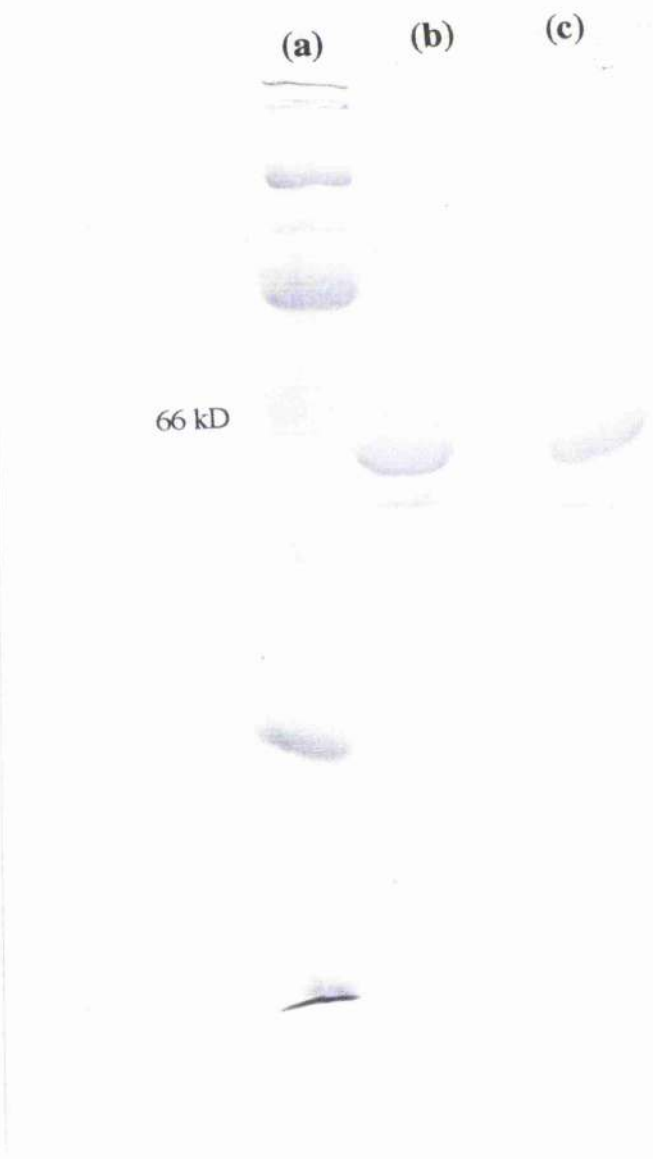


Figure 3-1: Streptokinase run on a 10 % SDS-PAGE gel. Track (a) shows molecular weight markers for constructing the standard curve and tracks (b) and (c) show streptokinase run non-reduced and reduced respectively.

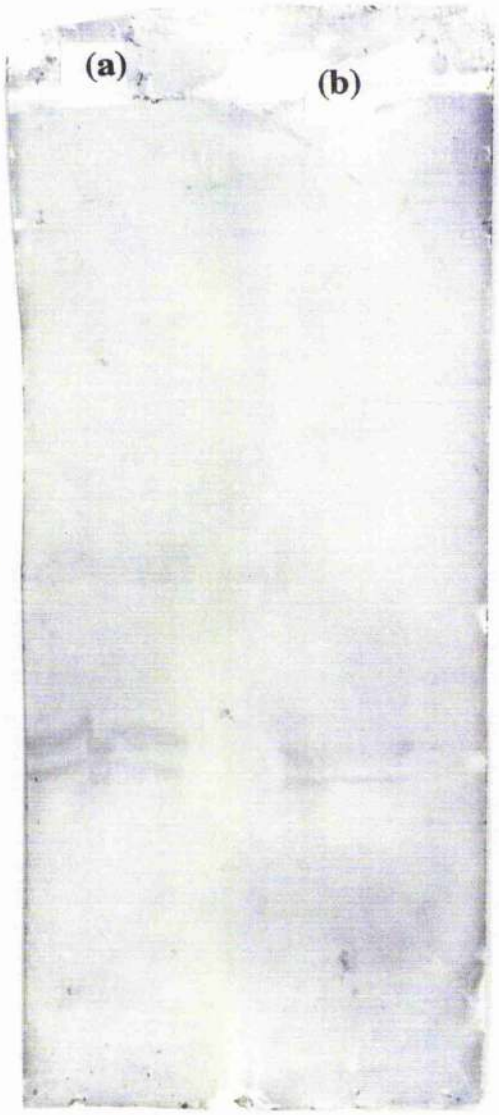


Figure 3-2: Immunoblot of streptokinase transformed from a 10 % SDS-PAGE gel and incubated with the human anti-streptokinase and HRP conjugated anti-human IgG (Tracks a and b non-reduced and reduced respectively).

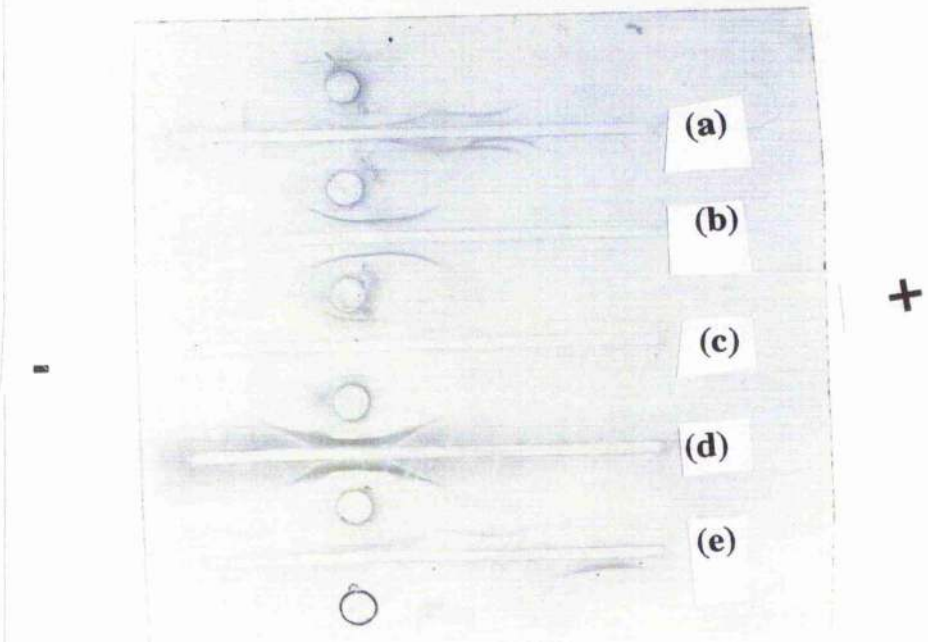


Figure 3-3: IEP showing purified human IgG anti-streptokinase run in wells and after electrophoresis, anti-whole human serum (troughs a and e), anti-IgA (b), anti-IgM (c) and anti-IgG (d) were applied to the plate.

density of the blank (OD_{490nm}= 0.06) (3-3) and one was chosen (OD_{490nm}=1.30) to construct a standard curve for measuring IgM in the unknown samples sera. IgM was initially purified from NHS by affinity chromatography (3-4), however, the amount of the positive serum was not sufficient for affinity chromatography purification (see protein recovery 3-4) as purification procedure resulted in a low yield of IgM (22%), therefore to construct standard curve for IgM anti-streptokinase the positive serum was used directly.

IgA was purified from a known positive sample with IgA anti-streptokinase activity by Jacalin-agarose affinity chromatography (3-4).The yield of IgA was 8.4 mg/ml and the purity of the sample was determined by IEP (Figure 3-4). The purified IgA was contaminated with IgG. To assess the amount of IgG, the level was determined by ELISA using HRP conjugated anti-human IgG and HRP conjugated anti-human IgA. The OD_{490nm} of IgA was 0.44 and IgG was 0.40. Due to large IgG contamination and the limited supply of IgA positive sera for constructing standard curve whole serum and not the purified IgA fraction was used.

Developing ELISA's for measuring isotype specific anti-streptokinase antibodies. For detecting anti-streptokinase antibodies different ELISA conditions were examined to find the optimum conditions for each assay.

As alkaline buffers may damage the biological activity of streptokinase (1-1), streptokinase was coated into ELISA plates at 5 μg/ml and 10 μg/ml in PBS pH 7.0 or in ELISA coating buffer pH 9.60 to assess the effect of concentration of streptokinase and the pH of coating buffer (3-5). Overnight coating of streptokinase in PBS (pH 7.0) resulted in more consistent standard curves than observed with alkaline buffer (Figures 3-5 and 3-6). In addition the sensitivity of the assay appeared to be greater with coating in PBS (Figures 3-5 and 3-6). The concentration of 5μg/ml streptokinase as opposed to 10μg/ml streptokinase did not significantly effect the standard curves (Figures 3-5 and 3-6), however, for economic reasons streptokinase at a concentration of 5μg/ml was used.

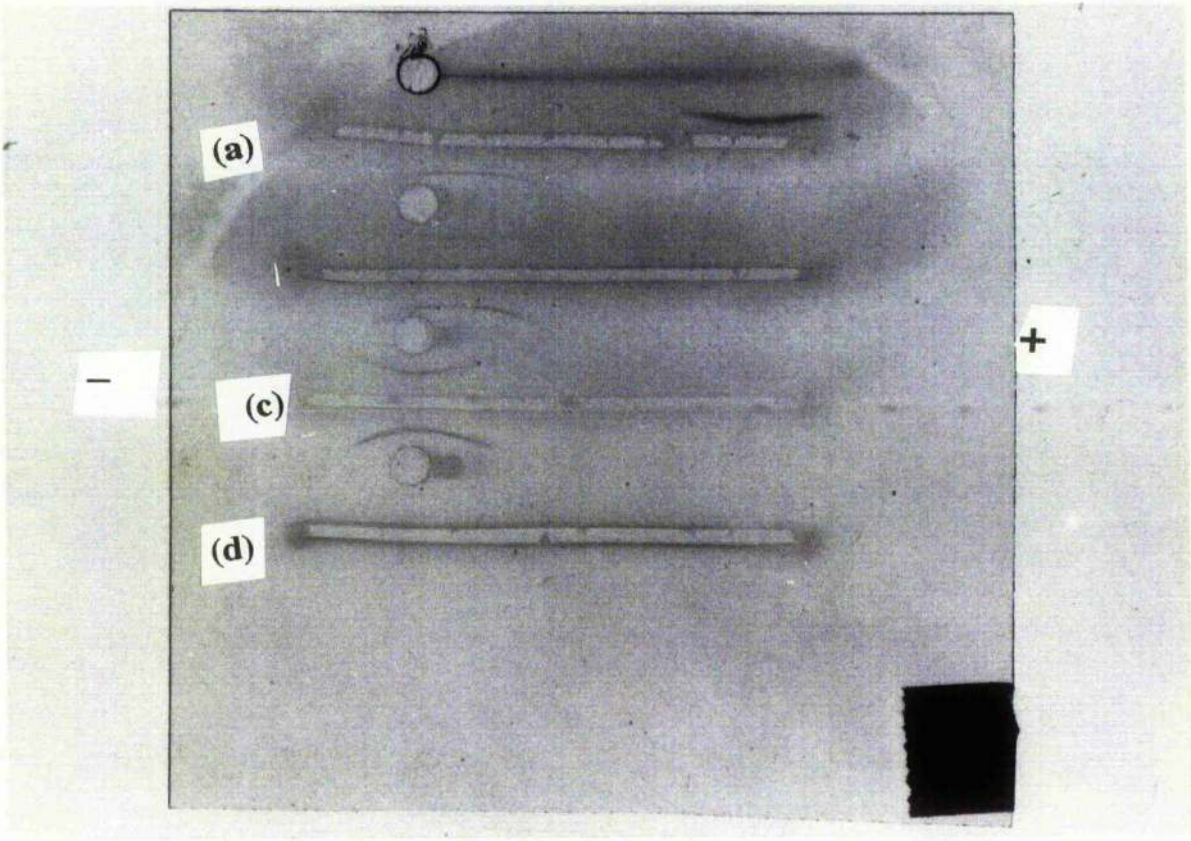
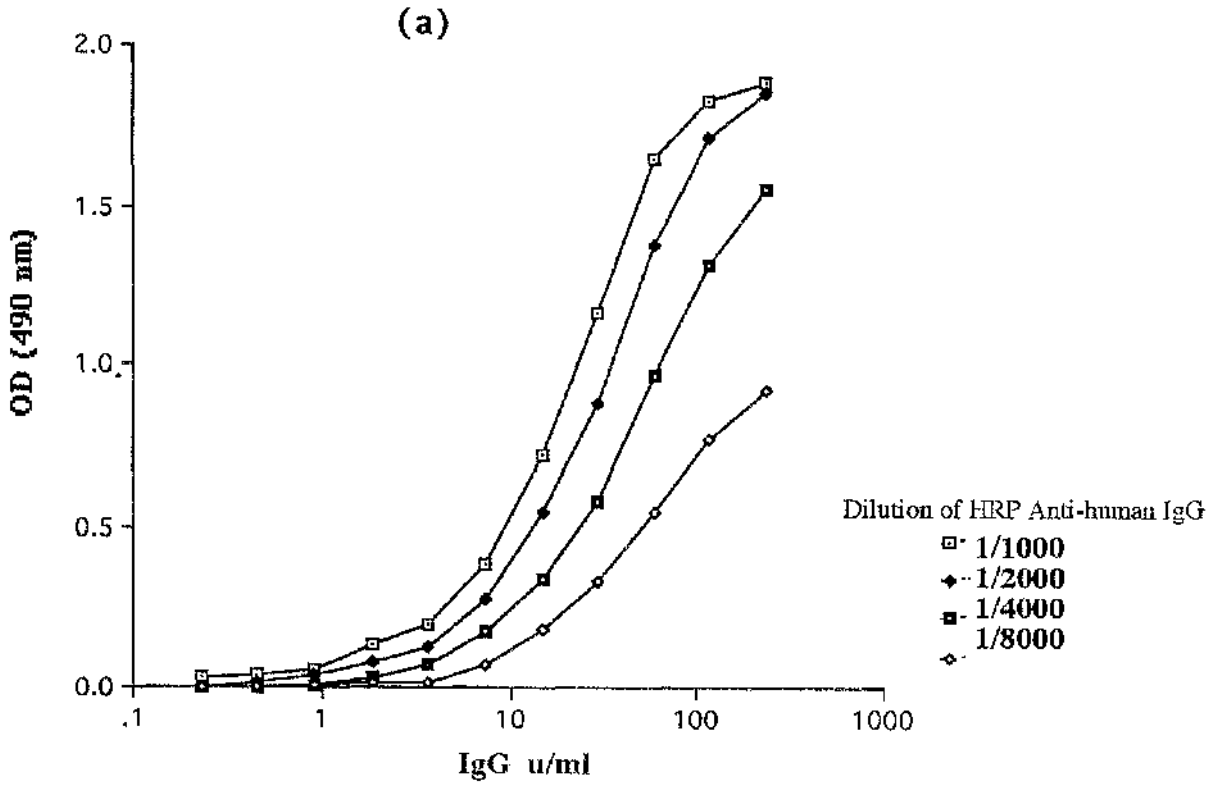


Figure 3-4: IEP showing purified human anti-streptokinase IgA by affinity chromatography run in wells and after electrophoresis, anti-human IgA (troughs a and b), anti-IgG (trough c) and anti-human IgM (d) were applied.



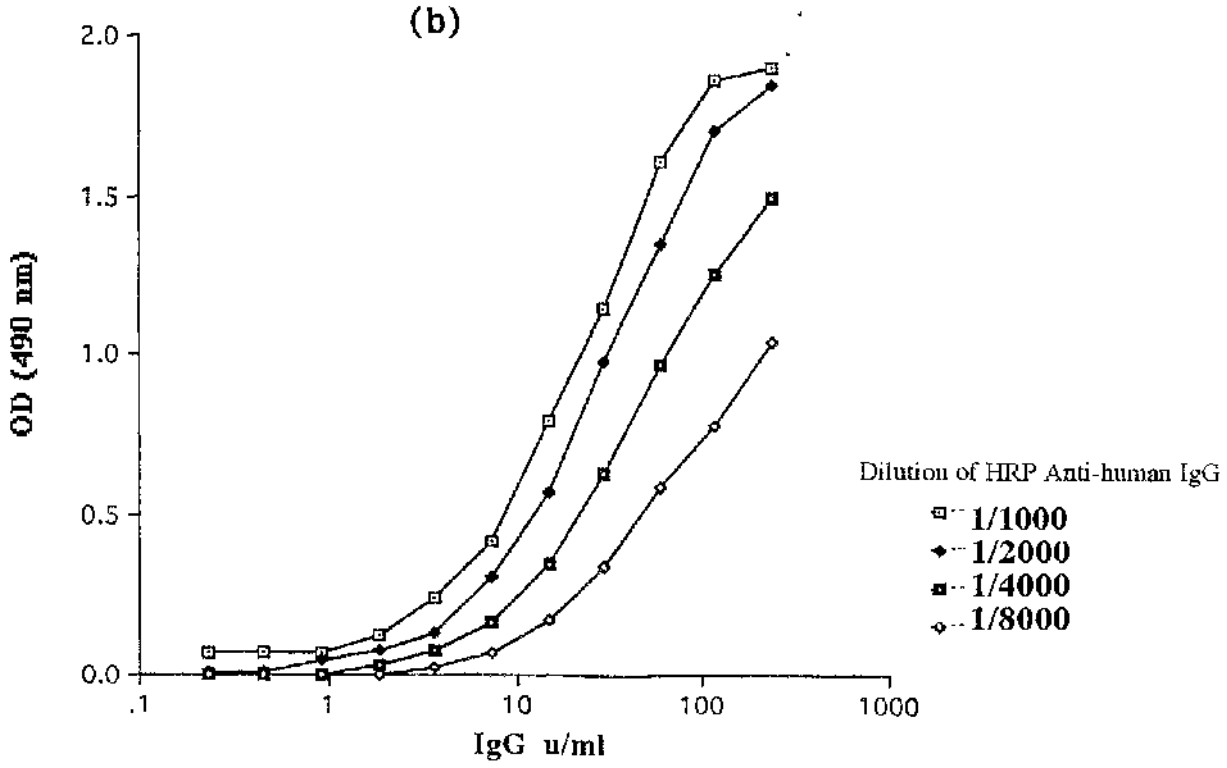
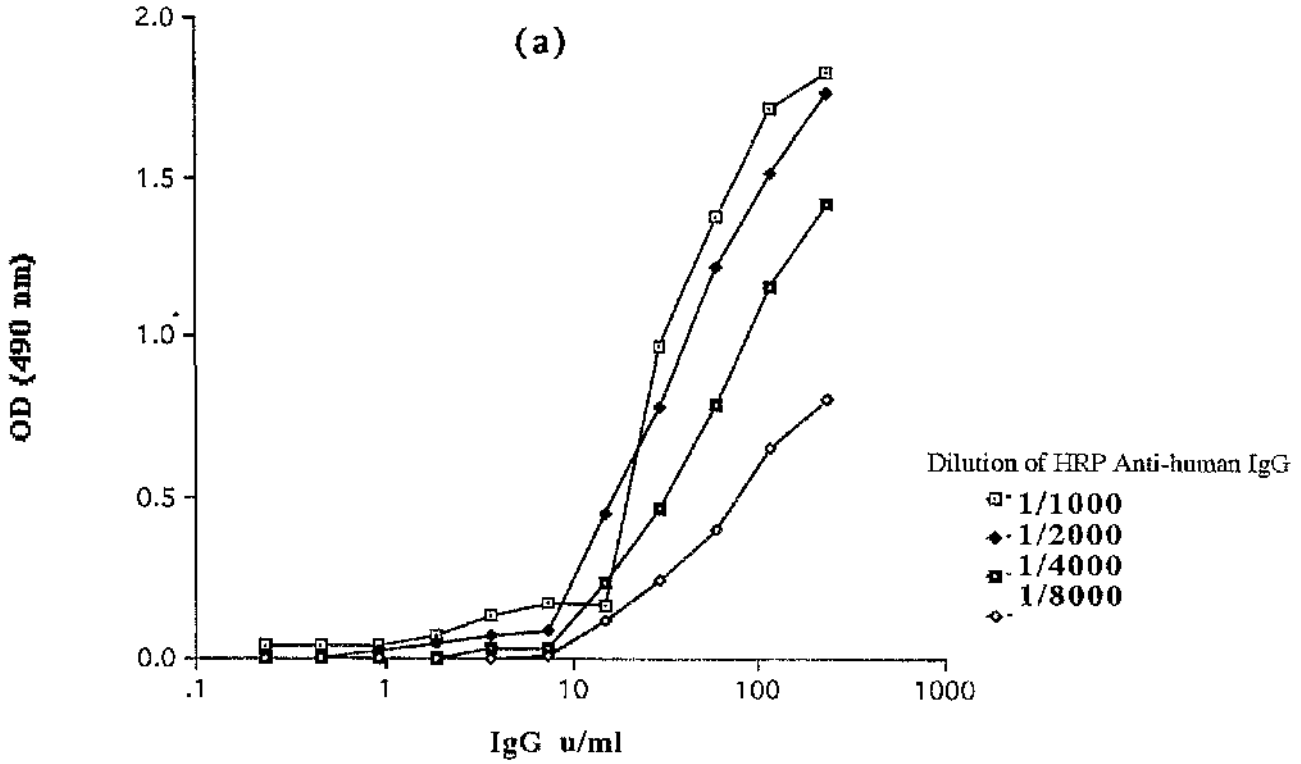


Figure 3-5: Standard curve for IgG anti-streptokinase.
(Streptokinase 5ug/ml (a) and 10ug/ml (b) coated in PBS, pH = 7)



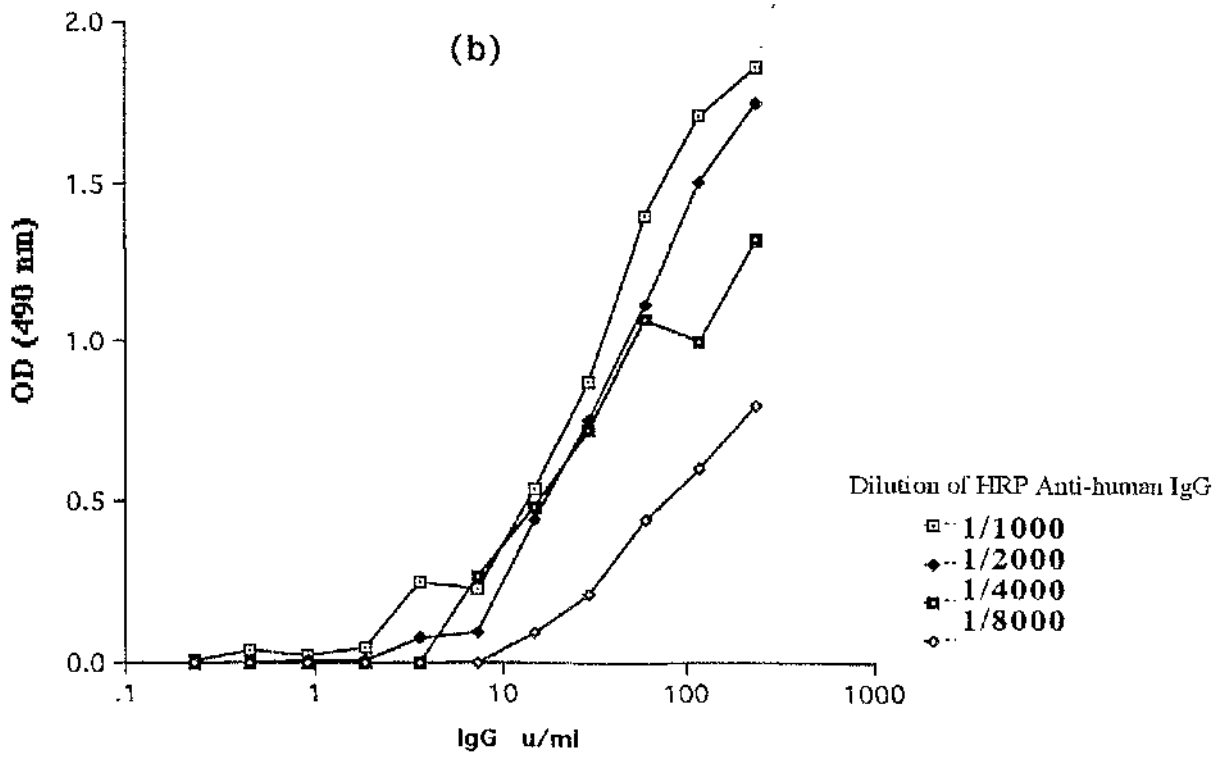


Figure 3-6: Standard curve for IgG anti-streptokinase. (Streptokinase 5 ug/ml (a) and 10 ug/ml (b) was coated in coating buffer, pH=9.6)

The HRP conjugated anti-human IgG was titrated from 1/1000 to 1/8000, the curves were linear from 5 units/ml to 100 units/ml with an optimal colour reaction with the 1/1000 dilution (Figures 3-5 and 3-6).

For IgM and IgA more consistent standard curves and optimal colour reaction was observed with 1/1000 dilution of HRP conjugated anti-human IgM and IgA (Data not presented). Standard curves for IgA and IgM anti-streptokinase were constructed with the positive sera as described (3-5) (Figure 3-7)

For IgE no standard curve was run, samples were expressed as positive or negative based on the OD of the blank.

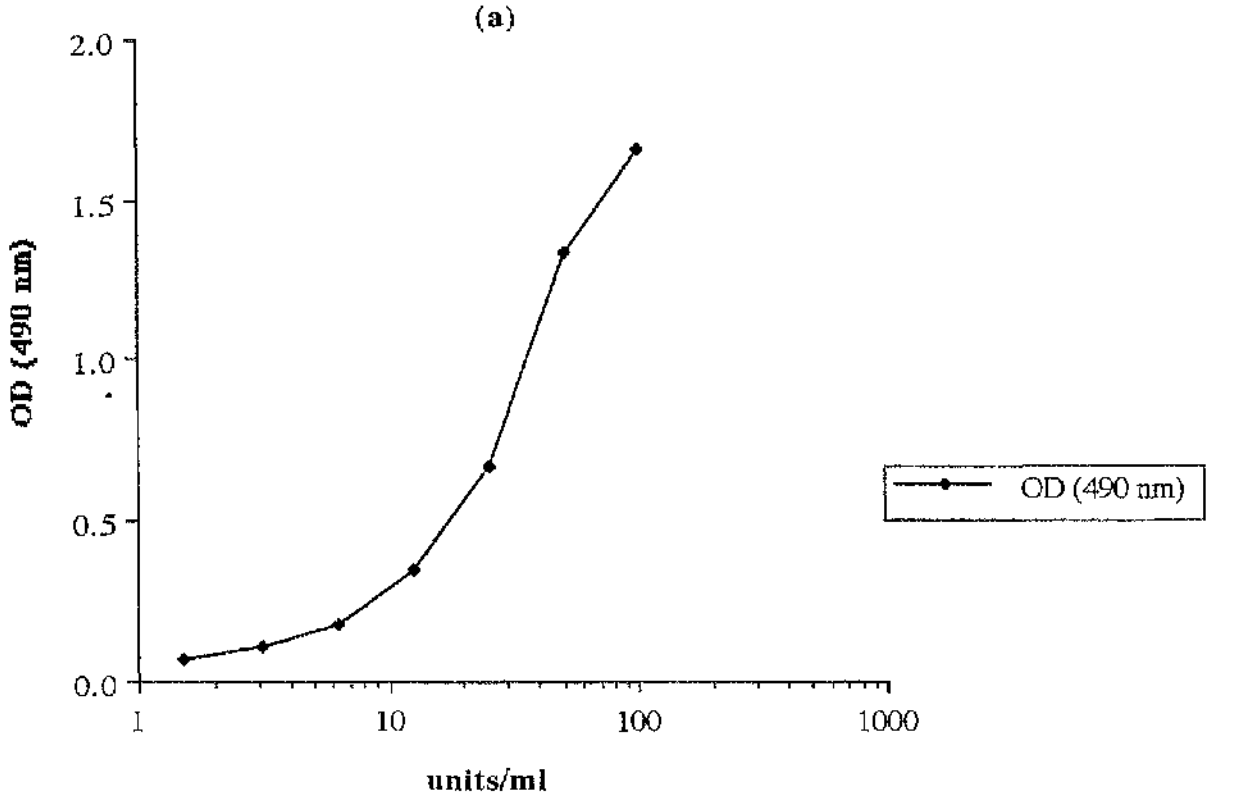
As result of the preceding analysis the following ELISA conditions were chosen: ELISA plates were coated with 5 µg/ml streptokinase in PBS pH 7.0 overnight at 4°C. After washing the plates in PBS-Tween the plates were blocked for one hour at room temperature with 200 µl per well of 0.01% gelatin in PBS.

The plates were washed and standard curves and samples run on the plates as described (3-5).and the plates were incubated for a period of 2 hours at room temperature. After washing horseradish peroxidase conjugated to human IgG , IgA (diluted 1/1000), IgM (For all conjugates diluted 1/1000) and IgE (diluted 1/500) were added and after a further one hour incubation the plates were washed and developed using o-phenylenediamine (OPD) as described (3-5).

Determination of intra-assay and inter-assay variations. The intra-assay and inter-assay variations of the methods employed for measuring anti-streptokinase antibodies were determined to assess accuracy and reproducibility of the methods (3-5).

The CVs for intra-assay variation in the ELISA techniques were between 11% and 20% and for neutralising assay (ASK-kit) the CV was 21% (Table 3-1a).

The CVs for inter-assay variations in the ELISA techniques were between 11% and 17% and for neutralising assay (ASK-kit) the CV was 20% (Table 3-1b).



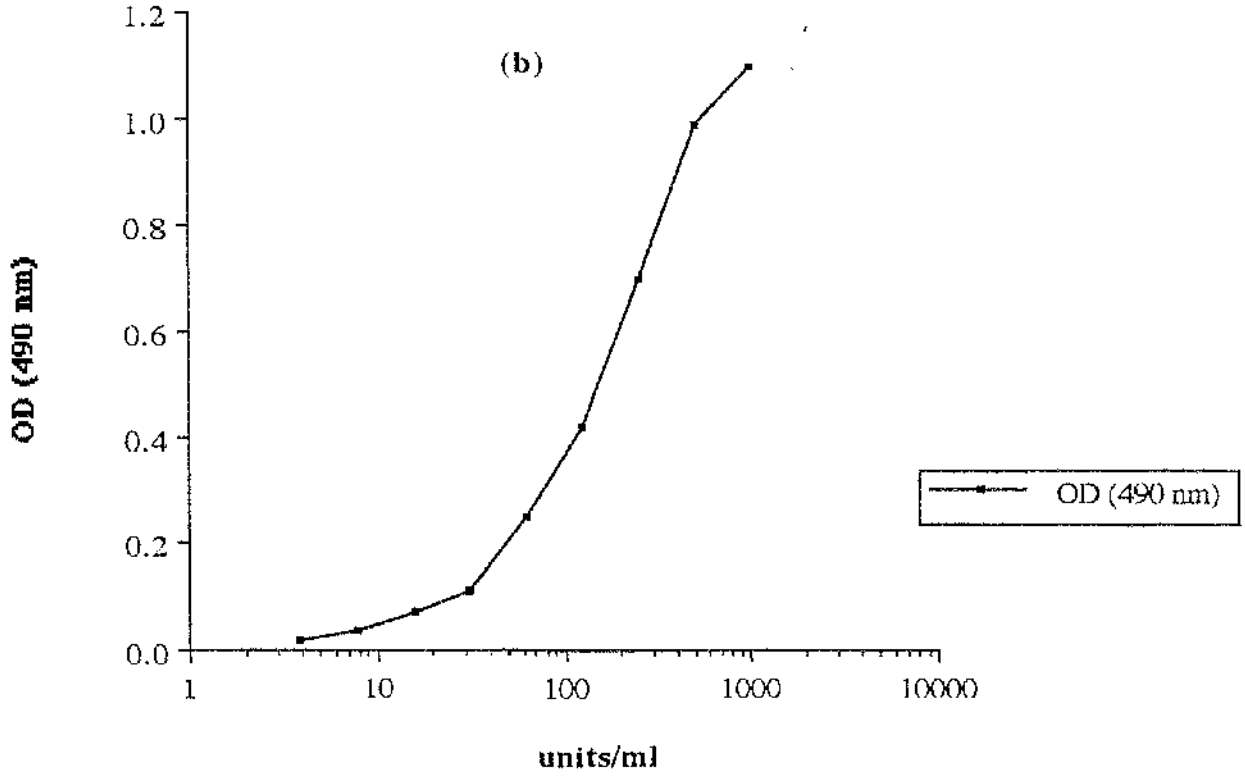


Figure 3-7: Standard curves for measring IgM (a) and IgA (b) anti-streptokinase antibodies by ELISA.

(a)

Isotype	Number of measurements	Mean	Median	S.D.	C.V.
IgG	12	9.21	9.2	1.91	20%
IgA	12	361.9	373.5	40.2	11%
IgM	13	13.8	13	1.88	14%
ASK	12	73.3	80	15.6	21%

Table 3-1a: Intra-assay variations in methods employed for measuring anti-streptokinase antibodies (The values are expressed in units/ml).

(b)

Isotype	Number of measurements	Mean	Median	S.D.	C.V.
IgG	8	2.0	1.9	0.2	11%
IgA	7	320	320	46.2	14%
IgM	7	25	24	4.3	17%
ASK	7	74.3	80	15	20%

Table 3-1b: Inter-assay variations in methods employed for measuring anti-streptokinase antibodies (The values are expressed in units/ml).

Intra-assay and inter-assay variations in the ELISA techniques were generally lower compared to the neutralising assay (ASK-kit) (Table 3-1).

Levels of isotype specific anti-streptokinase antibodies in a normal population. IgG, IgM, IgA and IgE anti-streptokinase antibodies levels were determined in 246 normal individuals (3-7). The values attained for all isotypes for the population studied were skewed in their distributions toward the lower end of the range (Figure 3-8a, b and c). To normalise the distributions, the values were transformed to Log^{10}. To transform the 0 values, they were assigned a value which was half of the lowest value obtainable from the standard curve for each isotype. The normal ranges of anti-streptokinase antibodies for each isotype were then established using mean ± 2SD (Table 3-2a, Figure 3-8a, b and c).

Most of the normal individuals had detectable levels of IgG and IgA anti-streptokinase antibodies (90%), however, only 1.68% and 2.7% respectively had levels above the normal ranges (Table 3-2, Figure 3-8a and c). In contrast 76% of the normal individuals did not have any detectable levels of IgM anti-streptokinase antibodies, 14.1% had levels within the normal ranges and 9.9% had levels above the normal range (Table 3-2, Figure 3-8b).The levels of the individual isotype responses did not correlate with one another.

None of the normal individuals tested showed any IgE anti-streptokinase activity (3-4).

Levels of neutralising anti-streptokinase antibodies in a normal population. The values of neutralising anti-streptokinase antibodies in the normals showed a skewed distribution. To normalise the data, the values were transformed to Log_{10}. To transform the 0 values, they were assigned as value of half of the lowest value obtained in the assay (i.e. 5 u/ml). The normal range for

(a)

Isotype	Mean	Median	Normal ranges
IgG	5.7	3.8	0.24 - 45.8
IgA	31.3	15.1	0.37 - 324
IgM	1.9	0	0.06 - 10.8
ASK	29.2	20	1.7 - 166

Table 3-2a: Mean, median and normal ranges of anti-streptokinase antibodies (Values are expressed in units/ml).

(b)

Isotype	Controls (n=240)	SK (n=10)	r-tPA (n=10)
IgG	1.68%	20%	0%
IgA	2.7%	10%	0%
IgM	9.9%	80%	50%
ASK	0.4%	10%	0%

Table 3-2b: Percentage of the individuals with elevated levels of anti-streptokiase antibodies in normal controls and patients treated with streptokinase (SK) or recombinant tissue plasminogen activator (r-tPA).

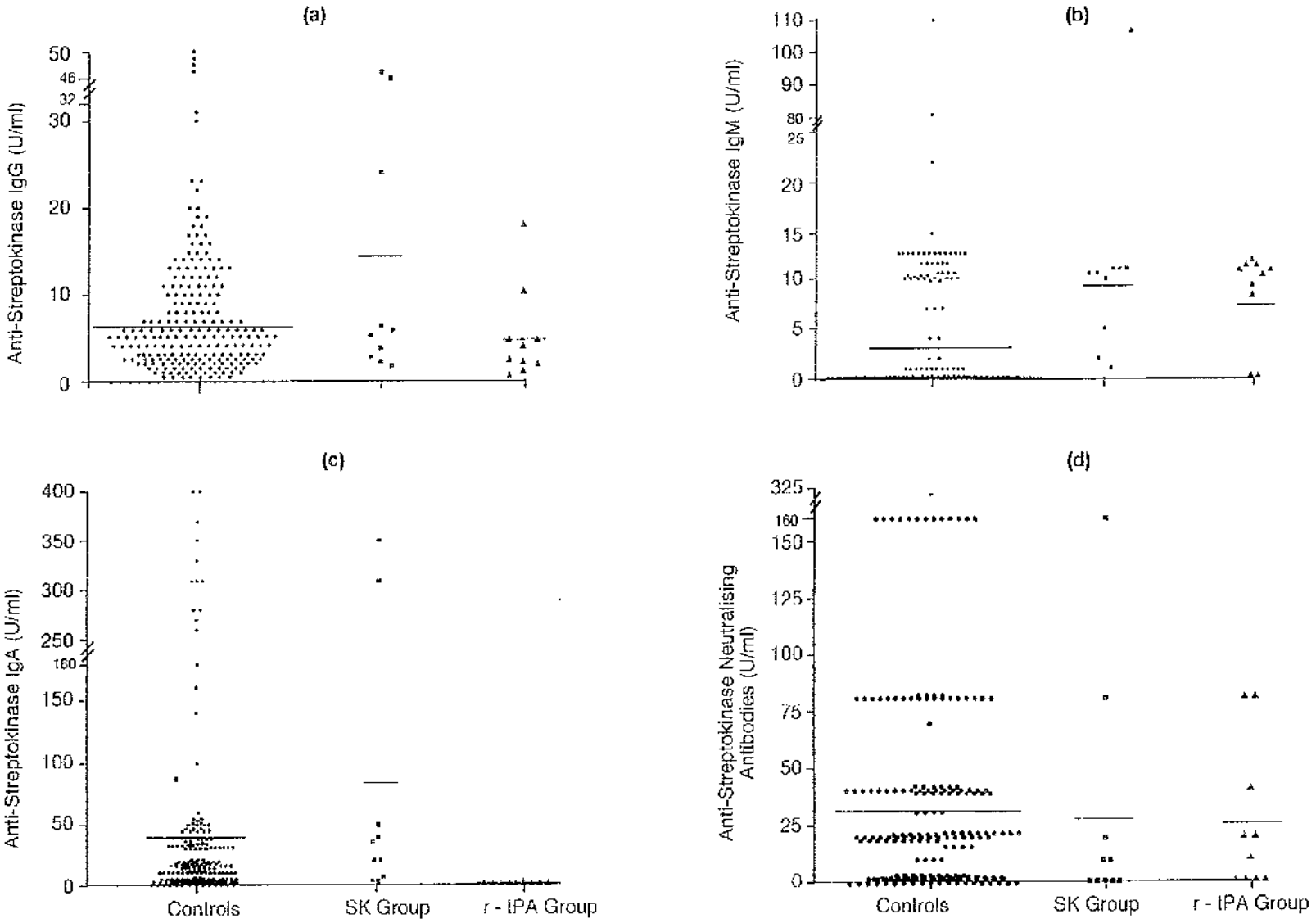


Figure 3-8: The levels of isotype specific and neutralising anti-streptokinase antibodies in

neutralising anti-streptokinase antibodies was then established using mean ± 2SD (Table 3-2a, Figure 3-8d).

In the population studied 39.8% of the normal individuals did not have any detectable levels of neutralising anti-streptokinase antibodies, 59.8% had levels within the normal ranges and only 0.4% had levels above the normal range (Table 3-2b, Figure 3-8d).

The levels of neutralising anti-streptokinase antibodies in the normal individuals showed a statistically significant positive correlation with the levels of IgG anti-streptokinase ($r = 0.553$, $p < 0.001$) but not with IgM ($r = 0.005$) or IgA ($r = 0.04$) levels.

Isotype specific response and neutralising anti-streptokinase antibodies in patients with acute myocardial infarction. Of the 20 patients studied, pre- and post-thrombolytic treatment for AMI, 10 were treated with r-tPA and 10 with streptokinase (Figure 3-8a, b, c and d).

The pre-treatment levels of isotype specific and neutralising anti-streptokinase antibodies in the 20 patients did not show statistically significant difference from the control population (Figure 3-8).

Based on the calculated normal ranges it was observed that 10-80 % of patients who were treated with streptokinase had levels of anti-streptokinase antibodies above the normal ranges (Table 3-2b) as following: for IgG 20% of patients had elevated levels of antibodies (Controls 1.68%), IgA 10% (Controls 2.7%), IgM 80% (Controls 9.9%) and neutralising antibodies 10% (Controls 0.4%) (Figure 3-8a, b, c and d, Table 3-2b).

In contrast to the normal control group the levels of IgG in the 10 patients, prior to administration of streptokinase did not correlate with neutralising antibodies, however, they correlated with the levels of IgA ($r=0.58$ $p<0.08$).(Table 3-3a). The levels of IgA in these patients also correlated with neutralising antibodies ($r=0.75$

a

Correlation	r value	Significant
IgG/ASK	0.4	N.S.
IgG/IgM	- 0.44	N.S.
IgG/IgA	0.58	$p < 0.08$
IgA/IgM	0.07	N.S.
IgA/ASK	0.75	p <0 .01
IgM/ASK	0.1	N.S.

b

Correlation	r value	Significant
IgG/ASK	0.67	p<0.03
IgG/IgM	0.06	N.S.
IgG/IgA	0.77	$p < 0.009$
IgA/IgM	0.4	N.S.
IgA/ASK	0.72	p <0 .01
IgM/ASK	-0.06	N.S.

Table 3-3: Correlation between the levels of anti-streptokinase antibodies prior to administration of thrombolytic agents in 10 AMI patients treated with streptokinase (a) and in 10 AMI patients treated with r-tPA (b).

p<0.01) (Table 3-3). The levels of IgG and IgM did not correlate with neutralising antibodies or one another (Table 3-3a).

In the group of the patients treated with r-tPA 50% had elevated levels of IgM anti-streptokinase antibodies (Controls 9.9%), however, none of them had elevated levels of IgG, IgA and neutralising anti-streptokinase antibodies (Figure 3-8 a b c and d, Table 3-2b).

Similar to the normal control group and in contrast to streptokinase patients group the levels of IgG anti-streptokinase in the 10 patients treated with r-tPA, prior to administration of r-tPA correlated with neutralising antibodies (r=0.67 p<0.03) (Table 3-3b). The levels of IgA correlated with the levels of IgG (r=0.77 p<0.009) and neutralising antibodies (r=0.72 p<0.01) (Table 3-3b).

An IgE response was not detectable in any of the patients treated with thrombolytic agents.

Out of ten patients given streptokinase two patients (Patients EM and CD) had elevated antibody levels of IgG anti-streptokinase antibodies (46 and 47 units/ml, normal range=0.24-45.8 units/ml) and had levels of IgA anti-streptokinase antibodies of 310 and 350 units/ml (Normal range=0.37-324), only CD's value for IgA levels was above the normal range. Only one patient (EM) had elevated levels of neutralising anti-streptokinase antibodies (160 units/ml) (Normal range=1.7-166).

The effect of administration of r-tPA on the levels of anti-streptokinase antibodies. The levels of isotype specific and neutralising anti-streptokinase antibodies in patients treated with r-tPA were studied during the time course of treatment till day 7. These patients did not show statistically significant changes in the levels of isotype specific anti-streptokinase antibodies, as measured by ELISA's (Table 3-4). However, quantitation of neutralising anti-streptokinase by the ASK-kit (3-1) showed a significant reduction in the levels of neutralising

Isotype	Pre-treatment (n=10)	Post-treatment (n=10)
IgG	5.1	5.6
IgA	0.4	0.5
IgM	8.3	9.4
ASK	28	2.2

Table 3-4: Pre- and post-treatment (at 10 minutes) mean values of IgG, IgA, IgM and neutralising anti-streptokinase antibodies (ASK) in patients treated with r-tPA (the values are expressed in units/ml).

anti-streptokinase in post-treatment samples compared to pre-treatment values at 10 minutes ($p < 0.01$) (Table 3-4).

In vitro effect of r-tPA on quantitation of neutralising anti-streptokinase antibodies. Since administration of r-tPA to patients, reduced the levels ASK titre at 10 minutes, the interference of r-tPA in this assay was examined (3-10). When serum with known elevated levels of anti-streptokinase antibodies (80 units/ml) was added to serial dilutions of r-tPA a sudden reduction in the measurable titre of neutralising anti-streptokinase antibodies was observed (Figure 3-9). The presence of 2.5 μg/ml of r-tPA (Therapeutic dose = 1000 μg/ml) dropped the titre from 80 units/ml to undetectable levels.

The effect of administration of streptokinase on the levels of anti-streptokinase antibodies in ten patients with AMI. The levels of isotype specific and neutralising anti-streptokinase antibodies in patients treated with streptokinase were studied serially every 15 minutes during the first two hours of treatment and then every day till day 7.

Administration of streptokinase in the group as whole resulted in an immediate fall in the levels of IgG anti-streptokinase antibodies within 15 minutes ($n=10$, $p < 0.01$), the levels remaining depressed until day 5 (Figure 3-10a). The levels of IgG anti-streptokinase antibodies then began to rise and reached values above the pre-treatment levels by the day 7 (Figure 3-10a). Analysis of the individual patients within the group showed the same general fluctuations for IgG, as seen when the mean values were compared (Figure 3-11a). Even those individuals with low levels of IgG showed a drop at 15 minutes after streptokinase administration (Figure 3-11a).

As described two patients treated with streptokinase had elevated levels of anti-streptokinase IgG prior to treatment (patients EM and CD), these patients in particular showed a dramatic reduction (50%) in the levels of IgG anti-streptokinase

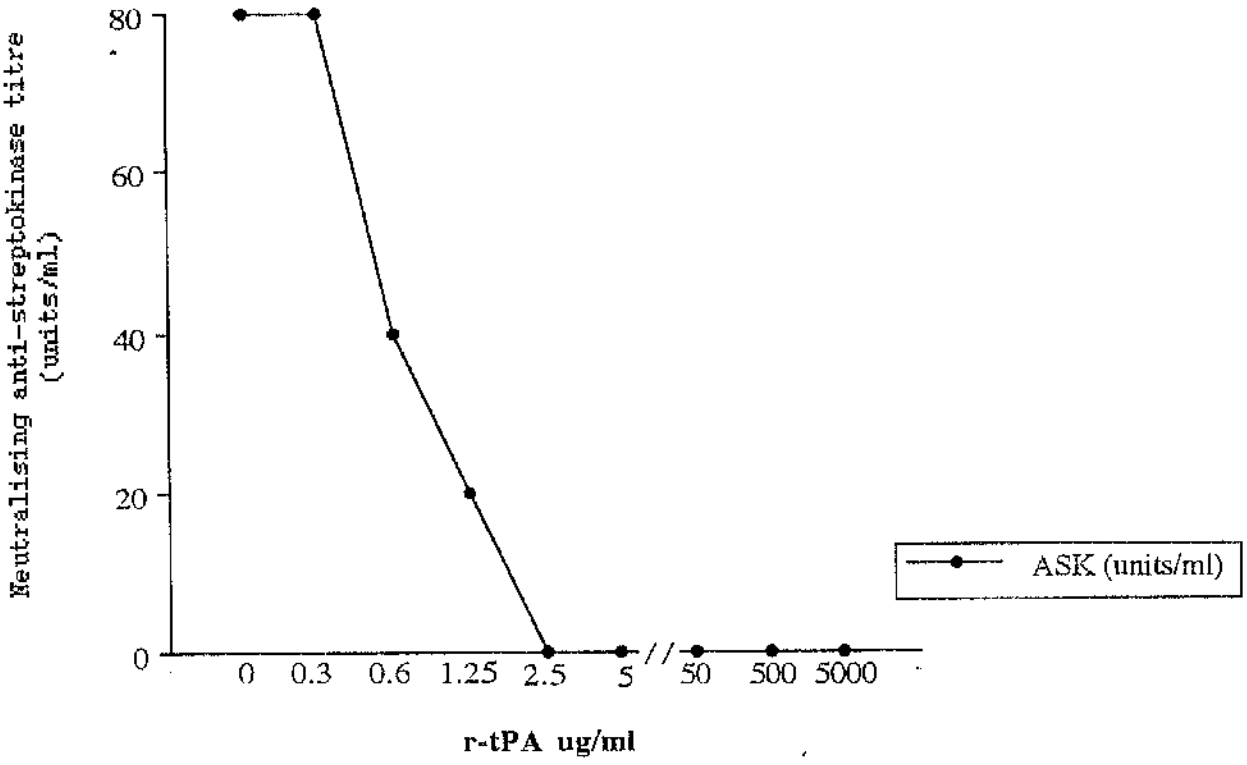


Figure 3-9 : In vitro effect of r-tPA on the titre of neutralising anti-streptokinase antibodies.

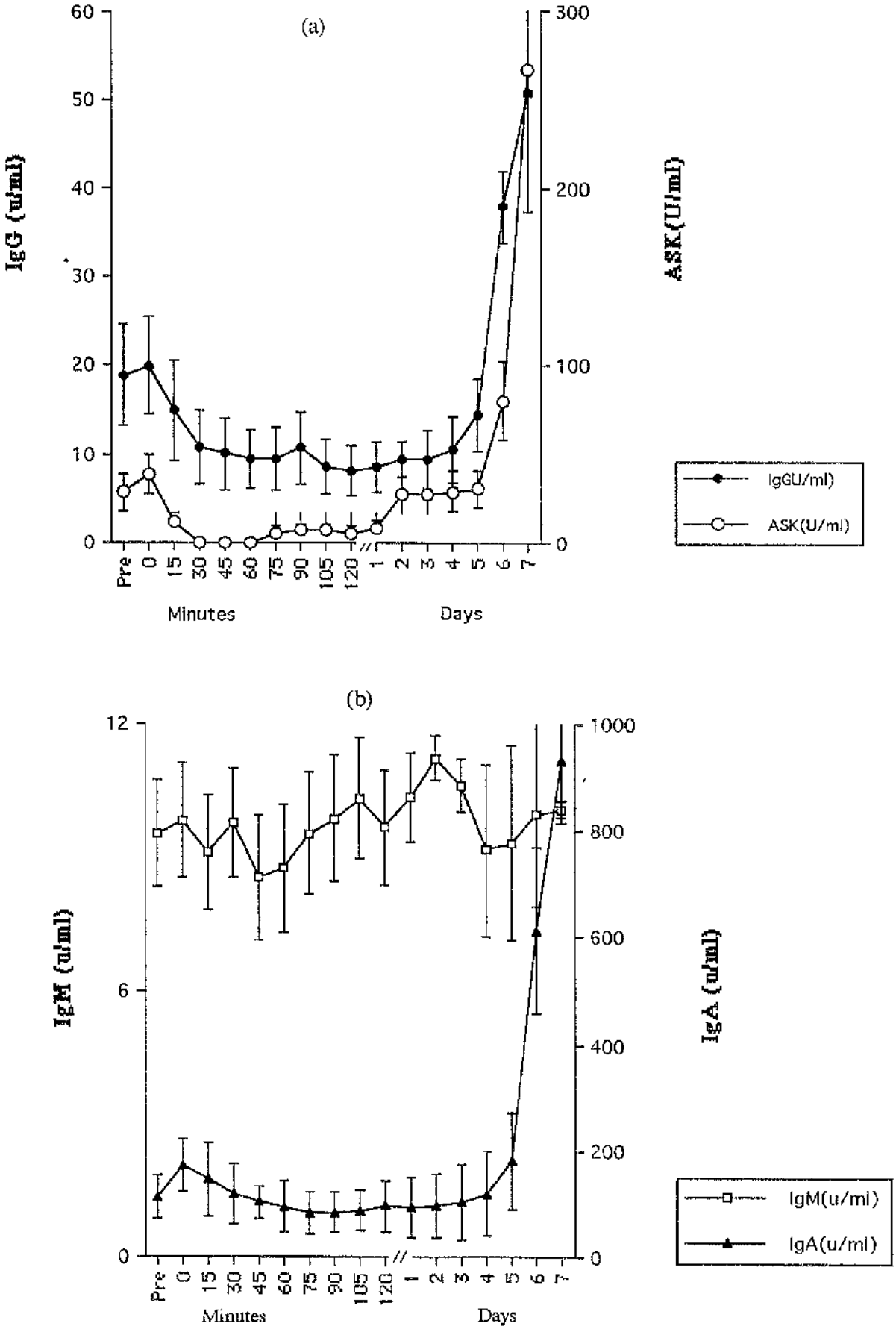


Figure 3-10: Fluctuations of the anti-streptokinase antibodies in 10 patients treated with streptokinase (Vertical bars represent SE).

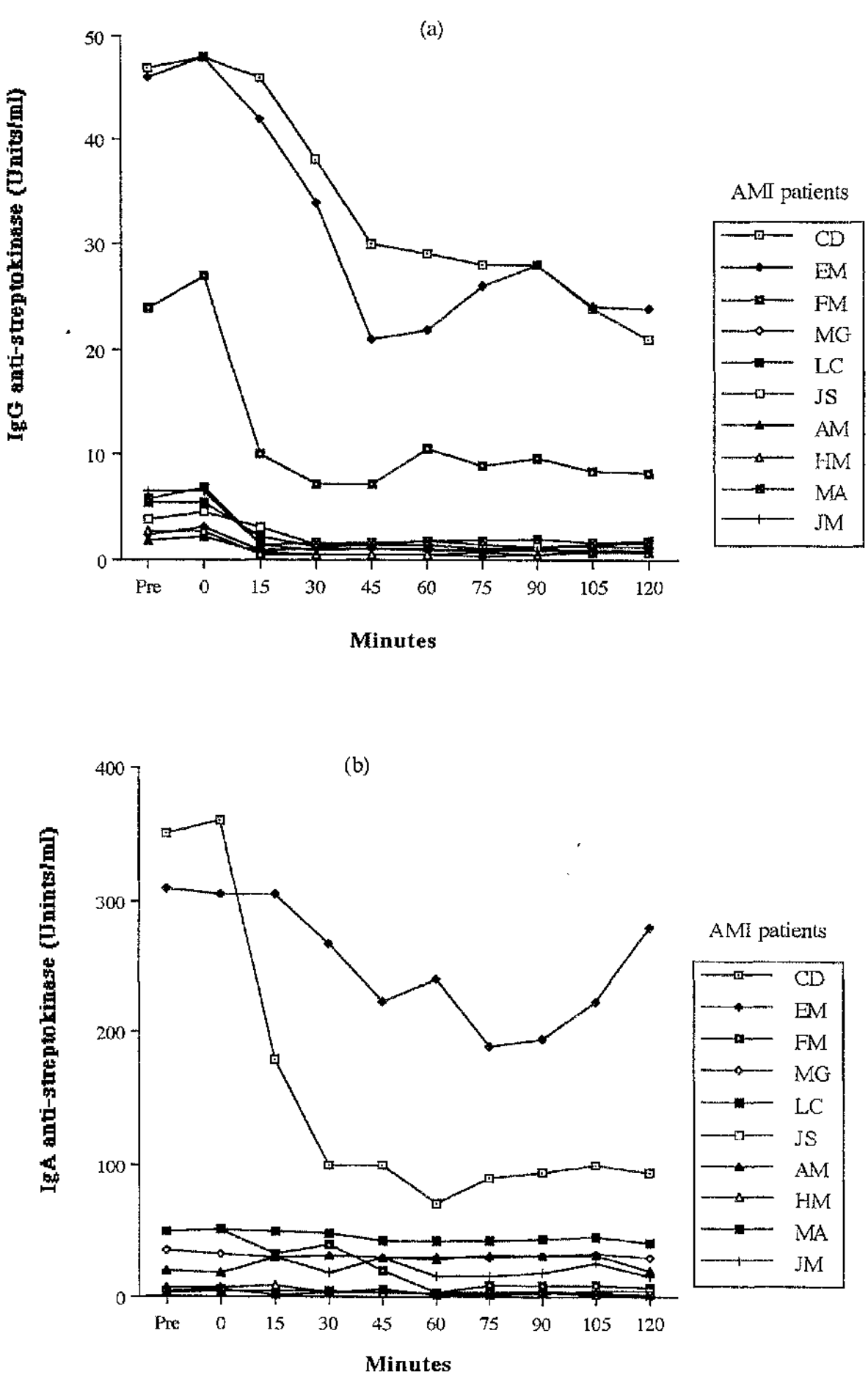


Figure 3-11: Fluctuations of IgG (a) and IgA (b) anti-streptokinase antibodies in 10 patients with acute myocardial infarction (AMI) during the first 2 hours of streptokinase treatment.

antibodies after streptokinase administration (Figure 3-11a), although the levels of IgG anti-streptokinase antibodies in these individuals (patients CD and EM) took longer to drop than those with lower levels (Figure 3-11a).

Administration of streptokinase in the group as a whole decreased the levels of IgA anti-streptokinase antibodies in 60 minutes and the levels remained depressed until day 6 (Figure 3-10b). The levels of IgA then began to rise and reached values above the pre-treatment levels by the day 7 (Figure 3-10b). Analysis of the individual patients within the group showed that administration of streptokinase in a patient with elevated levels of IgA (Patient CD) resulted in a significant fall (80% reduction at 30 minutes) in the levels of IgA (Figure 3-11b). The reduction in the levels of IgA in patient EM who also had high levels of IgA occurred more slowly and the other patients with the lower levels of IgA showed little variation over the time course (Figure 3-11b).

The levels of IgM in the 10 patients did not show any significant changes during and after streptokinase infusion (Figure 3-10b), nor were any significant changes seen when individual patients were studied (Figure 3-12b). Only one patient (Patient LC) in this group showed a significant reduction in the levels of IgM (90% at 45 minutes) after streptokinase administration (Figure 3-12b).

Administration of streptokinase resulted in an immediate fall in the levels of neutralising anti-streptokinase antibodies within the 30 minutes ($p < 0.01$), the levels remaining undetectable until day 2 (Figure 3-10a). The levels of neutralising anti-streptokinase antibodies then began to rise and reached the values above the pre-treatment levels by the day 7 (Figure 3-10a). Analysis of the individual patients within the group showed the same general fluctuations for neutralising antibodies, as when the means were compared (Figure 3-10a and 3-12a).

All the patients had elevated levels of specific antibodies to streptokinase by day 5, corresponding to an immune response to the administered antigen (Figure 3-10a,b).

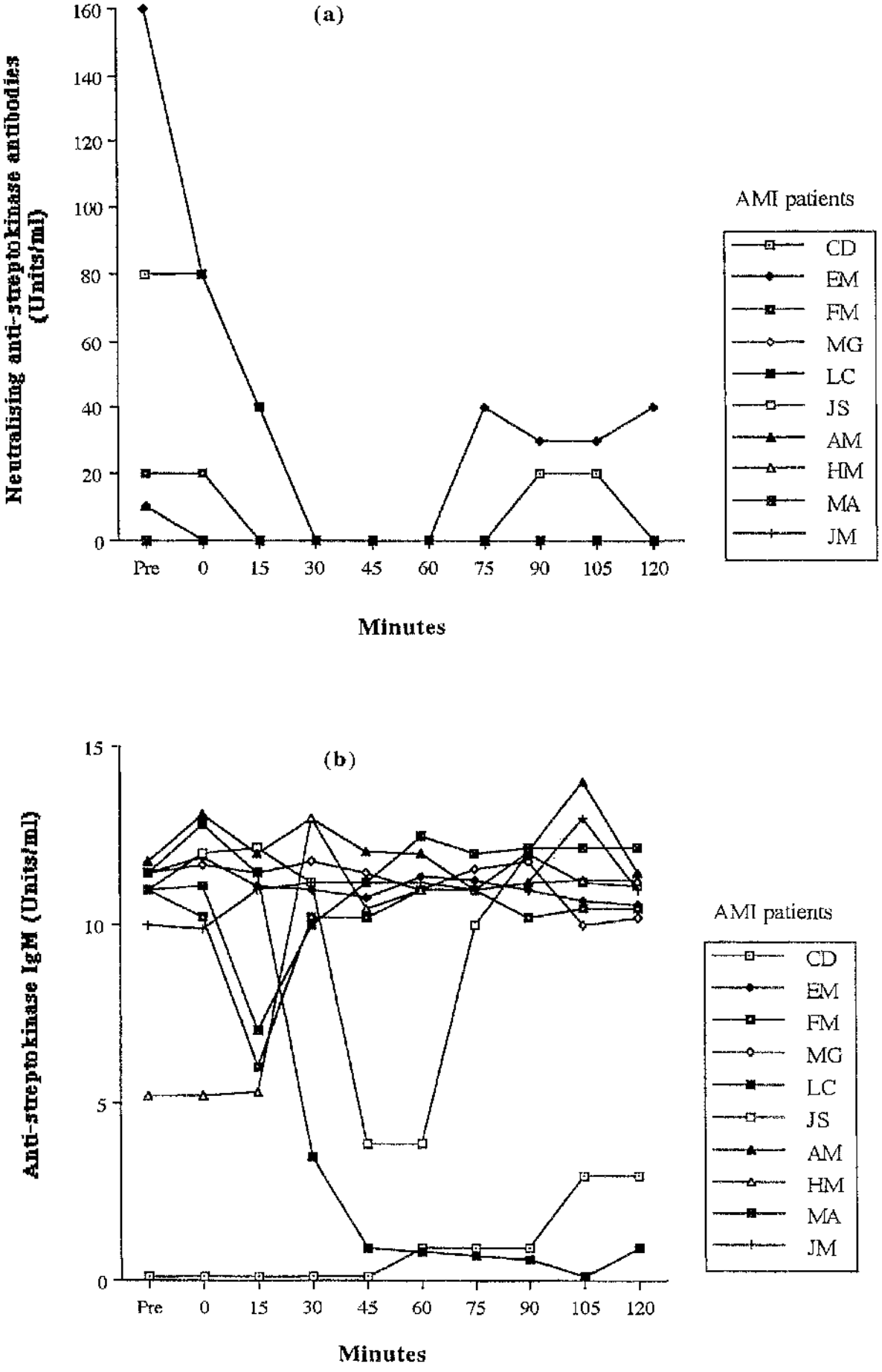


Figure 3-12: Fluctuations of neutralising (a) and IgM (b) anti-streptokinase antibodies in 10 acute myocardial infarction (AMI) patients during the first 2 hours treatment with streptokinase.

The levels of serum albumin during thrombolytic therapy. To determine whether clinical procedure and changes in plasma volume had any effect on the levels of proteins in patients with AMI the levels of serum albumin during streptokinase treatment were measured by single radial immunodiffusion (3-9). The administration of streptokinase did not cause any effect on the levels of serum albumin when post-treatment levels were compared with pre-treatment levels (Mann-Whitney test, U values = 23-46, not significant for all time points). The mean values of serum albumin levels of during streptokinase treatment are presented in Table 3-5 and Figure 3-13.

The effect of anti-streptokinase antibodies on clinical out-come of thrombolytic therapy. The patients were categorised into 3 groups according to their responses to thrombolytic therapy, early reperfusion group (less than 2 hours), late reperfusion group (less than 24 hours) and a group which showed no reperfusion.

Of 10 patients treated with r-tPA, 4 had an early reperfusion, 5 had late reperfusion and 1 did not reperfuse. Of 10 patients treated with streptokinase, 5 had early reperfusion, 3 had late reperfusion and 2 did not reperfuse (Table 3-6).

All of the patients (n=5) who reperfused within 2 hours of receiving streptokinase (early reperfusion group), had levels of IgG, IgA and neutralising anti-streptokinase antibodies which were within the normal ranges (Table 3-6). The mean values of IgG (8.2 units/ml), IgA (43 units/ml) and neutralising anti-streptokinase (10 units/ml) in this group were lower than late reperfusion group (17.5, 124 and 30 units/ml respectively) and the patients group which showed no reperfusion (26, 180 and 80 units/ml respectively) (Table 3-6, Figure 3-14).The levels of IgM anti-streptokinase antibody did not differ significantly in the 3 groups (Mean values for the 3 group in order were 9.8, 7.6 and 11.5 units respectively).

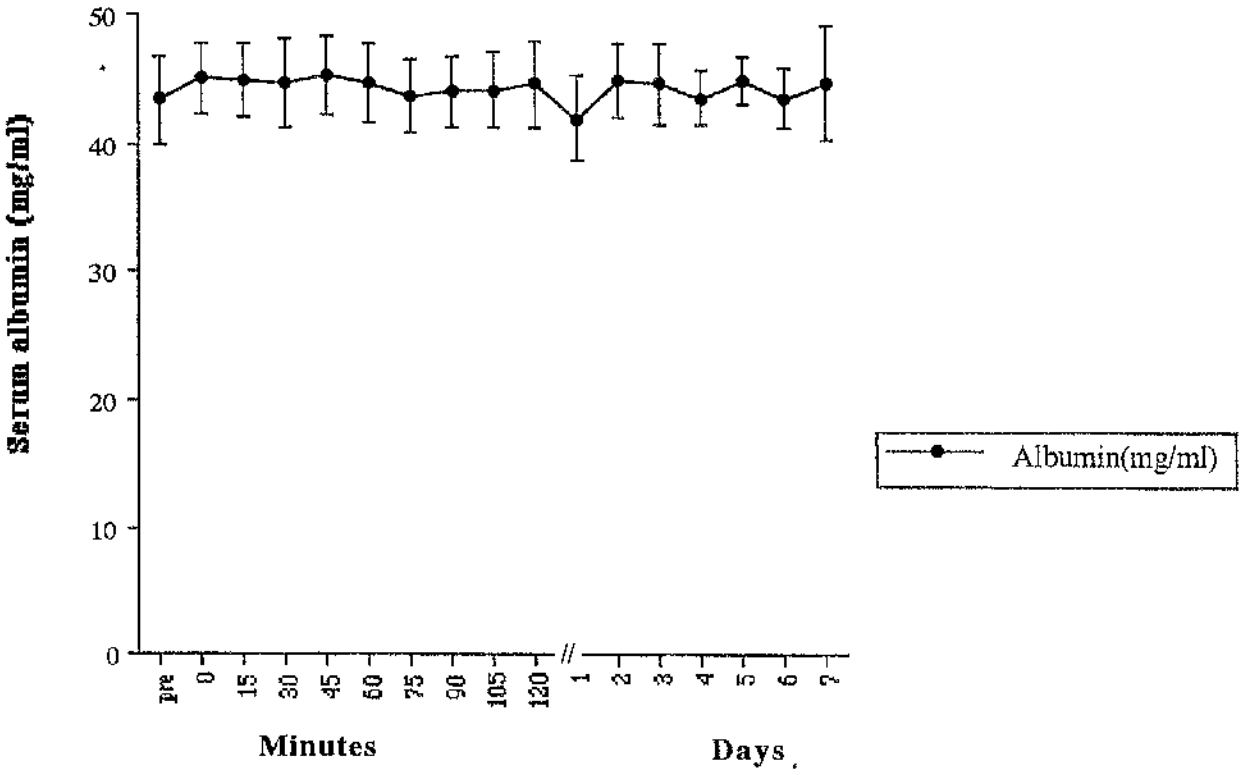


Figure 3-13: The levels of serum albumin during streptokinase administration in 10 patients with AMI.

Time (Minutes)	**Serum Albumin (mg/ml)**
Pre-treatment	40
0	45.1
15	45.5
30	45.8
45	43.4
60	44.9
75	43.9
90	44.1
105	4.3
120	44.8
Day 1	42.1
Day 2	44.3
Day 3	44.8
Day 4	43.7
Day 5	45
Day 6	43.6
Day 7	44.8

Table 3-5: Mean values of serum albumin levels fluctuations in ten patients with AMI treated with streptokinase.

(a)

Early	IgG	IgM	IgA	ASK
J.M.	6.4	10	20	0
M.G	2.4	11.5	36	10
M.A.	5.8	11.1	5	0
F.M.	24	11	39	20
H.M	2.7	5.2	7	0

(b)

Late	IgG	IgM	IgA	ASK
J.S.	3.8	11	3	0
A.M.	1.72	11.8	20	10
C.D.	47	0.1	350	80

(c)

Non	IgG	IgM	IgA	ASK
E.M.	46	11.5	310	160
L.C.	5.4	11.5	49	0

Table 3-6: The levels of anti-streptokinase antibodies in AMI patients treated with streptokinase that had early reperfusion (a), late reperfusion (b) and or did not reperfuse (c).The values are expressed in units/ml.

One of the 3 patients who reperfused late (patient CD) had elevated levels of IgG (47 units/ml) and IgA (350 units/ml) anti-streptokinase antibodies (Table 3-6). Two of the patients (patients EM and LC) did not respond to streptokinase treatment (Non-reperfusion group). Patient EM had elevated levels of anti-streptokinase antibodies (IgG=46 units/ml, IgA=310 units/ml and neutralising antibodies=160 units/ml) (Table 3-6). Patient LC had levels of IgG, IgM, IgA and neutralising anti-streptokinase antibodies that were within the normal ranges (IgG=5.4 units/ml, IgM=11.5 units/ml, IgA= 49 units/ml and neutralising antibodies=0 units/ml, Table 3-6), however, the levels of IgM anti-streptokinase in this patient after streptokinase administration reduced by 91% (Figure 3-12b).

Of the 10 patients given streptokinase, one patient (Patient CD, see above) developed adverse reaction to this agent. She developed fever, became unconscious and was admitted to I.T.U. After extensive bacteriology studies diagnosis of serum sickness was made, she was treated with corticosteriods, her condition improved and she was discharged from I.T.U. Patient EM who had also elevated levels of IgG anti-streptokinase antibodies (IgG= 46 units/ml, IgA= 310 units/ml and neutralising antibodies = 160 units/ml), did not develop adverse reaction to streptokinase administration, however, did not reperfuse.

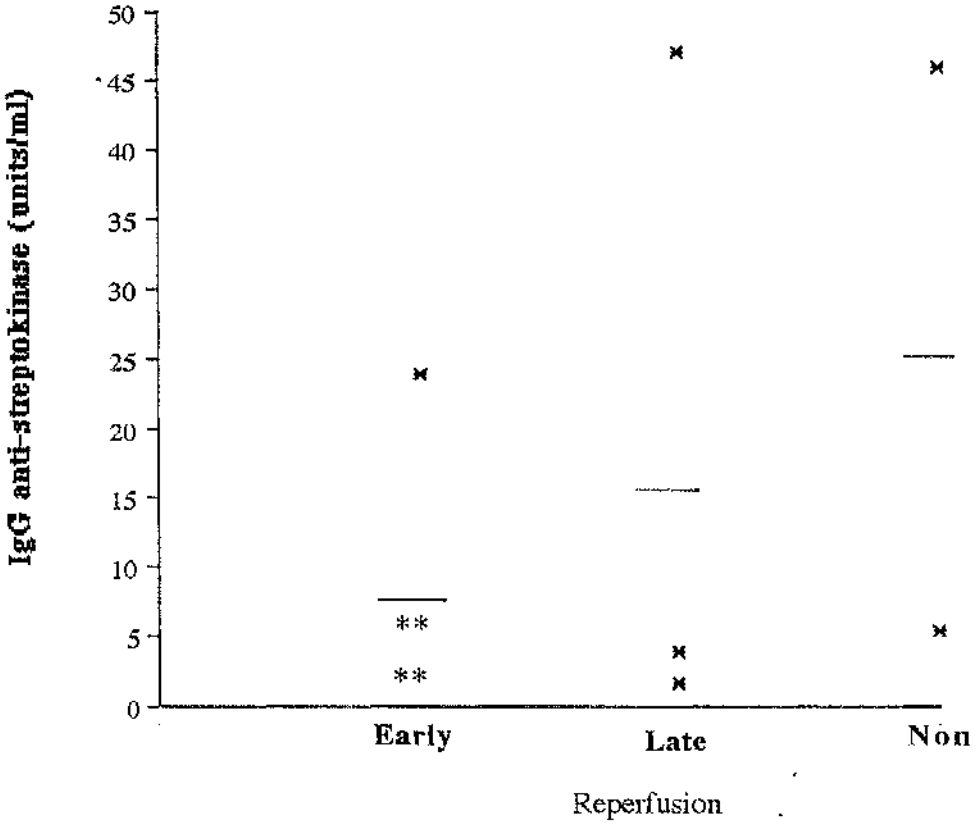


Figure 3-14: The effect of pre-existing IgG anti-streptokinase antibodies on reperfusion in 10 patients treated with streptokinase.

Discussion

Streptokinase is now a commonly administered thrombolytic agent in patients with AMI. By promoting clot lysis the mortality rate is reduced (White et al 1990, Anderson et al 991, Chouhan et al 1992), it has the same efficacy as r-tPA (GISSI-2, 1990, White et al 1990) but is often chosen preferentially as it is about ten times cheaper (Sharma & Sashara 1982). One of the main disadvantages of the streptokinase administration is that, as a foreign antigen it may provoke hypersensitivity reactions, through the presence of pre-existing anti-streptokinase antibodies in patients as a result of previous streptococcal infections. In addition pre-existing antibodies are thought to reduce the lytic effect of streptokinase (Garner & Tillet 1935, Lew et al 1984, Massel et al 1991, Fears et al 1992a, Bom et al 1993). To what extent these antibodies exist in the population and the role of isotype specific anti-streptokinase antibodies in triggering hypersensitivity reactions or reducing efficacy have not been fully studied.

Many types of methodology have been employed to measure anti-streptokinase responses. As described radio immunoassays have been employed to measure IgG and IgE anti-streptokinase antibodies, counter immuno-electrophoresis and radial immunodiffusion have been infrequently employed for detecting neutralising anti-streptokinase antibodies in patients sera (1-6). Enzyme-linked immunosorbent assay (ELISA) a sensitive and reproducible technique is now commonly used for antibody quantitation (Lynch et al 1991, Fears et al 1992, Elliot et al 1993), this technique is safe and cost effective compared to radioimmunoassays. In addition the ELISA technique can be adapted to measure individual antibody isotypes.

In this study ELISA's were developed to measure the levels of IgG, IgA, IgM, IgE and neutralising anti-streptokinase antibodies in a normal population and to find to what extent these antibodies exist in normal individuals. Serum samples were also analysed for neutralising anti-streptokinase antibodies using a commercial ASK-kit (3-1), as this methodology is still in common use.

The ELISA's were developed using commercial streptokinase used for AMI administration. Molecular weight and purity of the commercial streptokinase used in the different assays was first determined by SDS-PAGE (3-2). SDS-PAGE revealed two close bands with molecular weights of 47 kD and 50 kD (Figure 3-1). This is in agreement with previous reports of 2 close bands corresponding to streptokinase with a molecular weight of approximately 47 kD (Lynch et al 1993). The mobility of streptokinase as observed by this technique showed no difference between non-reduced and reduced conditions, this can be ascribed to the absence of the amino acids cysteine and cystine in the structure of this single chain polypeptide (DeRenzo et al 1967, Morgan & Henschen 1969, Brockway & Castellino 1974, Jackson & Tang 1982) (Figure 3-1). Immunoblotting with anti-streptokinase antiserum confirmed that these bands correspond to the streptokinase molecule at 47 and 50 kD (Figure 3-2). The presence of two bands has been observed by other investigators and reported to be due to the effect of de-amidation of the streptokinase molecule by alkaline buffer (Dillon & Wannamaker 1964, Hugh et al 1965, Einarsson et al 1979, Lynch et al 1993). ELISA coating buffers commonly used are alkaline in nature in particular for coating streptokinase therefore the effect of buffers on coating of streptokinase to the ELISA plates was examined (3-5). Streptokinase at pH 7.0 gave a better standard curve when compared with alkaline buffer(pH 9.6) (Figures 3-5 and 3-6). Therefore PBS was used for coating streptokinase onto ELISA plates.

The intra-assay and inter-assay variations in ELISA's techniques compared with quantitation neutralising assay (ASK-kit) showed a lower variations and better reproducibility (Table 3-1).

IgG, IgM, IgA and IgE anti-streptokinase antibodies levels were determined in 246 normal individuals (3-7) and normal ranges for each antibody isotype were established (Table 3-2, Figure 3-8a, b, c and d). The majority of the normal individuals had detectable levels of IgG and IgA anti-streptokinase antibodies (90%), however, only 1.68% and 2.7% had levels above the normal ranges. 24%

of the normal individuals had detectable levels of IgM (9.9% were above the normal range) and 60% had detectable levels of neutralising anti-streptokinase antibodies (0.4% were above the normal range) (Table 3-2).

Neutralising anti-streptokinase assay is a functional assay that measures the efficiency of antibodies to streptokinase to inhibit clot breakdown. In the present study the levels of IgG and neutralising anti-streptokinase antibodies correlated significantly ($r=0.55$ $p<0.001$) in normal control group. In contrast to the normal control group the levels of IgG in the 10 patients, prior to administration of streptokinase did not correlate with neutralising antibodies (Table 3-3), which may be explained by small population size studied. However, in 10 patients treated with r-tPA the correlation between IgG and neutralising antibodies was observed ($r=0.67$, $p<0.03$), in agreement with Hoffmann et al 1988 who demonstrated in a group of 61 individuals with AMI a significant correlation between the levels of IgG and neutralising anti-streptokinase antibodies ($r= 0.43$, $p<0.001$).

The group of normals that were studied here represent a group of European individuals, therefore the proportion of individuals with elevated anti-streptokinase antibodies in other normal population groups may vary according to the prevalence of streptococcal infections in their location. Therefore it does appear important that normal ranges are constructed which reflect the population being examined.

Studies to date document varying degrees of pre-existing anti-streptokinase antibodies. James (1970) in an early study using a streptokinase resistance test assay, showed that 8.3% of normals had a level of reactivity to streptokinase which fell above an arbitrary level which could completely neutralise 250000 units of streptokinase. As described the ubiquity of exposure to streptococcal antigen is demonstrated in my study by the numbers of the individuals with a detectable antibody response and is supported by Moran et al (1984) and Lynch et al (1991) who quote positivity rates of 80% and 75%.

Analysis of AMI group as a whole showed pre-treatment levels of anti-streptokinase antibodies in two groups of patients treated with streptokinase and r-tPA did not differ significantly from the normal population (Figure 3-8).

A break down of the 2 patient groups based on the normal ranges derived from the normal population studied showed that 50% of the patients treated with r-tPA had elevated levels of IgM anti-streptokinase antibodies (9.9% of normal controls were above the normal range) whilst IgG, IgA and neutralising antibodies were within the normal ranges (Figure 3-8a, b, c and d, Table 3-4). The larger incidence of elevated IgM anti-streptokinase antibodies in this group compared with normal individuals could be explained in two ways. 1) The majority of them may had a recent streptococcal infection, or 2) As fibronectin and streptokinase share a similar epitope (Gonzalez-Gronow et al 1993), in AMI patients, the presence of tissue damage antibodies against fibronectin (or fibronectin combined with other body constituents) may show cross-reactivity with streptokinase.

The levels of isotype specific anti-streptokinase antibodies (measured by ELISA's) in the patients treated with r-tPA did not change by the administration of this agent (Table 3-4). However, quantitation of neutralising anti-streptokinase (measured by ASK-kit) in these patients showed a significant reduction ($p < 0.01$) in the levels of detectable antibodies in post-treatment samples at 10 minutes (Table 3-4). Study of the effect of r-tPA on quantitation of neutralising anti-streptokinase assay in vitro (3-10) revealed that the presence of administered r-tPA in the patients sera was responsible for this observation (Figure 3-9). The effect of r-tPA which is through generation of plasmin and results in a false low neutralising anti-streptokinase antibody titre (ASK-kit) has not been reported before. This effect implies that the neutralising assay is not a suitable method for clinical diagnosis of anti-streptokinase antibodies levels in patients since any alteration to the coagulation system due to anticoagulant therapy i.e. heparin, r-tPA etc. may interfere with this assay.

Analysis of 10 patients treated with streptokinase based on the normal ranges derived from the normal population studied showed a larger incidence of elevated anti-streptokinase antibodies in particular for IgM (80%). Similar to r-tPA group this can be accounted for by recent streptococcal infection or cross reaction with fibronectin.

Administration of streptokinase in 10 patients studied, resulted in an immediate fall in level of IgG (15 minutes, $p<0.01$) and neutralising anti-streptokinase antibodies (30 minutes, $p<0.01$) (Figure 3-10). The observed reduction in the levels of the antibodies could not be accounted for by changes in plasma volume during the clinical procedure, as no changes were observed in serum albumin levels, or circulating IgM (Figures 3-10 and 3-13). The reduction of anti-streptokinase antibodies is therefore due to sequestering of specific anti-streptokinase antibodies into an immune complex with the administered streptokinase.

The decrease in levels of specific antibodies after streptokinase administration has been observed by other workers, Lynch et al (1991) showed IgG levels to drop at day 1 and increase to pre-treatment levels by day 4 with no change in IgM. Fears et al (1992) found antibody levels to drop, using a streptokinase resistance test.

An immune response in all the patients administered streptokinase was observed since all of them had elevated levels of specific antibodies to streptokinase by day 5 (Figure 3-10a and b). Similar to the present study after day 4 Fears et al (1992) found an elevation in the levels of antibodies corresponding to an immune response to the administered streptokinase.

There was no significant difference in reperfusion between patients given streptokinase and r-tPA. The patients who reperfused within 2 hours of receiving streptokinase, had levels of IgG, IgA and neutralising anti-streptokinase antibodies which were within the normal ranges (Table 3-6) and the mean values of anti-streptokinase antibodies in the patients who reperfused late or did not reperfuse were higher than patients who reperfused early (Table 3-6). This implies that elevated levels of antibodies prevent the thrombolytic effect of streptokinase.

reduced efficacy due to elevated antibodies has been reported by other investigators (Lew et al 1984, Massel et al 1991 and Bom et al 1993). Lew et al 1984 showed that elevated levels of anti-streptokinase antibodies can neutralise 1.5 million units administered streptokinase and cause failure of coronary artery reperfusion.

Furthermore, as described, two of the patients treated with streptokinase had elevated levels of anti-streptokinase IgG prior to treatment (patients EM and CD). These patients in particular showed a dramatic reduction (50%) in the levels of IgG anti-streptokinase antibodies after streptokinase administration (Figure 3-11a), although the levels of IgG anti-streptokinase antibodies in these individuals (patients CD and EM) took longer to drop than those with lower levels (Figure 3-11a). In individuals with large antibody levels the amount of streptokinase administered probably is not sufficient to bind to all available antibodies and as described the levels did not fall to undetectable levels as seen in those with low levels. Although the levels of IgM in group of 10 patients treated with streptokinase did not show any significant changes (Figures 3-10b, 3-12b) one patient in this group that showed a significant reduction in the levels of IgM (90%) did not reperfuse (None reperfusion group, Patient LC, Figure 3-12b). Patient EM with elevated levels of IgG and neutralising anti-streptokinase antibodies, did not develop adverse reaction to streptokinase administration but did not reperfuse (Table 3-6).

White et al 1990 showed that patients with AMI re-treated with streptokinase 3-5 days after initial treatment do not exhibit a better response, and immunological reactions are more common than after the initial dose. This is not surprising since as described at the same period the levels of antibodies are elevated due to immune response. It therefore appears that if streptokinase needs to be readministered due to failure of the first bolus, it would be advisable to give this therapy shortly after first bolus.

In this study three of the patients with normal levels of anti-streptokinase antibodies did not respond to streptokinase treatment and should be considered as 25% of

patients who fail to reperfuse (Anderson 1991, Chouhan 1992). The cause of this failure is due to other factors that prevent the thrombolytic effect of streptokinase i.e. elevated levels of lipoprotein-A (1-3). These factors have not been clearly identified. Lipoprotein-a has extensive homology with plasminogen but lacks the critical bond of Arg^{560}-Val^{561} that is required for the activity of plasminogen activators (1-2). Elevated levels of lipoprotein-a have been shown to inhibit the thrombolytic effect of streptokinase by competing with plasminogen for binding to streptokinase (Edelberg 1989).

Of the 10 patients given streptokinase, one patient (Patient CD, with levels of IgG and IgA above the normal range) developed serum sickness. Patient CD with elevated levels of IgA (350 units/ml) at 30 minute showed a significant reduction (80%) in the levels of IgA (Figure 3-11b). The reduction in the levels of IgA in patient EM with high levels of IgA (310 units/ml) and other patients was not as dramatic (Figure 3-11b). The observed reduction in the levels of IgA in patient CD can be explained by the high affinity and specificity of pre-existing IgA in this patient for the administered streptokinase. Although we have no clear past history of this patient the high specificity of IgG and IgA allows the assumption that this patient may have been exposed to a recent streptokinase treatment or group C streptococcal infection.

From the present study it is clear that elevated levels of the pre-existing anti-streptokinase antibodies are responsible for both failure of reperfusion and initiating adverse immunological reactions, and among many types of methodology of measuring anti-streptokinase responses, ELISA assays can predict both failure of treatment and adverse reactions, by measuring levels of IgG and IgA which seen to be important in determining the outcome.

Chapter 4

The levels of anti-streptokinase antibodies and anti-mycobacterial heat shock protein in patients with auto-immune diseases

Introduction

Autoimmune diseases are caused by the breakdown of the immune system which results in specific immune reactions to self antigens. There are a wide range of auto-immune disorders. Auto-immune thyroid disease is organ specific, where auto-immune process is confined to an individual organ. In non-organ specific diseases the auto-immune process is not confined to a specific organ, i.e. systemic lupus erythematosus (SLE). Lying between these two kinds of autoimmune disorders there are disorders that are localised but their auto-antibodies are not specific for the involved organ (i.e. Primary biliary cirrhosis).

Multiple mechanisms may lead to organ specific or systemic auto-immune disorders. It is known that multiple genes contribute to the development of auto-immune diseases (Argyrios 1995a), however, it is not clear how self tolerance mechanisms are broken down and auto-antibodies are formed. One theory is that the release of the hidden antigen (i.e. lens, sperm) may provoke auto-antibody formation. Another theory is the recognition of self antigens and MHC class II proteins complexes by helper T cells triggers immune responses. However, T cells do not usually normally respond to these complexes due to the effects of suppression mechanisms caused by T cells and macrophages, in addition clonal deletion mechanisms prevent such immune responses. The unresponsiveness to self antigen is known as tolerance and is altered by the break down of any of the mechanisms described and results in auto-immune diseases (Weir & Stewart 1993).

Many studies have tried to illuminate why self tolerance breaks down. Bacterial and viral infections have been suspected as antigens which may alter the immune system and produce auto-immune diseases. They may present antigen determinants similar to self components and produce cross-reactivity. Group A streptococci have similar antigens to human heart and glomeruli antigens and cause rheumatic fever or Henoch Schonlein purpura (HSP). Some bacterial endotoxins, lipopolysaccarides of gram negative bacteria and infections (Epstein Barr virus) provide a non-specific signal for B cells and trigger polyclonal activation of them without the mediation of helper T cells.

Streptococcal infections and the following immune response has been associated with auto-immune diseases (rheumatic fever). The presence of anti-streptokinase antibodies in sera is a result of previous streptococcal infections (Anderson et al 1948, Flute 1973, Buchalter et al 1992). To investigate whether streptococcal infections have any role to play in initiating the auto-immune disorders the levels of anti-streptokinase antibodies were measured in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), thyroiditis and Henoch Schonlein purpura (HSP) patient groups.

Heat shock proteins (HSPs) were first shown to be released by cells cultured at temperatures above 37°C. Heat shock proteins (HSPs) are a ubiquitous and highly conserved group of proteins and are a major antigenic component of many bacteria (Argyrios 1995a). The structural homology between foreign and self HSPs may lead through cross reactivity to an organ specific auto-immune disorder (DeNagel & Pierce 1993), however, it is not clear how this molecular similarity could lead to auto-immune diseases.

IgG anti-mHSP65 (mycobacterial heat shock protein) antibody levels were measured in the above mentioned auto-immune patients groups and a group of normal individuals, to find if the present of this antibody has any relationship with immune responses to streptococcal infections.

Materials and methods

4-1 Rheumatoid arthritis patients. The levels of isotypes IgG, IgA, IgM, IgE (3-5) and neutralising anti-streptokinase (3-1) and IgG anti-mycobacterial heat shock protein (anti-mHSP$_{65}$, 4-6) in fifteen patients with rheumatoid arthritis (RA) were studied.

These patients were selected carefully according to their clinical diagnosis and laboratory findings. The levels of rheumatoid factor (RF) and anti-nuclear factor (ANF) were determined in the Immunopathology laboratory, Western Infirmary, Glasgow University. The levels of RF were measured by ELISA's for IgG, IgA, IgM isotypes and by nephelometery. They all had elevated positive RF and a negative ANF.

4-2 Affinity chromatography elution of rheumatoid factor. To find whether the presence of rheumatoid factor (RF) has any effect on the levels of detectable anti-streptokinase antibodies, rheumatoid factor from sera of patients with rheumatoid arthritis was eluted by affinity chromatography using Sepharose coupled to human IgG.

Buffers

10^{-3}M HCl: 43 ml concentrated HCl was added to 800 ml water and final volume was made up to 1 litre with water (1.0 M HCl). 1.0 M HCl was then diluted 1/1000 to give 10^{-3} M HCl.

0.1M $NaHCO_3$ + 0.15 M NaCl: 8.4g $NaHCO_3$ and 5.8g NaCl were dissolved in 800 ml water and the final volume was made up to 1 litre with water.

0.1M Tris-HCl (pH 8.0): Concentrated HCl was added to 800 ml 0.1M Tris till pH 8.0 was achieved and then the total volume was made up to 1 litre with water.

0.1 M Acetate buffer (pH 4.0)+ 1.0 M NaCl: 0.1M Sodium acetate (8.2g/L) and 0.1M acetic acid (5.7 ml glacial acetic acid/L) were mixed together till pH 4.0 achieved and sodium chloride was then added to give final concentration of 1.0 M (58.8g/L) .

0.1M Borate buffer (pH 8.0) + 1.0M NaCl: 0.1M Boric acid (6.18 g/L) and 0.1M sodium tetraborate (borax) (9.54 g/L) were mixed together till pH 8.0 and sodium chloride was added to give final concentration of 1.0 M (58.8g/L).

0.1 M Glycine-HCl buffer (pH 2.5) 7.5g glycine was dissolved in 800 ml water and pH was made to 2.5 with concentrated HCl, the final volume was made up to 1 litre with water.

Method: Sepharose 4B-cyanogen bromide was supplied by Pharmacia in a stable form and reactivated by swelling with 10^{-3}M HCl and washed for 15 minutes in same buffer. The coupling material, human IgG, was dialysed into 0.1M $NaHCO_3$ + 0.15 M NaCl at 4°C overnight and 10 mg IgG was added per 1 gram of gel and mixed at 4°C overnight. The mixture was then spun at 4°C for 10 minutes. The supernatant was removed and the amount of protein was determined (2-4) to estimate the efficiency of binding of IgG to Sepharose. The Sepharose was washed once in 0.1M $NaHCO_3$+0.15 M NaCl and then mixed with 0.1M Tris-HCl (pH 8.0) for 2 hours at room temperature on a Matbuen mixer. This buffer reacts with any remaining active sites on the Sepharose and renders then inactive. The Sepharose was washed with 0.1 M Acetate buffer (pH 4.0)+ 1.0 M NaCl, and then with 0.1M Borate buffer (pH 8.0) + 1.0M NaCl. After repeating this washing cycle once the Sepharose was ready to use.

For this experiment sera from 13 patients with rheumatoid arthritis were used. The sera were diluted 1/2 in PBS and the levels of RF and anti-streptokinase antibodies prior to affinity chromatography were measured. The sera were then added to an equal volume of Sepharose-IgG and mixed for one hour at room temperature. The Sepharose-IgG-sera suspension were then spun, the supernatants were then removed and the levels of anti-streptokinase antibodies and RF were measured (4-1).

4-3 Systemic lupus erythematosus patients. The levels of isotypes IgG, IgA, IgM, IgE (3-5) and neutralising anti-streptokinase (3-1) and IgG anti-mycobacterial heat shock protein (anti-mHSP$_{65}$, 4-6) were measured in twenty patients with systemic lupus erythematosus (SLE). These patients were selected according to their clinical diagnosis and laboratory findings. They all had positive ANF, positive Crithidia test and negative RF as determined in the Immunopathology laboratory, Western Infirmary, Glasgow University.

4-4 Thyroiditis patients. The levels of isotypes IgG, IgA, IgM, IgE (3-5) and neutralising anti-streptokinase (3-1) and IgG anti-mycobacterial heat shock protein (anti-mHSP$_{65}$, 4-6) were determined in twenty patients with thyroiditis. These patients were selected according to their clinical diagnosis and laboratory findings. They all had a high titre of anti-thyroglobulin and anti-thyroid microsomal antibodies as determined in the Immunopathology laboratory, Western Infirmary, Glasgow University.

4-5 Henoch Schonlein Purpura patients. Twelve patients with known Henoch Schonlein Purpura (HSP) were studied for the presence of anti-streptokinase antibodies (IgG, IgA, IgM, IgE and neutralising antibodies) (4-1) and IgG anti-mycobacterial heat shock protein (anti-mHSP$_{65}$) (4-7). These patients were selected according to their clinical diagnosis.

4-6 Measuring IgG anti-heat shock protein (HSP65) in patient groups. This method was developed by Dr. David Burnie, Department of Cardiology, Western Infirmary, Glasgow University.

Materials

Sources of materials used here are described in 2-1.

ELISA plates (Dynatech, Immulon 4)

Recombinant mycobacterial mHSP65 (mycobacterial bovis BCG)

Horseradish peroxidase (HRP) conjugated goat anti-human IgG

o-phenylenediamine (OPD)

Buffers: Prepared as described in 2-2.

Phosphate buffered saline (PBS)

1% BSA in PBS

ELISA coating buffer pH 9.60

ELISA wash buffer (0.05% Tween in PBS)

0.2M citric acid

ELISA enzyme substrate

4N H_2SO_4

Method. ELISA plates were coated with 100 µL per well of recombinant mycobacterial mHSP65 solution containing 1µg/ml in coating buffer overnight at 4°C. The plates were washed with PBS-Tween and blocked with 200 µL per well of 1% BSA in PBS for one hour at room temperature in a humidified chamber.

To construct a standard curve, a sample contained 6.85 mg/ml of IgG anti-HSP65 was double diluted in 1% BSA in PBS from 1/30, and 100 µl per well applied in duplicates to the plates. The first dilution was assigned a value of 220 arbitrary units/ml. Serum samples, diluted 1/400 in 1% BSA in PBS were applied to the plates (100 µl per well in triplicate) and incubated for 2 hours at room temperature. The plates were washed and HRP conjugated goat anti-human IgG (1/3000 in 1% BSA in PBS) was added (100 µl per well) and incubated at room temperature for 1 hour. The plates were washed and the colour developed using o-phenylenediamine (OPD) and the reaction stopped using 4N H_2SO_4. Colour reaction was read at 490 nm on a Dynatech ELISA reader. The

antibody concentrations in unknown serum samples were obtained by interpolation from the standard curve and the values expressed in the arbitrary units/ml.

4-7 Statistical analysis. Statistical analysis of data was performed using Spearman rank correlation test, Wilcoxon rank test and Student t-test (paired).

Results

The levels of RF and anti-streptokinase antibodies in Rheumatoid Arthritis patients. The levels of IgG, IgA and IgM rheumatoid factor (RF) were measured by ELISA's and by nephelometry (Table 4-1a). The levels of RF as measured by nephelometry, IgG-RF, IgM-RF as measured by ELISA's were above the normal ranges, IgA-RF levels fell within the normal range (Table 4-1a). The levels of RF measured by nephelometry correlated with the levels of IgM-RF measured by ELISA's ($r=0.51$, $p<0.04$), but did not correlate with IgG-RF ($r=0.25$). The levels of IgM-RF) and IgG-RF measured by ELISA's did not correlate with one another ($r=0.28$).

The levels of IgG, IgM, IgA and neutralising anti-streptokinase antibodies in these patients were measured (4-1) and the values compared to the established normal ranges (Table 4-1b Figures 4-1 and 4-2).

The levels of IgG, IgA and neutralising anti-streptokinase antibodies in this group were skewed towards the lower end of the range, on the other hand, the levels of IgM anti-streptokinase antibodies showed a normal distribution (Figures 4-1 and 4-2).

All the patients in this group had levels of IgG (Patients mean=10.5 units/ml, normals mean= 5.7 units/ml) and neutralising anti-streptokinase antibodies (Patients mean=28.7 units/ml, normals mean= 29.2 units/ml) that fell within the normal ranges (Figures 4-1

Rheumatoid Factor	Mean	Median	Normal range
IgG (ELISA)	201	139	0-20 (u/ml)
IgM (ELISA)	5440	4720	0-800 (u/ml)
IgA (ELISA)	7.5	7.5	0-7.5 (u/ml)
Nephelometry	602	470	0-22 (IU/ml)

Table 4-1a: The mean, median and normal ranges of rheumatoid factor (RF) in 15 patients with rheumatoid arthritis measured by ELISA's and nephelometry. Values for IgG, IgA, IgM rheumatoid factor measured by ELISA are expressed in units/ml and values for rheumatoid factor measured by nephelometry are expressed in I.U./ml.

Isotype	RA	SLE	Thyroiditis	HSP
IgG	10.5	8.8	10.6	27
IgA	157	87	44.2	141
IgM	60.3	14.6	14.8	16.5
ASK	28.7	15.5	6.75	36

Table 4-1b: The mean levels of anti-streptokinase antibodies in 20 patients with Systemic Lupus Erythematosus (SLE), 15 patients with rheumatoid arthritis (RA), 20 patients with thyroiditis and 12 patients with Henoch Schonlein Purpura (HSP). The values are expressed in units/ml, normal ranges for IgG=0-45.8, for IgA=0-324, for IgM=0-10.8 and for neutralising anti-streptokinase antibodies=0-166 units/ml.

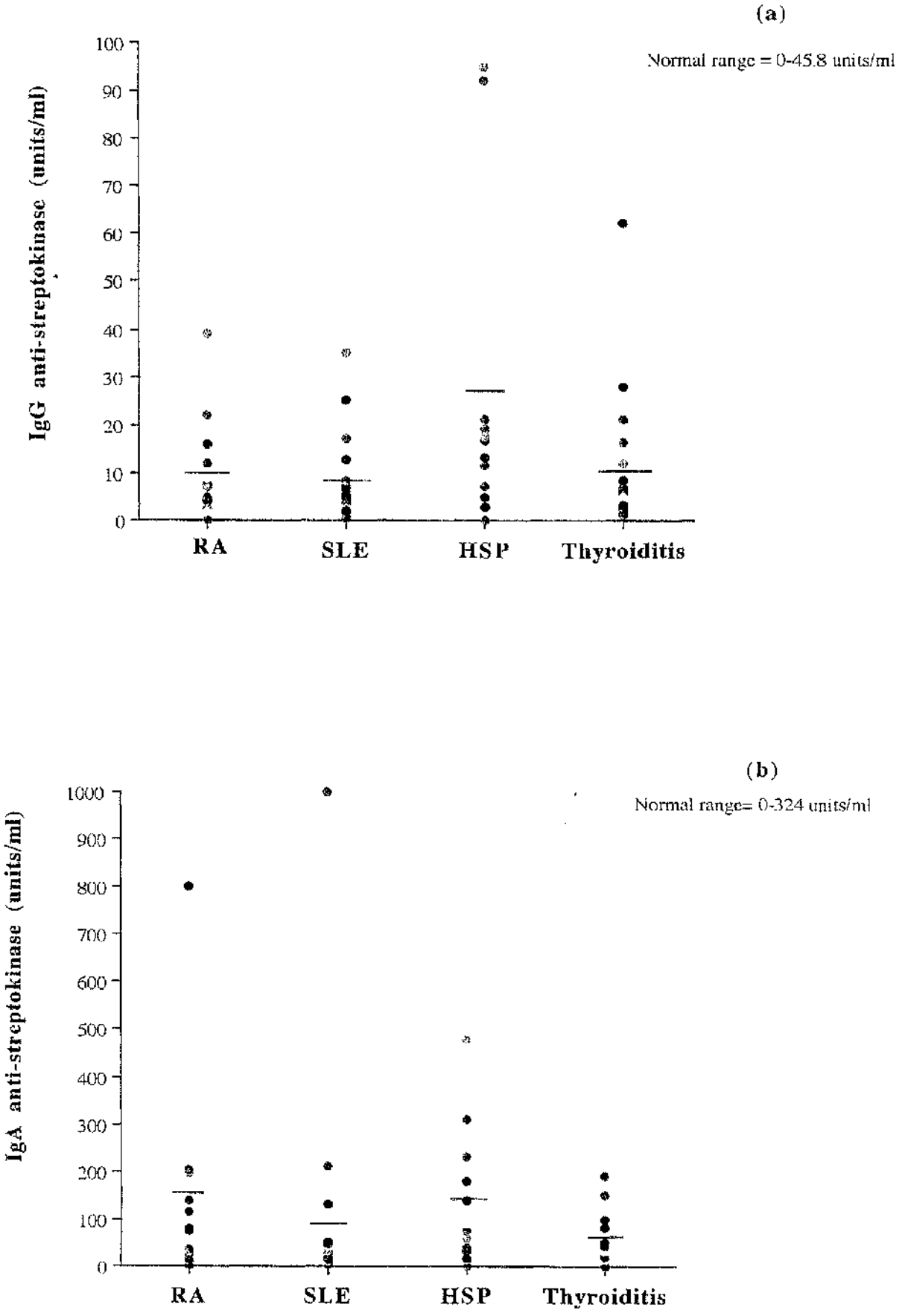


Figure 4-1: The levels of IgG (a) and IgA (b) anti-streptokinase in patients with rheumatoid arthritis (RA, n=15), systemic lupus erythematosus (SLE, n=20), Henoch Schonlein Purpura (HSP, n=12) and thyroiditis (n=20).

and 4-2, Table 4-1b). All the patients had detectable levels of IgA anti-streptokinase antibodies (Patients mean=157 units/ml, normals mean= 31.3 units/ml), 13.3% had levels which were above the normal range (2.7% normals had elevated levels) (Figure 4-1, Table 4-1b) and all of the patients had levels of IgM anti-streptokinase antibodies (Patients mean= 60.3 units/ml, normals mean= 1.9 units/ml) which were above the normal range (9.9% of normals had elevated levels) (Figures 4-2, Table 4-2).IgE anti-streptokinase antibodies were detectable in all of the RA patients when the optical densities (OD) were compared with the OD of the blank (Figure 4-3), this did not follow the finding in normal population studied (Chapter 3).

In this group the levels of anti-streptokinase antibodies correlated significantly with one another (Table 4-2) but did not correlate with the levels of RF measured by nephelometry or by ELISA's.

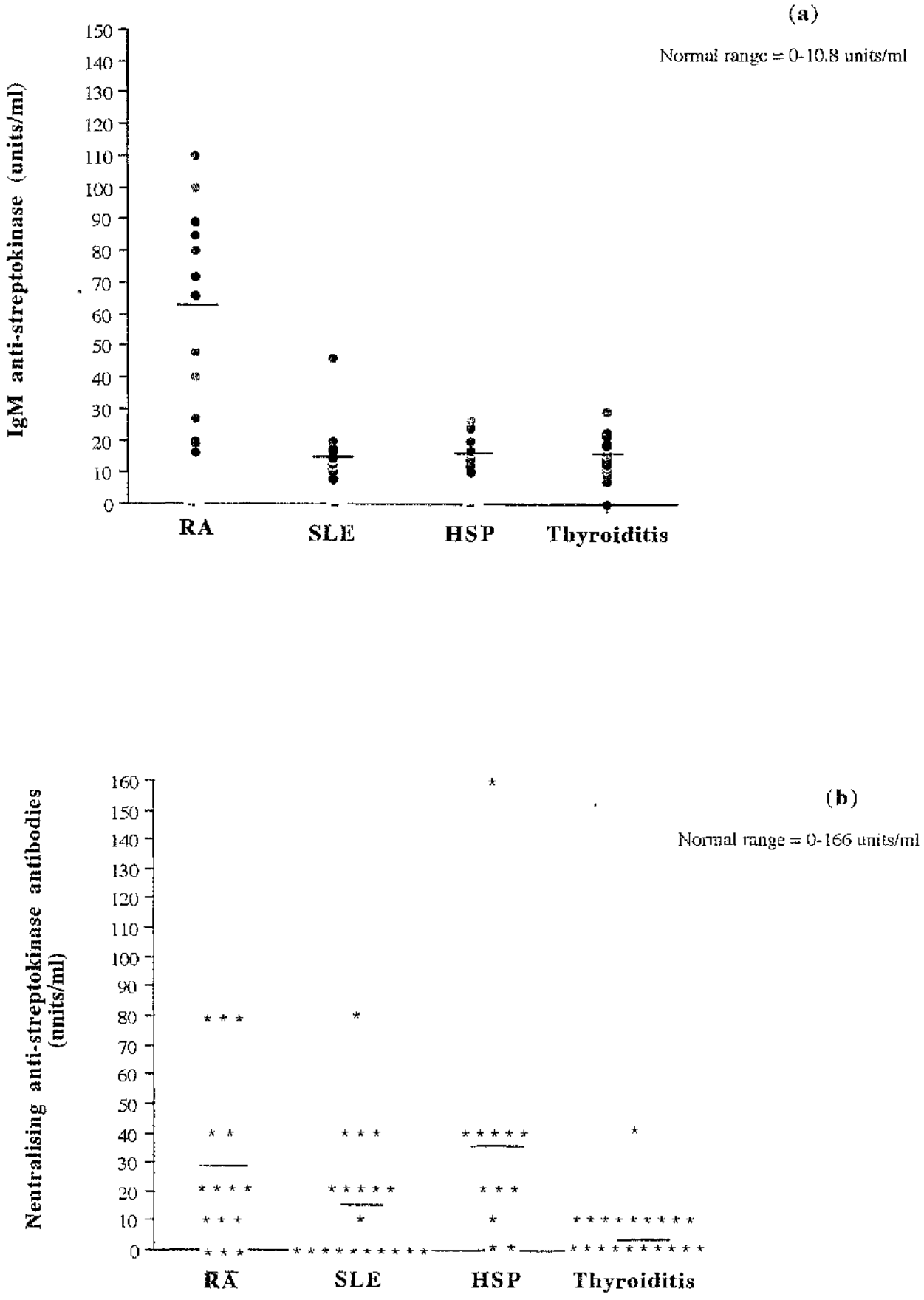
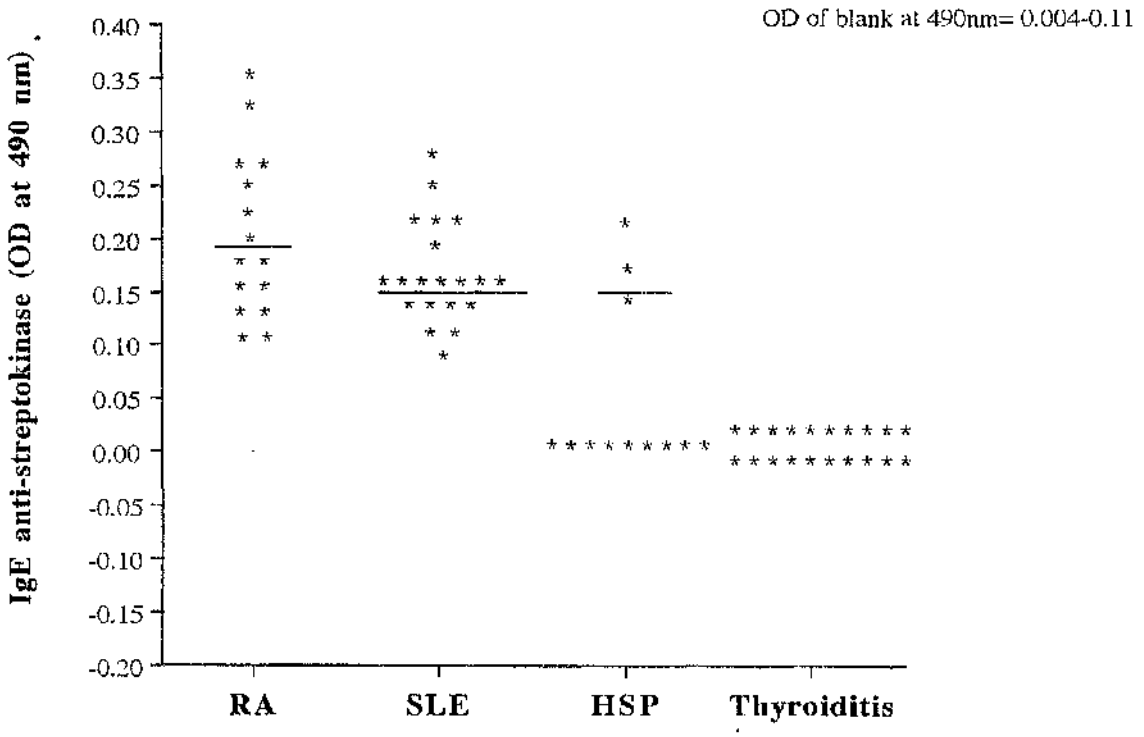


Figure 4-2: The levels of IgM (a) and neutralising anti-streptokinase antibodies (b) in patients with rheumatoid arthritis (RA, n=15), systemic lupus erythematosus (SLE, n=20), Henoch Schonlein Purpura (HSP, n=12) and thyroiditis (n=20).



Figutre 4-3: The levels of IgE anti-streptokinase in patients with rheumatoid arthritis (RA, n=15), systemic lupus erythematosus (SLE, n=20), Henoch Schonlein Purpura (HSP, n=12) and thyroiditis (n=20).

Affinity chromatography elution of rheumatoid factor. To determine whether the presence of rheumatoid factor (RF) had any effect on the levels of detectable anti-streptokinase antibodies, rheumatoid factor from the sera of a second group of patients with rheumatoid arthritis (n= 13) was eluted by affinity chromatography (4-2). The levels of anti-streptokinase antibodies and RF in the 13 patients prior to affinity chromatography were measured and compared with the post-purification values (4-2).

Rheumatoid Factor (RF) was successfully eluted from the sera of patients as measured by nephelometry and affinity chromatography with a 73% reduction in the mean value of RF (Paired student t-test statistic=6.6, $p<0.001$) (Table 4-3, Figure 4-4a). However, many of the individuals still had RF levels above the normal ranges (Figure 4-4a).

The mean values of IgM, IgG and IgA anti-streptokinase antibodies after affinity chromatography were reduced by 66%, 53% and 64% respectively (For IgG, paired student t-test statistic=6.6 $p<0.001$, IgA statistic=3.14 $p<0.008$ and IgM statistic=4.3 $p<0.001$) (Figures 4-4 and 4-5, Table 4-3). The reduction in the levels of RF post-purification was to higher extent (7-20 %) compared to anti-streptokinase antibodies and the levels of IgM anti-streptokinase antibodies in many of the individuals were still above the normal range (Figures 4-4 and 4-5, Table 4-3).

Correlation	Statistic (r value)	Significance (p value)
IgG/IgA	0.7	0.003
IgG/IgM	0.85	0.001
IgG/ASK	0.86	0.001
IgA/IgM	0.51	0.04
IgA/ASK	0.6	0.01
IgM/ASK	0.8	0.002
IgE/IgG	0.85	0.001
IgE/IgA	0.71	0.003
IgE/IgM	0.86	0.001
IgE/ASK	0.78	0.001

Table 4-2: Correlation between levels of anti-streptokinase antibodies (IgG, IgA, IgM, IgE) and streptokinase neutralising antibodies in 15 patients with rheumatoid arthritis.

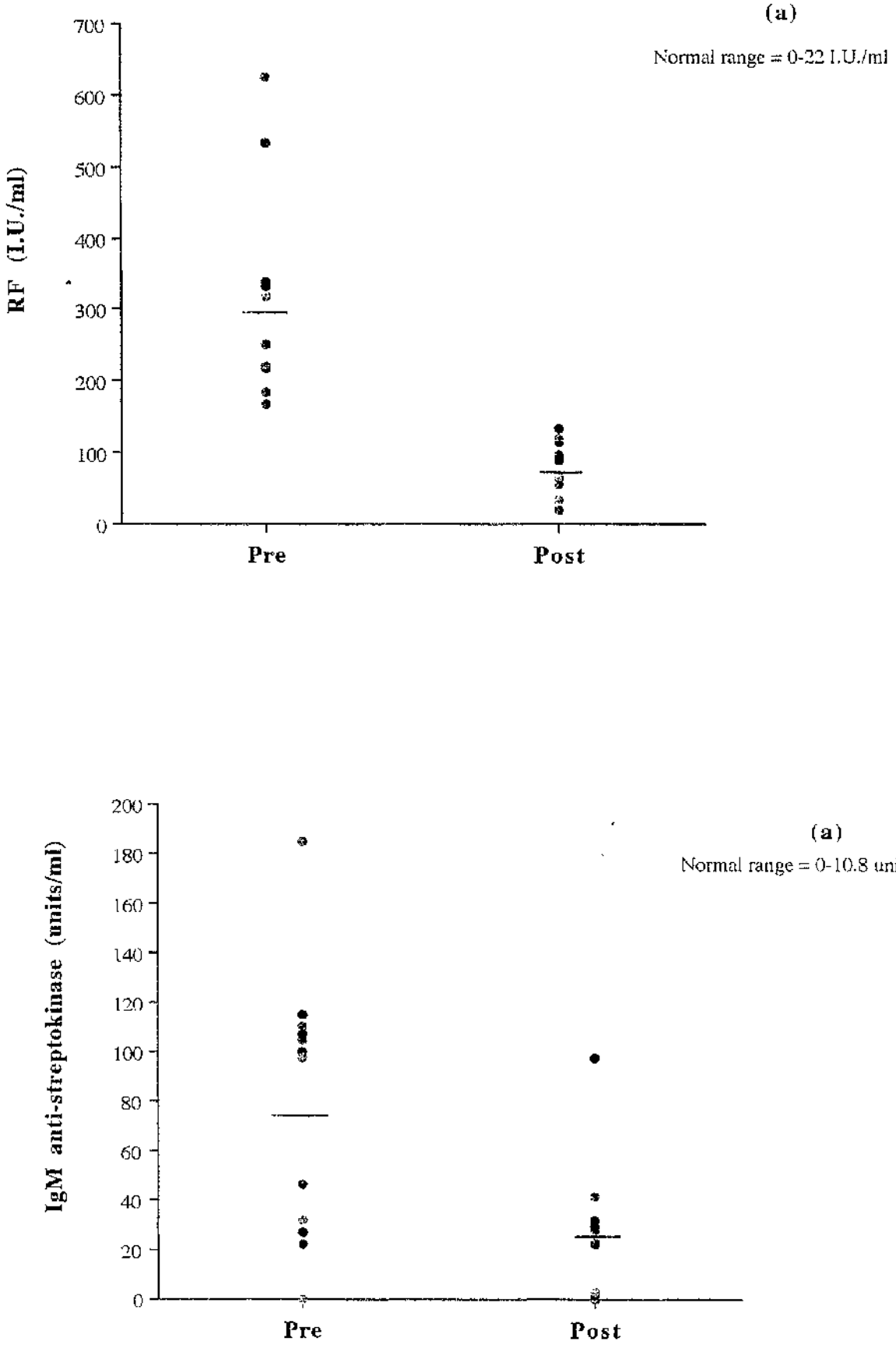


Figure 4-4: The levels of rheumatoid factor (a) measured by nephelometry and IgM anti-streptokinase antibodies (b) in patients with rhumatoid arthritis (n=13) pre- and post-affinity chromathography for elution of RF.

The levels of anti-streptokinase antibodies in Systemic Lupus Erythematosus patients. The levels of IgG, IgM, IgA and neutralising anti-streptokinase antibodies in the Systemic Lupus Erythematosus (SLE) patients were measured (4-3) and the values compared to the established normal ranges (Table 4-1b Figures 4-1 and 4-2). The individuals in this group had levels of IgG anti-streptokinase antibodies which fell within the normal ranges (Table 4-1a, Figure 4-1a), 5% and 85% had levels of IgA and IgM anti-streptokinase antibodies respectively above the normal ranges (2.7% and 9.9% of normal controls had elevated levels of IgA and IgM) (Table 4-1b, Figures 4-1b and 4-2a). The levels of neutralising anti-streptokinase antibodies in this group were within the normal ranges (Figure 4-2b). IgE anti-streptokinase antibodies were detectable in the majority of individuals in this group (Figure 4-3), this did not follow the finding in normal population studied (Chapter 3). The mean value of this group (OD_{490}=0.15) was lower than RA patients group (OD_{490}=0.2).

The levels of IgM anti-streptokinase antibodies showed a normal distribution, however, the levels of IgG, IgA and neutralising anti-streptokinase antibodies were skewed towards the lower end (Figures 4-1 and 4-2). The levels of IgG and neutralising anti-streptokinase antibodies in this group correlated significantly ($r = 0.7$, $p < 0.001$). The levels of IgM, IgA and neutralising antibodies did not correlate with one another.

The levels of anti-streptokinase antibodies in thyroiditis patients. The levels of IgG, IgM, IgA and neutralising anti-streptokinase antibodies in the thyroiditis patients were measured (4-3) and the values compared to the established normal ranges (Table 4-1b Figures 4-1 and 4-2). All the patients in this group had detectable levels of IgG anti-streptokinase antibodies, however, only one individual (5%) had an elevated value (1.68% of normal controls had elevated levels of IgG) (Figures 4-1a Table 4-2),

RF	Mean	Median	Normal Range
Pre	297	250	0-22
Post	79	77	0-22

IgG anti-SK	Mean	Median	Normal Range
Pre	4.9	4.8	0-45.8
Post	2.3	1.9	0-45.8

IgA anti-SK	Mean	Median	Normal Range
Pre	111	52	0-324
Post	52	29	0-324

IgM anti-SK	Mean	Median	Normal Range
Pre	74	100	0-10.8
Post	25	23.5	0-10.8

Table 4-3: The levels of rheumatoid factor (RF) measured by nephelometry and IgG, IgA and IgM anti-streptokinase antibodies in patients with rheumatoid arthritis (n=13) pre- and post-affinity chromatographic elution of RF. The values of anti-streptokinase antibodies are expressed in units/ml and the value of RF are expressed in I.U.

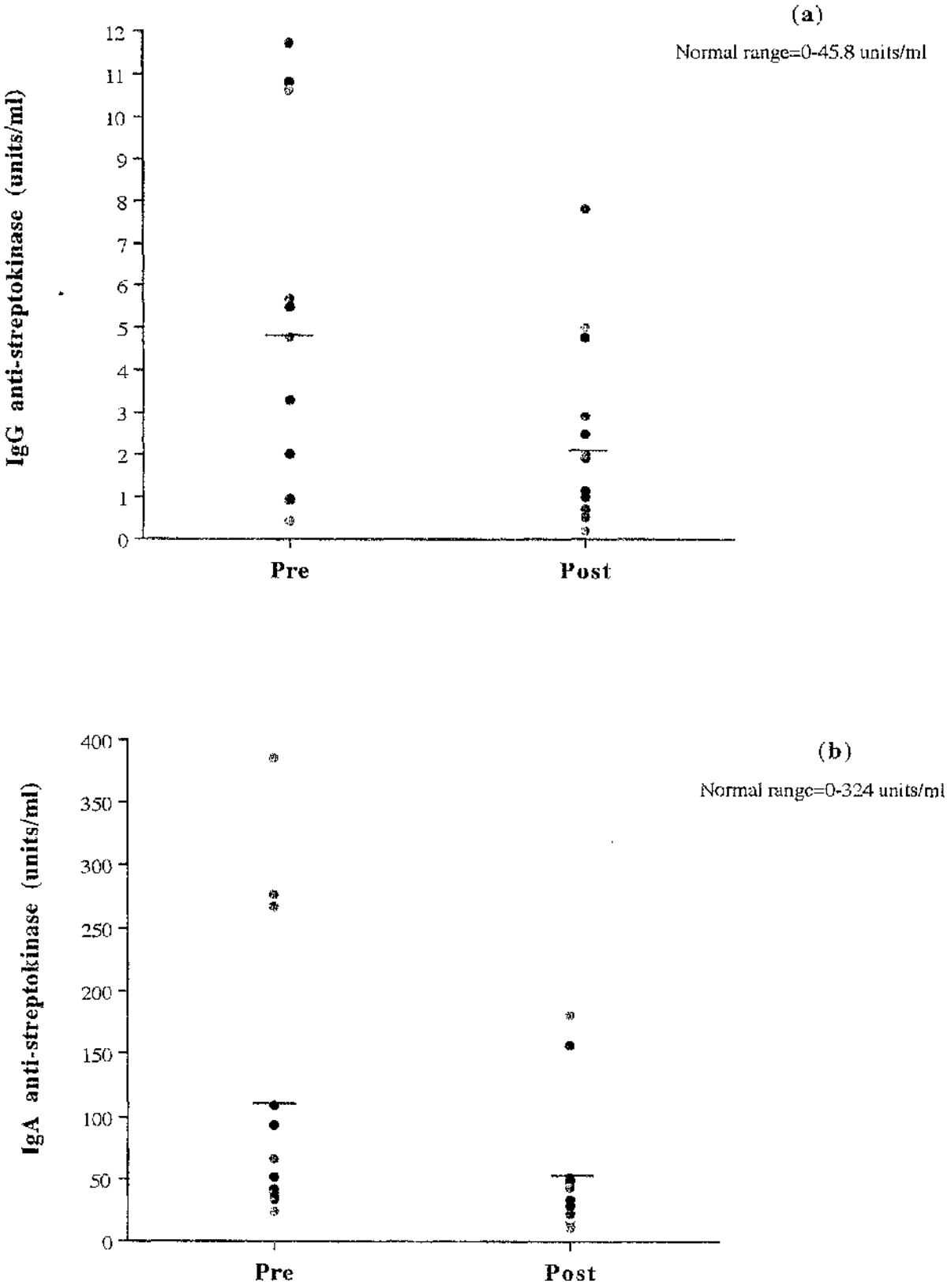


Figure 4-5: The levels of IgG (a) and IgA (b) anti-streptokinase antibodies in patients with rheumatoid arthritis (n=13) pre- and post affinity chromathography for elution of RF.

most of the patients (90%-95%) had IgA and IgM levels within the normal ranges (Table 4-2, Figures 4-1b and 4-2a). An IgE anti-streptokinase response was not observed in any of the patients (Figure 4-3).

The levels of neutralising anti-streptokinase antibodies detectable in 50% of the patients correlated significantly with the levels of IgG and IgA ($p < 0.001$) but did not correlate with the levels of IgM (Table 4-4, Figure 4-2). The levels of IgM showed a negative correlation with the levels of IgA ($r = -0.6$, $p < 0.002$).

The levels of IgM anti-streptokinase antibodies showed a normal distribution and the levels of IgG, IgA and neutralising anti-streptokinase antibodies skewed towards the lower end of the distributions (Figures 4-1 and 4-2).

The levels of anti-streptokinase antibodies in Henoch Schonlein Pupura patients. The levels of IgG, IgM, IgA and neutralising anti-streptokinase antibodies in the Henoch Schonlein Pupura (HSP) patients were measured (4-3) and the values compared to the established normal ranges (Table 4-1b Figures 4-1 and 4-2).

All of the individuals had detectable levels of IgG, IgA and IgM anti-streptokinase antibodies, two patients (16.6%) had levels of IgG and IgA above the normal ranges (1.68% and 2.7% of normal controls had elevated levels of IgG and IgA respectively) (Figures 4-1 and 4-2, Table 4-2). Eleven patients (92%) had levels of IgM anti-streptokinase antibodies which fell above the normal range (9.9% of normal controls had elevated levels of IgM anti-streptokinase antibodies) (Figure 4-2a, Table 4-2). Ten patients (83.3%) in this group had detectable levels of neutralising anti-streptokinase antibodies, only one (8.3%) of them was above the normal range (0.4% of normal controls had elevated levels of neutralising anti-streptokinase antibodies) (Figure 4-2b, Table 4-2).

Three of the patients in this patient group (25%) had an IgE anti-streptokinase antibody

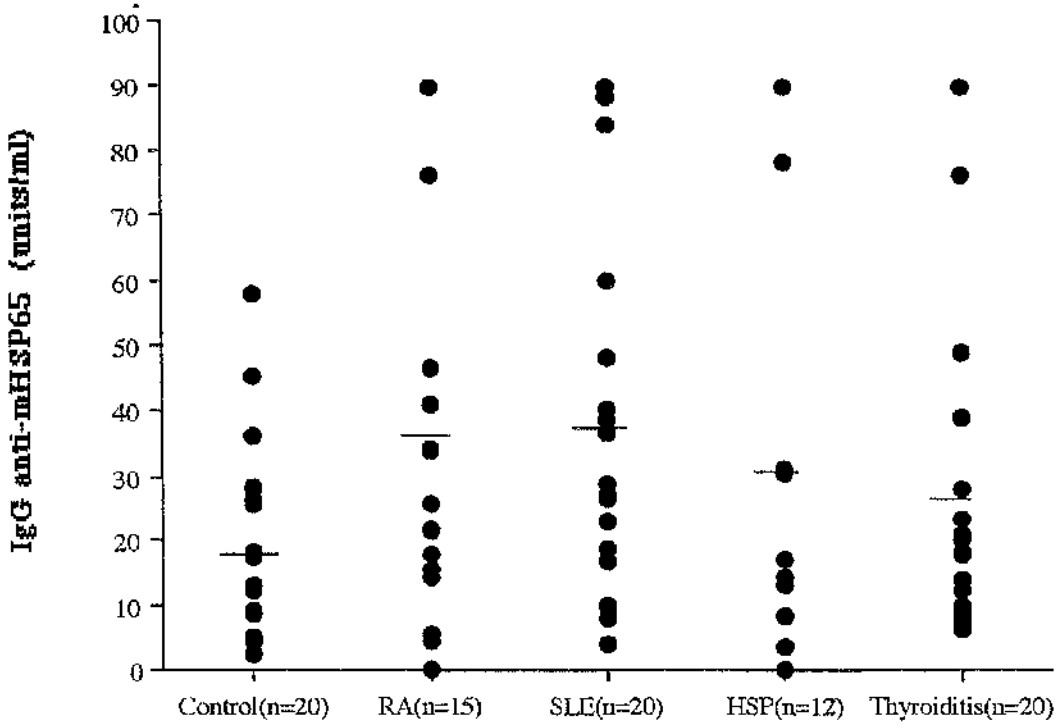
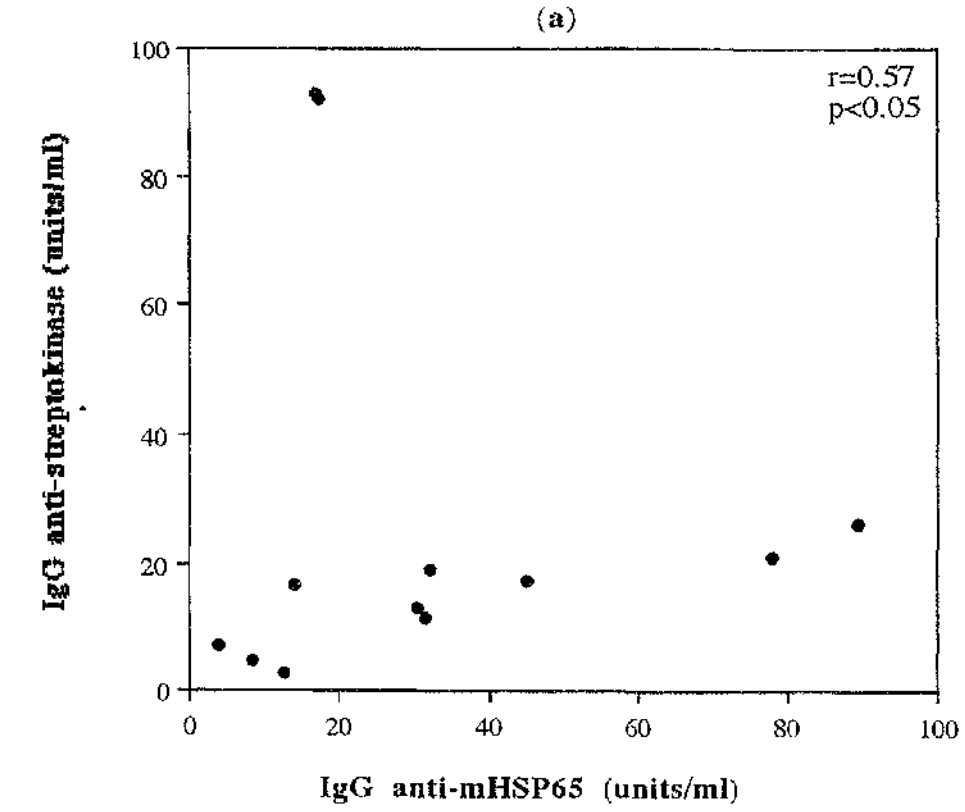


Figure 4-6: Levels of IgG anti-mHSP65 in RA, SLE, HSP, thyroiditis patients and a group of normal individuals.



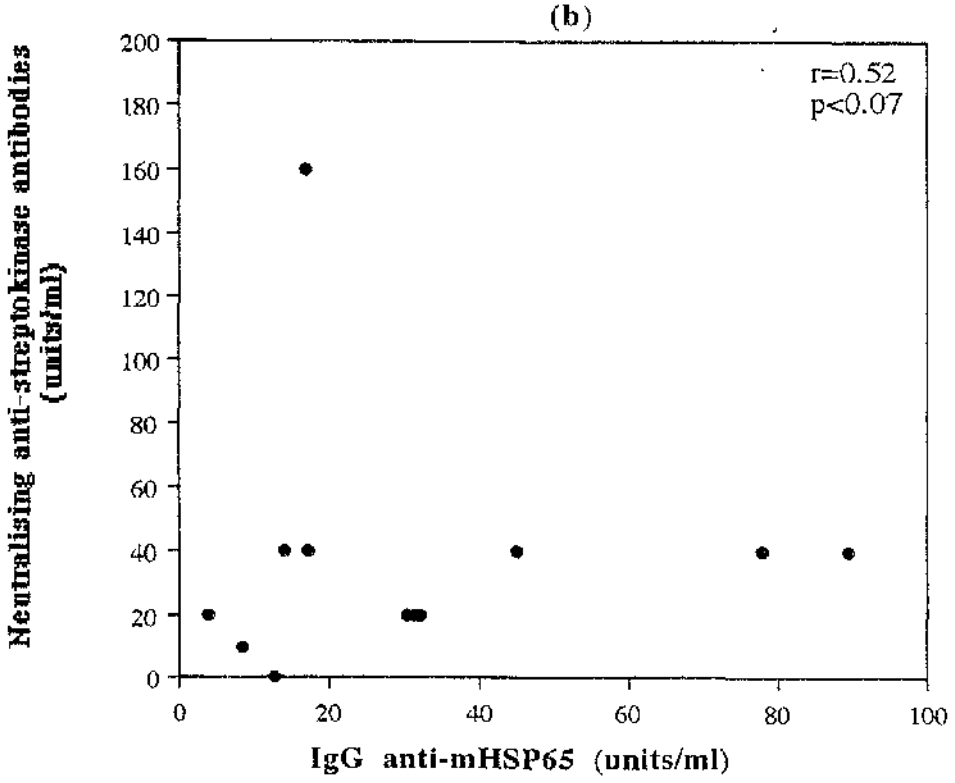


Figure 4-7: Correlation between the levels of IgG (a) , neutralising (b) anti-streptokinase antibidies and IgG anti-mHSP65 in 12 patients with Henoch Shonlein Pupura.

response when the optical densities (OD) of samples compared with the OD of blank (OD of samples were 0.15, 0.16, 0.21 respectively and OD of blank was 0.004) (Figure 4-3).

The levels of IgM anti-streptokinase antibodies showed a normal distribution and the levels of IgG, IgA and neutralising anti-streptokinase antibodies skewed towards the lower end of the distributions (Figures 4-1 and 4-2).

The levels of neutralising anti-streptokinase antibodies correlated significantly with IgG ($r = 0.93$, $p < 0.001$) and IgA ($r = 0.63$, $p < 0.04$). The levels of IgG and IgM correlated significantly ($r = 0.75$, $p < 0.1$) but did correlate with the levels of neutralising antibodies.

The mean value of IgG and neutralising anti-streptokinase antibodies in the HSP patients was higher when compared with the mean values in SLE, thyroiditis and RA patients (Table 4-1b, Figures 4-1a and 4-2b). The mean value of IgA anti-streptokinase antibodies in these patients was higher than the mean values in SLE and thyroiditis but did not differ from the RA patients (Table 4-1a, Figure 4-1b). The mean value of IgM anti-streptokinase antibodies in these patients did not differ from the mean values in SLE and thyroiditis patients, however it was substantially lower than RA patients (Table 4-1b, Figure 4-2a).

The levels of IgG anti-heat shock protein (mHSP65) in auto-immune patients. IgG anti-mHSP65 levels were measured in RA (n=15), SLE (n=20), Thyroiditis (n=20), HSP (n=12) patients and a group of normal individuals (n=20) (4-6).

The mean values of IgG anti-mHSP65 levels in the 4 patients groups (RA=35.7 units/ml, SLE=37.1 units/ml , Thyroiditis= 27.9 units/ml and HSP=30.17 units/ml) were higher than the mean value in the normal individuals (18.9 units/ml), although this

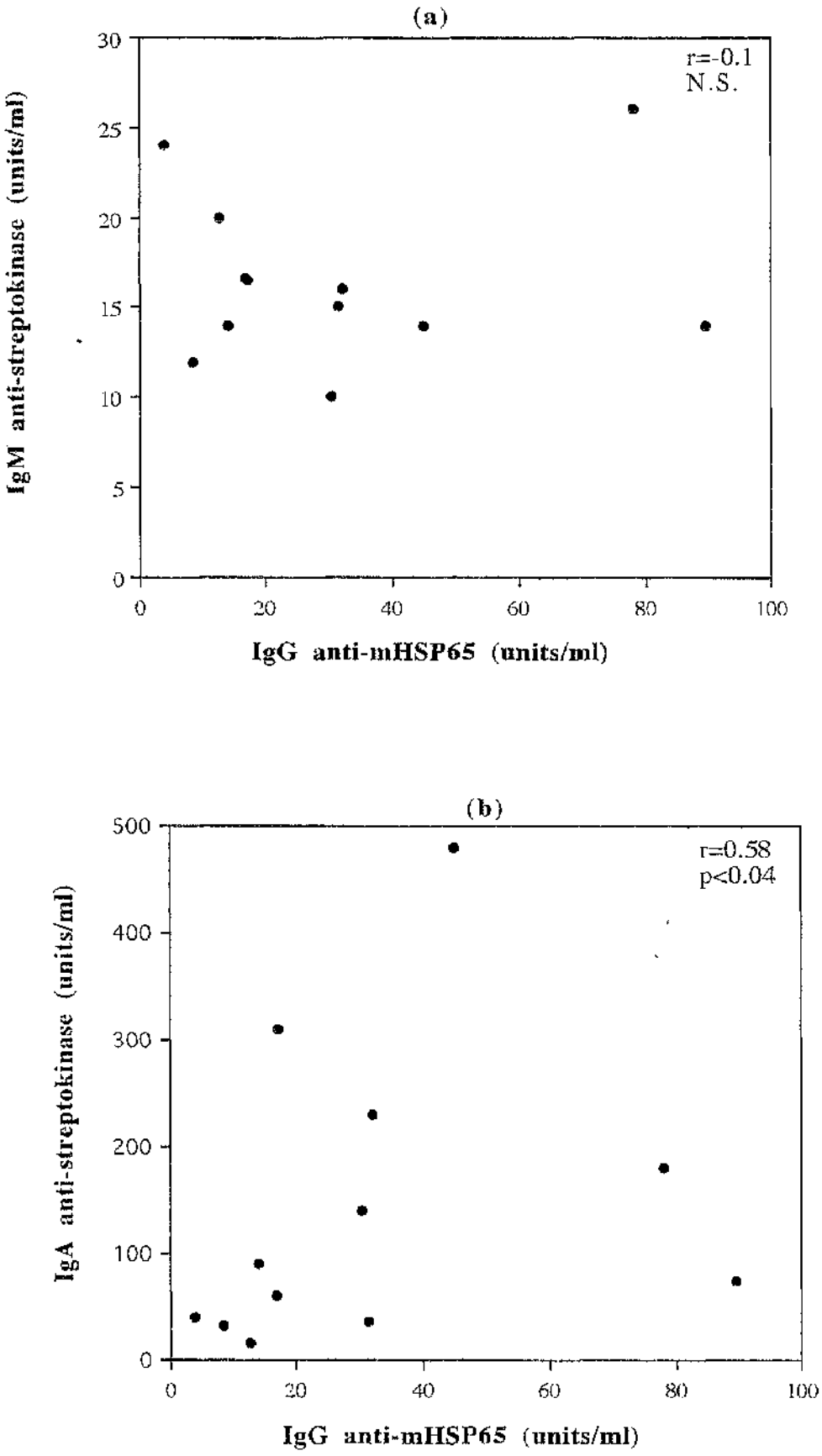


Figure 4-8: Correlation between the levels of IgM (a) , IgA (b) anti-streptokinase antibidies and IgG anti-mHSP65 in 12 patients with Henoch Shonlein Pupura.

was not statistically significant (Figure 4-6).

The levels of IgG, IgA, IgM, neutralising anti-streptokinase antibodies in RA, SLE and thyroiditis patients did not correlate with the levels of IgG anti-mHSP65. The levels of IgG anti-mHSP65 in HSP patients (n=12) correlated significantly with the levels of IgG anti-streptokinase antibodies ($r=0.57$, $p < 0.05$), IgA anti-streptokinase antibodies ($r=0.58$, $p < 0.04$) and neutralising anti-streptokinase antibodies ($r = 0.52$ $p < 0.07$), however, the levels of IgG anti-mHSP65 did not correlate with the levels of IgM anti-streptokinase antibodies (Figures 4-7 and 4-8).

Discussion

There is abundant evidence to indicate that auto-immune diseases are immunologically driven. Certain bacterial and viral infections (i.e. group A streptococci, mycobacteria) are suspected agents in the initial aetiology of autoimmune diseases. Among microbial antigens, heat shock proteins, have been the focus of attention of many investigators (Argyrios 1995a).

In this section the levels of isotype specific antibody responses to streptokinase, the antigen released during infection with the common bacterium streptococcus were measured in patients with a spectrum of auto-immune diseases (RA, SLE, thyroditis and HSP). In addition the specific IgG response to mycobacterium heat shock protein 65 (mHSP65) a possible superantigen for auto-immune diseases was investigated.

Rheumatoid arthritis is an auto-immune disease in which IgM antibody to human IgG called rheumatoid factor (RF) is present in the serum of the patients. The role of the rheumatoid factor in the pathogenesis of this disease is not clear. It has been suggested that infections such as mycoplasma or chronic bacterial agents modify the immune system which results in failure of normal tolerance. Systemic lupus erythematosus

(SLE) is a non-organ specific auto-immune disease in which auto-antibodies are not confined to a specific organ. In SLE auto-antibodies are against DNA and other cellular components of damaged cells, this results in immune complexes formation and deposition in various sites that ultimately produces a generalised inflammation. Thyroiditis is an organ specific auto-immune disease which includes Grave's disease (Hyperthyroidism), Hashimoto's disease (Hypothyroidism) and myxoedema (No thyroid function). Henoch Schonlein Purpura (HSP) is an auto-immune disease which in small blood vessels in a number of organs are involved (vasculitis). It is usually a disease of children age between 4 and 10 years. The biopsy of the affected areas i.e. glomeruli, skin shows the deposition of IgA, C3. A variety of infections could be associated in the pathogenesis of the diseases.

Since 1940's the involvement of infectious agents in the pathogenesis of RA has been the subject of controversial debates. The possibility of heat-shock proteins acting as superantigens and triggering autoimmune disorder, has re-opened these debates.

The general population are commonly exposed to streptokinase as this antigen is released during streptococcal infections (throat, skin, nasal, ears, etc.). After analysis of a normal population and a group of AMI we were keen to establish what levels were like in patients with auto-immune diseases.

The levels of IgG, IgM, IgA and neutralising anti-streptokinase antibodies in the patient groups described were measured and the values compared to the established normal ranges (Table 4-1b Figures 4-1, 4-2 and 4-3). Although the levels of anti-streptokinase antibodies in these patients were not statistically elevated a significant proportion of the patients had elevated levels of the antibodies compared with the normal population. In addition the levels of anti-streptokinase antibodies in these patients generally correlated with one another.

Patients with RA had significantly elevated levels of specific IgM anti-streptokinase antibody levels (Figure 4-2, Table 4-2). The mean value of IgM anti-streptokinase

antibody in this group (60.3 units/ml) was about 60 fold greater than the mean value of normal individuals (1.9 units/ml). The levels of IgM anti-streptokinase did not correlate with RF measured by nephelometry or ELISA. However, to determine whether the presence of rheumatoid factor (RF) had any effect on the levels of detectable IgM anti-streptokinase antibodies, rheumatoid factor was removed from the sera by affinity chromatography (Sepharose-IgG). The levels of IgM anti-streptokinase post-purification were still above the normal range (Figures 4-3 and 4-4, Table 4-4).

The elevated anti-streptokinase antibodies in R.A. patients could be attributable to: a) the association of streptococcal infections in the pathogenesis of the R.A similar to participation of several infections in induction of auto-immune diseases i.e. carditis with group A streptococci, b) the effect of immuno-suppressive therapy on these patients making these individuals more susceptible to infectious diseases, c) as fibronectin and streptokinase share a similar epitope (Gonzalez-Gronow et al 1993), in this chronic inflammatory disease, the presence of autoantibodies against fibronectin (or fibronectin combined with other body constituents) may show cross-reactivity with streptokinase and d) the presence of excess amounts of rheumatoid factor in the sera of theses patients (IgM isotype) enhances the results which are obtained by ELISA's. The observed elevation of anti-streptokinase antibodies may renew the idea of the streptococcal infections association in the pathogenesis of the Rheumatoid Arthritis, however, this infection does not seem to be related to any concomitant rise in the super antigen mHSP65.

Heat shock proteins are a ubiquitous, highly conserved group of proteins used in a variety of cellular activities. They constitute a major antigenic component of mycobacterium and are considered as a major trigger for auto-immunity. Elevated levels of anti-mHSP65 have been demonstrated in RA patients (Bahr et al 1988). I could not support this observation in the RA patients, or patients with SLE, thyroiditis or HSP as

the levels of IgG anti-mHSP65 in this group were not statistically elevated compared to the normal individuals (Figure 4-5).

On the other hand, in patients with Henoch Schonlein Purpura there was a correlation between IgG anti-mHSP65 levels and IgG, IgA and neutralising anti-streptokinase antibodies levels (Figures 4-6 and 4-7). Although this was not highly significant and based on a small number of patients, it does however, raise the possibility that streptococcal infections and the release of heat shock proteins may play an important role in the aetiology of HSP.

From this data it can be concluded that streptococcal infections have an association in the pathogenesis of Rheumatoid Arthritis and Henoch Schonlein Purpura.

Chapter 5

Streptokinase and Complement System Recruitment

Introduction

Streptokinase treatment provides a natural model for the study of immune complex formation where pre-existing anti-streptokinase antibody levels and complement activation products can be monitored serially following administration of the thrombolytic agent. The extent of pre-existing antibodies to streptokinase in a normal population and patient groups has been examined as well as the changes of these antibodies during thrombolytic therapy, to study the important role of pre-existing anti-streptokinase antibodies in the poor response to thrombolytic treatment or adverse immune response (Chapter 3). This study showed that during streptokinase administration, immune complexes appear to be formed and patients with elevated levels of pre-existing anti-streptokinase antibodies develop adverse immunological reactions, however, elevated levels of anti-streptokinase antibodies do not always resulted in such reactions. This in part may be explained by the interaction between the complement system and the immune complexes (1-8). There are few studies to date studying the role of complement in streptokinase therapy (1-10).

There are multiple methods for assessing complement activation, Nephelometry, rocket electrophoresis, radial immunodiffusion, enzyme-linked immunosorbent assay (ELSIA) and radioimmunoassay (RIA) quantify antigenic levels of complement components. Haemolytic assays, the classical pathway CH50, the alternative pathway CH50 and specific haemolytic assays are used to determine the functional activities of the complement system. More recently, measurement of complement activation products has become a popular means of assessing ongoing complement activation, particularly in patients with immune complex diseases (Auda et al 1990). Activation of

classical, alternative and terminal pathways can be assessed by the levels of generated C1s:C1-INH, C3b-P and C5b-9 complexes respectively. C1s:C1-INH complex is formed during classical pathway activation by covalent binding of C1-INH and activated C1s, the complexes C3b-P and C5b-9 are formed during alternative and terminal pathways activation respectively (1-7) (Figure 5-1). Another product of complement activation which is measured in routine measurements is C3d, a product of Factor I mediated cleavage of C3b. The advantages of these assays is their sensitivity and specificity for ongoing complement activation.

In this chapter complement system recruitment in AMI patients treated with streptokinase and the influence of pre-existing anti-streptokinase antibodies on this recruitment is studied. The antigen streptokinase is somewhat unusual in that it may trigger complement activation by two mechanisms: 1) Through plasminogen activation and generation of plasmin. Plasmin is thought to activate the complement system in two ways, firstly, plasmin like trypsin and kallikrein can activate C1s in absence of immune complexes and secondly it can activate the alternative pathway by acting as Factor D and degrading Factor B (1-0). 2) The more classical mechanism of the complement system activation by streptokinase is through immune complex formation and activation of the classical pathway (1-10).

In vitro effects of streptokinase and streptokinase immune complexes, thyroglobulin and thyroglobulin immune complexes on complement system were studied by measuring the levels of C1s:C1-INH, C3b-P, C5b-9. Novel ELISA's were developed to measure free C3d and erythrocyte bound C3d. The effect of plasmin on this in vitro system was also investigated.

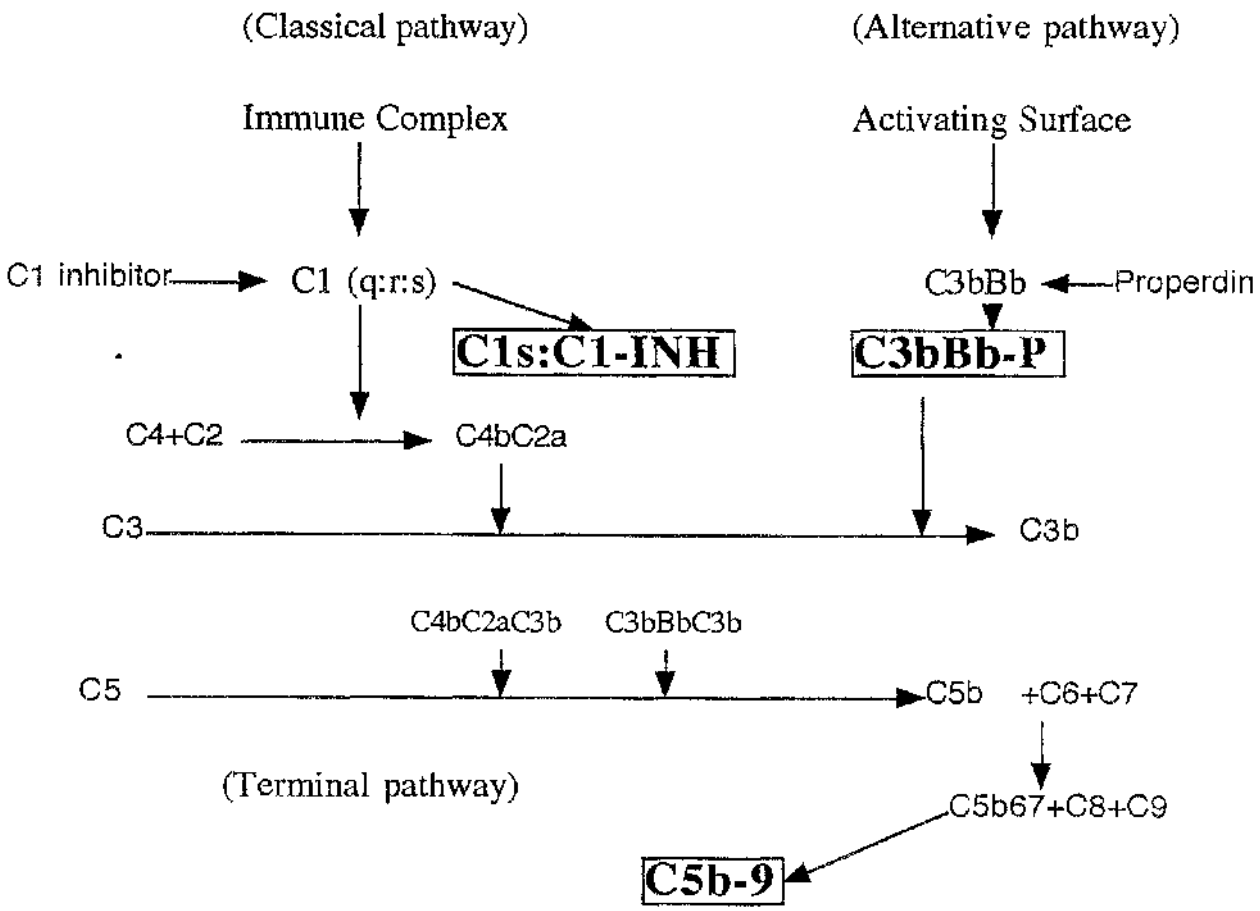


Figure 5-1: Schematic diagram of the formation of, C1s:C1-INH, C3b-P and C5b-9, studied as indicators of complement pathways activation.

5-1 Ion exchange chromatography for purification of human anti-streptokinase IgG. Diethylaminoethyl cellulose (DE52, Whatman) binds to negative charged ions and the extent of binding depends on the degree of charge. The serum to be used is dialysed into the column buffer (pH 7.6), under those conditions IgG with positive charge does not bind to the DE52 but the remaining proteins bind and IgG can be eluted. Human sera were screened by ELISA procedure to collect positive samples with anti-streptokinase antibody activity (3-3) and IgG was then purified from the pooled sera with elevated levels of anti-streptokinase activity.

Materials

Buffer: 0.01M sodium phosphate buffer pH 7.6 (Conductivity at 4°C, 0.5- 0.8 mS) (2-2 and 2-3).

Column: 1.5 X 9 cm column (60 ml syringe barrel) containing DE52 extensively equilibrated with the buffer.

Sample: 20 ml pooled sera with elevated anti-streptokinase antibodies (protein concentration 90 mg/ml) were dialysed into the buffer.

Methods. After dialysis the pooled sera were applied to the column and the column was washed with buffer, 3 ml fractions were collected, the protein concentration of fractions was measured at 280 nm. Fractions 3 to 10 containing the protein peak were pooled. The elution profile of collected fractions by the DE52 columns from pooled sera with elevated anti-streptokinase antibodies is shown in Figure 5-2.

The purified IgG was run on a 7.5% SDS-PAGE gel and three bands with molecular weights of 150kDa, 105 kDa and 66 kDa were observed. Total concentration of the purified IgG was 11.5 mg/ml.

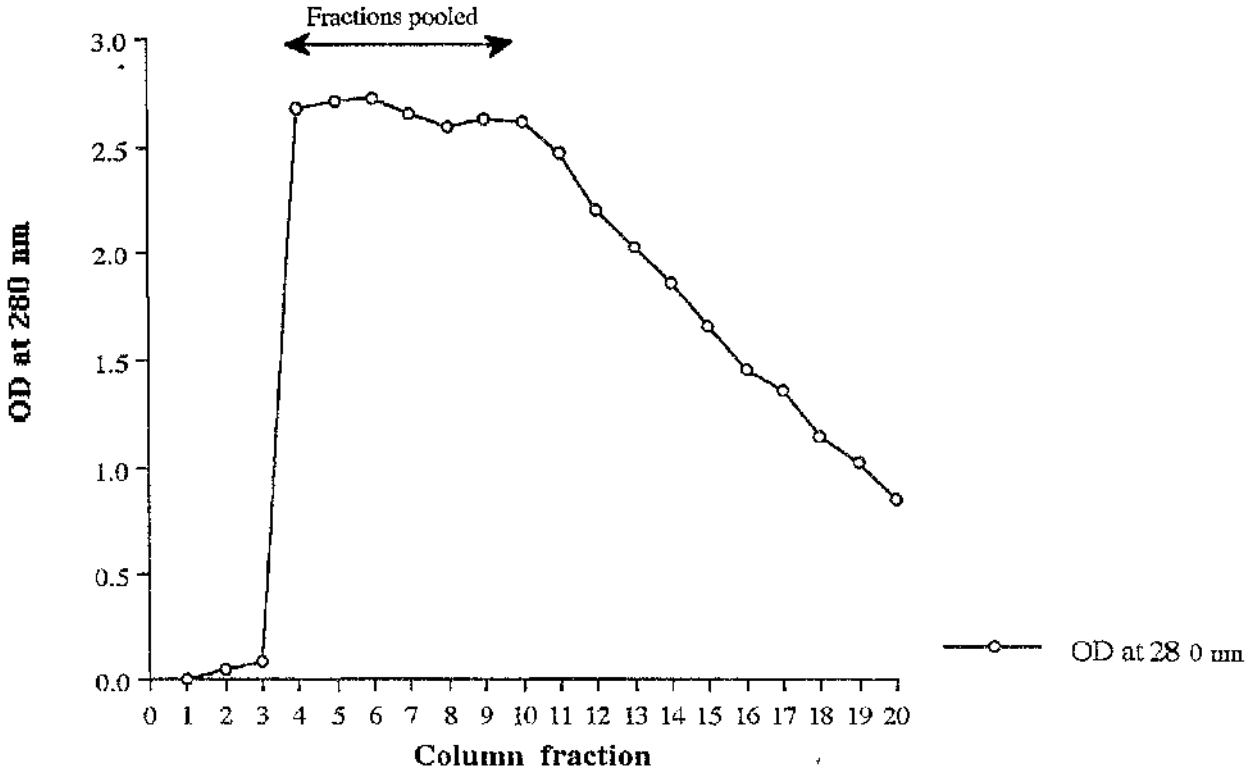
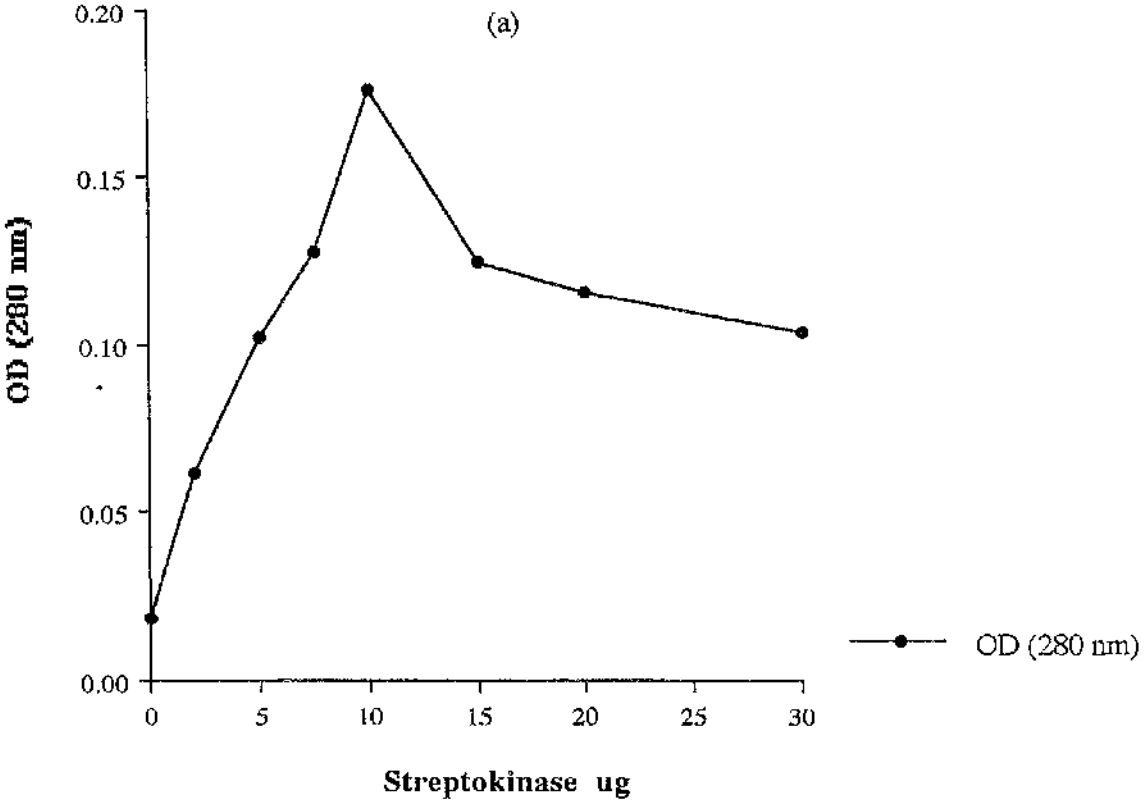


Figure 5-2: Elution profile of fractions collected by a DE52 column from pooled sera with elevated levels of anti-streptokinase antibodies.

5-2 Preparation of immune complexes. Rabbit anti-streptokinase serum was prepared as described before (2-13, 2-14), and double immunodiffusion confirmed antiserum specificity to streptokinase (2-7, Figure 2-3). Rabbit anti-streptokinase IgG was then purified from this serum by caprylic acid and total concentration of purified IgG was 13 mg/ml (2-19). The purified rabbit anti-streptokinase IgG was run on a 7.5% SDS-PAGE gel (2-11) and 3 bands with molecular weights of 160kDa, 94kDa and 62 kDa were observed. Rabbit anti-thyroglobulin IgG was purified from the immunised serum (a kind gift of Dr.E.Holme) by caprylic acid (2-19) and total concentration of purified IgG was 5 mg/ml.

Quantitative precipitin test were performed for both antisera sources (2-18) by adding increasing amount of antigens (streptokinase or bovine thyroglobulin) to a constant amount of antibody (human anti-streptokinase 11.5 mg/ml, rabbit anti-streptokinase IgG 13 mg/ml, rabbit anti-thyroglobulin IgG 5 mg/ml) to determine the equivalence points. The equivalence point for rabbit anti-streptokinase IgG was 10 μg streptokinase and 1300 μg IgG (Figure 5-3a), for human anti-streptokinase IgG was 10 μg streptokinase and 287.5 μg IgG (Figure 5-3b) and for rabbit anti-thyroglobulin IgG was at 10 μg thyroglobulin and 100 μg IgG (Figure 2-4).

5-3 Measurement of complement activation products (C1s:C1-INH, C3b-P and C5b-9). C1s:C1-INH, C3b-P and C5b-9 were measured by indirect ELISA's using a modification of the techniques developed by Auda et al 1990. In this technique C1s:C1-INH, C3b-P and C5b-9 complexes are captured by relevant antibodies and using a labelled second antibody which reacts with a different component of the complex the bound complex is quantified (Table 5-1). As the antibodies react with different components of the complexes, the intact components (C1-INH, C3 and C5) also present in serum samples do not interfere with the assay.



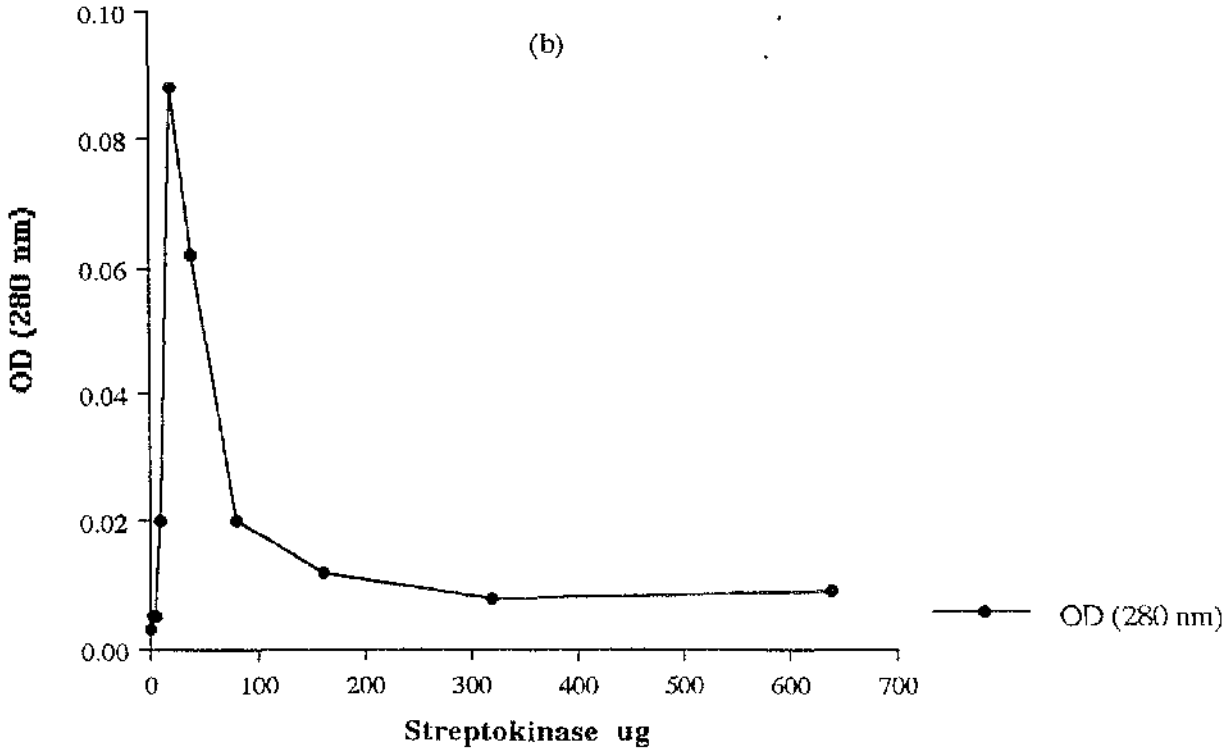


Figure 5-3: Quantitative precipitin test for rabbit (a) and human (b) IgG anti-streptokinase.

Condition	C1s:C1-INH	C3b-P	C5b-9	C3d
Standard curve, coating antibody	anti-C1-INH (10μg/ml)	anti-C3 (5μg/ml)	anti-C5 (10μg/ml)	anti-C3d (5μg/ml)
Samples, coating antibody	anti-C1s (10μg/ml)	anti-properdin (10μg/ml)	anti-C9 (10μg/ml)	anti-C3d (5μg/ml)
Blocking buffer	0.1% Gelatin in PBS	0.1% Gelatin in PBS	0.1% Gelatin in PBS	0.1% BSA in PBS
Initial standard dilution	1/1000	1/10000	1/1000	1/5000
Initial standard concentration	180 ng/ml	130 ng/ml	70 ng/ml	200 units/ml
Sample dilution	1/10 or 1/80	1/10 or 1/80	1/10 or 1/80	1/80 or 1/600
Biotin-conjugate dilution	anti-C1-INH (1/6000)	anti-C3 (1/10000)	anti-C5 (1/6000)	anti-C3d (1/10000)
HRP-Avidin dilution	1/5000	1/5000	1/5000	1/5000

Table 5-1: ELISA conditions for measuring complement activation products. The first dilution for samples is the dilution used to dilute patients sera and the second dilution is the dilution used for in vitro assays.

Materials. Sources of materials used here are described in 2-2.

ELISA plates (Immulon 4, Dynatech)

Goat anti-human C3 (IgG fraction)

Goat anti-human Properdin (IgG fraction)

Goat anti-human C1 (esterase) inhibitor (IgG fraction)

Goat anti-human C1s (IgG fraction)

Biotin N-hydroxy-succinimide ester

Horse radish peroxidase Avidin-D

Buffers were made as described in 2-3.

Phosphate buffered saline (PBS)

ELISA coating buffer (pH 9.60)

ELISA blocking buffer (0.1% gelatin in PBS)

ELISA wash buffer (PBS-Tween)

ELISA enzyme substrate

4N H_2SO_4

20 mM isotonic EDTA

Method. The ELISA conditions for measuring the complement activation products are summarised in Table 5-1.

The first two rows of ELISA plates were coated with goat anti-human C1-INH, anti-C3 or anti-C5 to construct standard curves (Table 5-1). The remaining wells were coated with goat anti-human C1s, anti-properdin, anti-C9 or rabbit anti-human C3d in ELISA coating buffer (Table 5-1). The plates were incubated at 4°C overnight and washed extensively with PBS-Tween. Non-specific binding sites were blocked with 0.1%

gelatin in PBS (200 µl per well) for 1 hour at room temperature in a humidified chamber (Table 5-1). The plates were washed and fresh NHS, diluted in 20mM PBS-EDTA as described in Table 5-1, was applied in the first two rows to construct the standard curves (100 µl per well). Diluted samples were applied to remaining wells and incubated for 1 hour at room temperature (Table 5-1). After washing 100 µl of the relevant biotin-conjugated antibody (2-22) was added to each well and incubated for half an hour (Table 5-1). The plates were washed and 100 µl Avidin-horseradish peroxidase was applied per well and incubated for 20 minutes at room temperature (Table 5-1). The plates was washed and the conjugates were developed using O-phenylene diamine as the substrate (2-2). The reaction was stopped with 4N H_2SO_4 50 µl per well and the optical densities were measured at 490 nm on a Dynatech ELISA reader. Intra-assay and inter-assay variations of complement activation products were determined as described before (3-6).

5-4 Measurement of serum C3d levels. Complement activation can also be assessed by measuring serum levels of C3d, the degraded fragment of C3b. An indirect ELISA's was developed for measuring serum C3d levels as,follows.

Materials. Sources of materials used here are described in 2-2.

ELISA plates (Immulon 4, Dynatech)

Rabbit anti-human C3d

Biotin N-hydroxy-succinimide ester

Horse radish peroxidase Avidin-D

Buffers were made as described in 2-3.

Phosphate buffered saline (PBS)

ELISA coating buffer (pH 9.60)

ELISA blocking buffer (0.1% BSA in PBS)

ELISA wash buffer (PBS-Tween)

ELISA enzyme substrate

4N H_2SO_4

20 mM isotonic EDTA

Method. The ELISA conditions for measuring C3d are summarised in Table 5-1. The ELISA plates were coated with rabbit anti-human C3d (Table 5-1). The plates were incubated at 4°C overnight and washed extensively with PBS-Tween. Non-specific binding sites were blocked with 0.1% BSA in PBS (200 µl per well) for 1 hour at room temperature in a humidified chamber (Table 5-1). The plates were washed and 100 µl per well of an activated serum containing C3d, diluted in 20mM PBS-EDTA as described in Table 5-1, was applied in the first two rows to construct the standard curve. Diluted samples were applied to remaining wells and incubated for 1 hour at room temperature (Table 5-1). After washing 100 µl of biotin-conjugated rabbit anti-human C3d (2-22) was added to each well and incubated for half an hour (Table 5-1). The plates were washed and 100 µl Avidin-horseradish peroxidase was applied per well and incubated for 20 minutes at room temperature (Table 5-1). The plates was washed and the conjugates were developed and the optical densities were measured at 490 nm as described (5-3). Intra-assay and inter-assay variations of C3d were determined as described before (3-5).

5-5 Validity of ELISA used for measuring serum C3d levels. The presence of large amount of intact C3 in serum may interfere with the C3d assay. Therefore this cross-reactivity was studied by the following experiments.

Materials. Sources of materials used here are described in 2-2.

ELISA plates (Immulon 4, Dynatech)

Rabbit anti-human C3d

Goat anti-human C3

Biotin N-hydroxy-succinimide ester

Horse radish peroxidase Avidin-D

Buffers as described in 5-4.

Methods. a) The effect of changes in antibody conditions on the measurement of free C3d: To capture C3d, ELISA plate was coated with rabbit anti-human C3d or goat anti-human C3 (5μg/ml in coating buffer) at 4°C overnight. The plate was washed and non-specific binding sites were blocked by 0.1% BSA in PBS for 1 hour. After washing the plates, activated NHS (By immune complexes, contained C3d and C3, 5-9) and naive NHS (non-activated NHS contained only intact C3) diluted 1/600 in 0.1% BSA-PBS were applied to the plates and incubated for 1 hour at room temperature. The plates were washed and biotin-conjugated anti-C3d or anti-C3 (2-22, diluted 1/10000) was added and incubated for half an hour. The plate was washed and Avidin-horseradish peroxidase diluted 1/5000 was added and incubated for 15 minutes. After washing the conjugate was developed using OPD and the optical densities measured at 490 nm (5-4).

b) The effect of dilution on the detectable levels of C3d: Activated NHS (By immune complexes, contained C3d and C3) and naive NHS (Contained C3) were diluted 1/50 or 1/600 and the levels generated C3d were measured (5-4) to determine whether by diluting the serum samples the effect of C3 on the assay could be minimised.

c) Kinetics of C3 and C3d during complement activation: The levels of C3d generated during complement activation (By immune complexes) was studied over a period of 30

minutes at 37°C (5-4) and compared with the levels of C3. The levels of C3 was measured by modified technique used for measuring C3b-P in which anti-properdin was replaced with anti-C3 (5 μg/ml) to capture C3 and the values of C3 were read against C3 standard curve (5-3).

5-6 Detection of erythrocytes bound complement components by a modified ELISA technique. This assay was developed to evaluate erythrocyte bound complement components during complement activation.

Materials. Sources of materials used here are described in 2-2.

Round-bottomed microplates

Rabbit anti-human C3d

Goat anti-human C3

Goat anti-human Properdin

Goat anti-human Factor B

Human erythrocytes

Buffers were made as described in 2-3.

Phosphate buffered saline (PBS)

ELISA coating buffer (pH 9.60)

1% and 0.1% BSA in PBS

ELISA wash buffer (PBS-Tween)

Methods. a) Evaluation of erythrocytes bound C3, properdin and Factor B: To capture the bound C3, properdin and Factor B on erythrocytes, an ELISA plate (Immulon 4, Dynatech) was coated with goat anti-human C3, anti-human properdin or anti-human

Factor B (5μg/ml) in ELISA coating buffer respectively and incubated at 4°C overnight. The plates were washed with PBS-Tween and non-specific binding sites were blocked with 0.1 % BSA in PBS for 1 hour at room temperature. Erythrocytes (E's) from an activated blood sample (By immune complexes, 5-9) or naive blood sample (unactivated) were washed with 1% BSA and reconstituted to $1X\ 10^8$ cells/ml (1-17). The erythrocytes were then added to the plate (100 μl / well, in triplicates) and incubated at room temperature for 2 hours. To remove the unbound E's the plates were washed very gently by 1% BSA (200μl/well X3) using a multi-channel pipette. After washing bound erythrocytes were lysed by adding of 100 μl of water per well and the optical densities of lysed cells measured at 405 nm on a Dynatech ELISA reader.

b) Evaluation of erythrocytes bound C3d: To capture the bound C3d on erythrocytes, ELISA plates were coated with rabbit anti-human C3d (Dakopatt) from 0.625 μg to 80μg/ml in ELSA coating buffer and incubated at 4°C overnight. Erythrocytes (E's) from an activated blood sample (By immune complexes) or an unactivated blood sample were washed with 1% BSA and reconstituted to $1X\ 10^8$ cells/ml, added to the plate as described and the plates incubated at room temperature for 2 hours. To remove the unbound E's the plates were washed with 1% BSA as described before and the bound erythrocytes were lysed with 100 μl of water per well, the optical densities of lysed cells measured at 405 nm on a Dynatech ELISA reader. Intra-assay and inter-assay variations of erythrocytes bound C3d were determined as described before (3-6).

5-7 The effect of thrombolytic therapy on complement activation in patients with AMI. The levels of complement activation products in these patient were studied in conjunction with Dr.E.Holme University Department of Immunology, Western Infirmary, Glasgow. Twenty patients with AMI who received thrombolytic treatment were studied (3-8). Blood samples were taken prior to administration of streptokinase and at timed intervals after treatment and kept at -70°C. The following

activation products were measured, C1s:C1-INH, C3-b-P and C5b-9. The fluctuations of complement activation products and the relationship of these products with the anti-streptokinase antibodies were studied.

5-8 Effects of dose and kinetics of streptokinase on the complement activation. Normal human serum (NHS) was prepared from venous blood of 10 healthy volunteers (2-15). As pre-existing elevated levels of anti-streptokinase antibodies in the NHS may interfere with this assay by forming of immune complex when streptokinase is added, the levels of anti-streptokinase antibodies in the 10 normal individuals were measured (3-1 and 3-5) and only sera with levels of anti-streptokinase antibodies within the normal ranges were used.

Materials. Sources of materials used here is described in 2-2.

Streptokinase

NHS

Buffers: prepared as described in 2-3.

Phosphate buffered saline (PBS)

Isotonic 20 mM EDTA

Methods. a) The effect of streptokinase on NHS at the therapeutic concentrations: To study the in vitro effects of streptokinase on complement activation, 20 μg, 40μg and 100 μg streptokinase were added to the 200 μl NHS. The volumes were equalised with PBS to 300 μl to give a final concentration of 66 μg/ml, 133 μg/ml and 333 μg/ml streptokinase in the mixtures (The dose of streptokinase administered to AMI patients is 5000-7500 I.U./ml = 66-100 μg/ml). After incubation at 37°C the assays were sampled at 0, 5 15 and 30 minutes. Complement activation was stopped by adding an equal

volume of ice cold isotonic 20 mM EDTA (2-2) and complement activation products were measured as described (5-3).

b) The effect of streptokinase beyond the therapeutic concentrations: The dose response of streptokinase in concentrations above the therapeutic concentrations was also studied. The levels of anti-streptokinase antibodies in the NHS used for this experiment were within the normal ranges (IgG= 1.7 units/ml, IgA, IgM and neutralising anti-streptokinase were not detectable).

Streptokinase 20 μg to 6000 μg was added to 500 μl NHS and the volume of the mixtures equalised to 800 μl with PBS, to give a final concentration from 25 μg/ml to 7500 μg/ml of streptokinase in the mixtures. The mixtures were then incubated at 37°C for 30 minutes and complement activation products were measured as described (5-3).

5-9 The effect of streptokinase and thyroglobulin immune complexes on complement activation. Preformed and nascent immune complexes were prepared to study the in vitro effects of immune complexes on complement activation. To prepare nascent immune complexes, antigen and antibody were added simultaneously to NHS and incubated at 37°C. Preformed immune complexes were prepared by incubating antigen and antibody at 37°C for 30 minutes prior to experiment.

Materials. Sources of materials used here is described in 2-2.

Human and rabbit anti-streptokinase IgG

Streptokinase

Rabbit anti-thyroglobulin IgG

Thyroglobulin

NHS

Buffers: prepared as described in 2-3.

Phosphate buffered saline (PBS)

Isotonic 0.086M EDTA

0.1 M $MgCl_2$ EGTA

20 mM EDTA

Methods. a) Kinetics and dose response of rabbit streptokinase immune complexes: The effect of rabbit streptokinase immune complexes (preformed and nascent, see above) on complement activation was studied. Immune complexes at equivalence, antigen excess or antibody excess were prepared (5-2) by adding 1μg to 80 μg streptokinase to a constant amount of rabbit anti-streptokinase IgG (1300 μg) and the volumes equalised to 380 μl with PBS. The mixtures were then incubated with 200 μl NHS at 37°C for 30 minutes. The assay sampled at 0, 5 15 and 30 minutes, complement activation was stopped by addition of equal volume ice cold isotonic 20 mM EDTA to the reaction mixture (2-2) and complement activation products were measured as described (5-3).

b) The effect of human streptokinase immune complexes on the complement activation: The effect of human streptokinase immune complexes (preformed) on complement activation was studied. Immune complexes at equivalence, antigen excess or antibody excess were prepared (5-2) by adding 5 μg to 320 μg streptokinase to a constant amount of human anti-streptokinase IgG (1150 μg) and the volumes were equalised to 670 μl with PBS, and then incubated with 250 μl NHS at 37°C for 5 minutes. The assay sampled at 5 minutes and complement activation stopped by addition of an equal volume of ice cold isotonic 20 mM EDTA to the reaction mixture (2-2) and complement activation products were measured as described (5-3).

c) Kinetics and dose response of rabbit thyroglobulin immune complexes: The effect of rabbit thyroglobulin immune complexes (preformed and nascent, see above) on complement activation was studied. Immune complexes at equivalence, antigen excess or antibody excess was prepared (5-2) by adding 3μg to 200 μg thyroglobulin to a

constant amount of rabbit anti-thyroglobulin IgG (500 μg) and the volumes equalised to 450 μl with PBS, the mixtures were then incubated with 200 μl NHS at 37°C for 30 minutes. The assay sampled at 0, 5 15 and 30 minutes, complement activation was stopped by addition of an equal volume of ice cold isotonic 20 mM EDTA to the reaction mixture (2-2) and complement activation products were measured as described (5-3).

Controls: 1) The levels of complement activation products in NHS (In absence of activating factors, antigens, antibodies or ICs) at 37°C was used as control throughout the experiments, and control for spontaneous complement activation.

2) To inhibit complement activation, NHS was incubated with isotonic 0.086M EDTA to give a final concentration of 10 mM (2-2) and to inhibit alternative pathway activation, NHS was incubated with 0.1 M $MgCl_2$ EGTA to give a final concentration 6 mM (2-2). NHS treated with EDTA or Mg^{++}, EGTA were incubated with immune complexes at 37°C and complement activation products were measured as described (5-3).

5-10 The effect of erythrocytes on complement activation. The effects of erythrocytes on complement activation were studied as they may influence the levels of generated complement activation products, through either the action of CR1, an essential cofactor for Factor I or acting as a surface upon which these products can be deposited. The effect of erythrocytes on complement activation products was studied.

Materials. Sources of materials used here is described in 2-2.

Human and rabbit anti-streptokinase IgG

Streptokinase

Rabbit anti-thyroglobulin IgG

Thyroglobulin

NHS

erythrocytes

Mouse monoclonal anti-CR1

Buffers: prepared as described in 2-3.

Phosphate buffered saline (PBS)

1% BSA in PBS

20 mM isotonic EDTA

Methods. a) The effect of erythrocytes on the complement activation products: Nascent thyroglobulin immune complexes at equivalence (25 μg antigen and 250 μg IgG, 5-2) were incubated with 200 μL NHS in the presence or absence of 200 μl packed E's and the volumes were equalised to 470 μl with 1% BSA in PBS, the mixtures were then incubated at 37°C for 30 minutes. The assay mixture was sampled at 0, 5 15 and 30 minutes, complement activation was stopped by addition of equal volume of 20 mM EDTA to the reaction mixture (2-2) and complement activation products were measured (5-3).

Preformed rabbit streptokinase immune complexes at equivalence (10 μg streptokinase and 1300 μg IgG, 5-2) were incubated with 500 μL NHS in presence or absence of 100 μl packed E's and the volumes were equalised to 750 μl with 1% BSA in PBS, the mixtures were then incubated at 37°C for 15 minutes. The assay sampled at 0, 5 and 15 minutes, complement activation was stopped by addition of equal volume of 20 mM EDTA to the reaction mixture (2-2) and complement activation products, C1s:C1-INH, C3b-P, free C3d and erythrocytes bound C3d were measured (5-3, 5-4 and 5-6).

A limited study was undertaken with preformed human streptokinase immune complexes at equivalence (40 μg antigen and 1150 μg IgG, 5-2) incubated with 250 μL NHS in presence or absence of 50 μl packed E's, the volumes were equalised to 440 μl

with 1% BSA in PBS, the mixtures were then incubated at 37°C for 5 minutes. The assay mixture was sampled at 5 minutes and complement activation was stopped by addition of equal volume of 20 mM EDTA to the reaction mixture (2-2) and complement activation products were measured as described (5-3).

b) The effect of CR1 receptors on the complement activation products: To study the effect of the CR1 receptors, 150 µL of packed E's were incubated with 75 µl of monoclonal anti-CR1 (Dakopatt, 135 µg/ml) at 37°C for 30 minutes and the cells were washed with 1% BSA in PBS. 100 µl of packed naive (untreated with anti-CR1) E's or 100 µl of packed E's treated with anti-CR1 were added to preformed rabbit streptokinase ICs at equivalence (10 µg streptokinase and 1300 µg IgG, 5-2) and 500 µL NHS, the volumes were equalised to 750 µl by 1% BSA in PBS. The mixtures were then incubated at 37°C for 15 minutes. The assay sampled at 0, 5 and 15 minutes, complement activation was stopped with 20 mM EDTA as described and the levels of C1s:C1-INH, C3b-P, free C3d and erythrocytes bound C3d were measured (5-3, 5-4 and 5-6).

5-11 The effect of plasmin on the levels of complement activation products. As described plasmin can interact with complement system (see introduction) these interactions therefore may influence the detectable levels of complement activation products.

Materials. Sources of materials used here is described in 2-2.

Plasmin

Streptokinase

Rabbit anti-thyroglobulin IgG

Thyroglobulin

NHS

Buffers: prepared as described in 2-3.

Phosphate buffered saline (PBS)

20 mM isotonic EDTA

Methods. a) The effect of plasmin on the levels of complement activation products: Direct effect of plasmin on the generated levels of complement activation products by rabbit thyroglobulin immune complexes was studied. Thyroglobulin ICs were used as streptokinase may affect the assay. Plasmin from 4μg to 256μg (Total protein) was added to a constant amount of nascent thyroglobulin immune complexes at equivalence (25 μg antigen and 250 μg IgG, 5-2) and 200 μl NHS, the volumes were equalised to 475 μl with PBS. The mixtures were then incubated at 37°C for 30 minutes. The assay mixture was sampled at 30 minutes and complement activation was stopped by addition of an equal volume of 20 mM EDTA to the reaction mixture (2-2) and complement activation products were measured as described (5-3).

Controls: 256 μg plasmin + 200 μl NHS, 200 μL NHS alone, thyroglobulin ICs + 200 μl NHS in absence of plasmin (Total volumes were equalised to 475 μl with PBS), at 37°C for 30 minutes were used as controls.

b) Kinetics of complement activation by rabbit thyroglobulin immune complexes in presence of streptokinase: To study the indirect effect of streptokinase through the generation of plasmin on the levels of complement activation products generated by thyroglobulin immune complexes, serial amount of streptokinase from 50 μg/ml to 400 μg/ml (Therapeutic dose = 66-100 μg/ml) were added to a constant amount of nascent thyroglobulin immune complexes at equivalence (25 μg thyroglobulin and 250 μg IgG, 5-2) and 200 μl NHS, the volumes were equalised to 475 μl with PBS. The mixtures were then incubated at 37°C for 30 minutes. The assay mixture was sampled at 0, 5 15

and 30 minutes, complement activation was stopped by addition of equal volume of 20 mM EDTA to the reaction mixture (2-2) and complement activation products were measured as described (5-3).

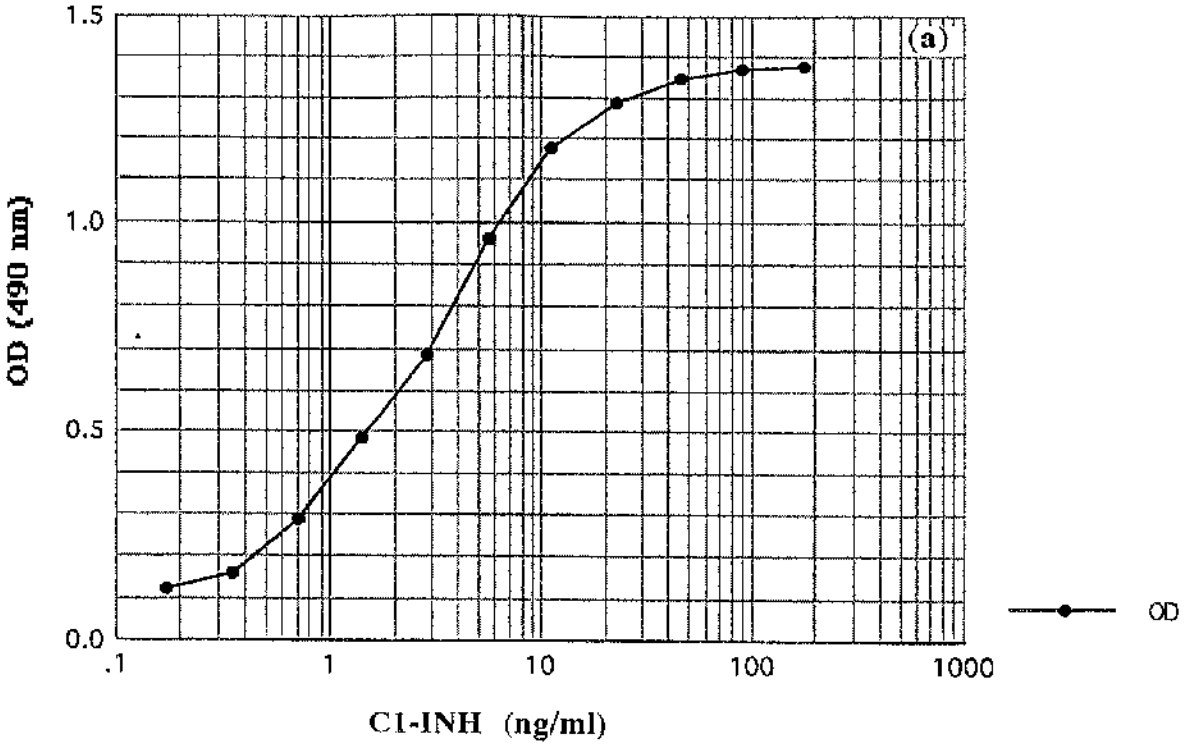
Controls: ICs + NHS, streptokinase + NHS and NHS alone (Total volumes were equalised to 475 µl with PBS) at 37°C for 30 minutes were used as controls.

5-12 Statistical analysis. Statistical analysis of data was performed using Spearman rank correlation test, Wilcoxon signed rank test and Mann-Whitney test.

Results

Measurement of complement activation products C1s:C1-INH, C3b-P and C5b-9. C1s:C1-INH, C3b-P and C5b-9 were measured by indirect ELISA's using a modification of the techniques developed by Auda et al 1990. These modifications were 1) The time of incubation for the samples, biotin-labelled antibodies and Avidin-HRP were reduced (5-3, Table 5-1), 2)Pooled sera from 40 individuals to construct standard curves to measure the levels of the complement activation products in unknown serum samples (5-3, Table 5-1).

Dilutions of samples (Table 5-1) corresponded to the linear part of the standard curves and the optical densities were read off the standard curves and the values expressed in ng/ml (Figures 5-4 and 5-5). The intra-assay variations of the C1s:C1-INH and C3b-P were 12% and 6% respectively (Table 5-2). C1s:C1-INH assay showed a better reproducibility since the inter-assay variations (9%) for this assay was 10% lower than C3b-P assay (19%) (Table 5-2). For C5b-9 the intra-assay and inter-assay variations were not determined.



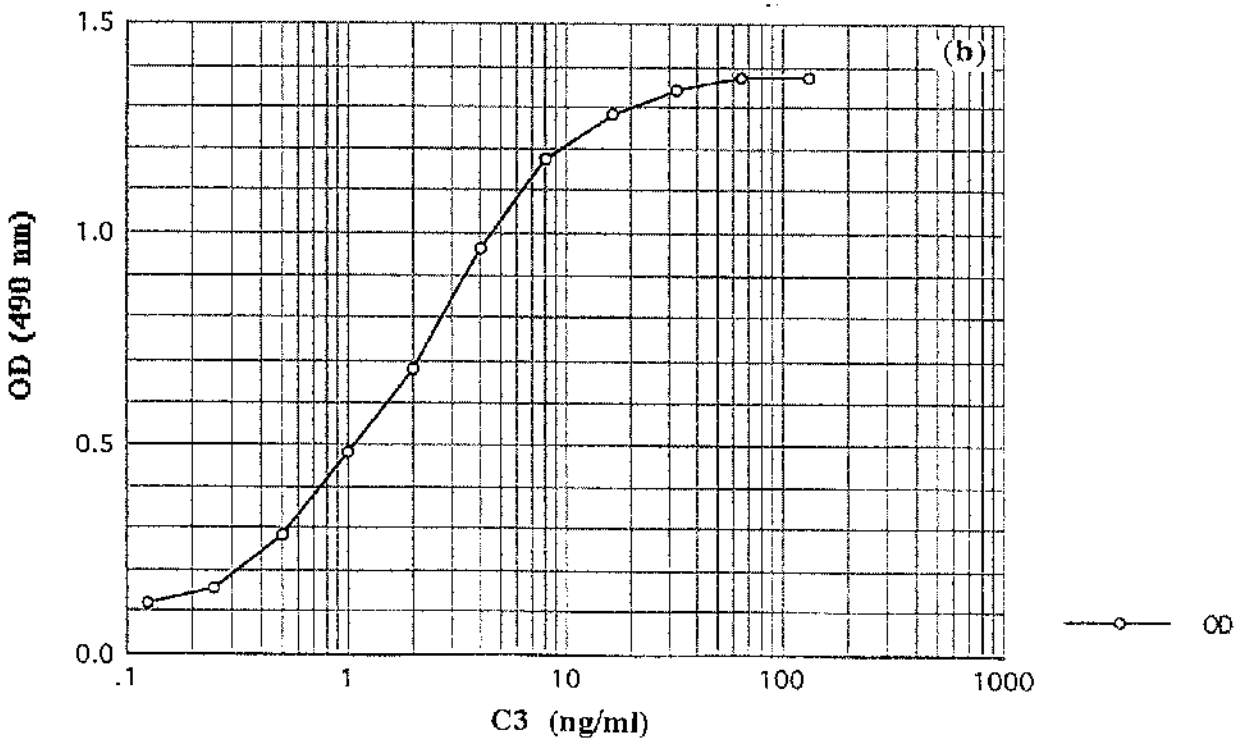
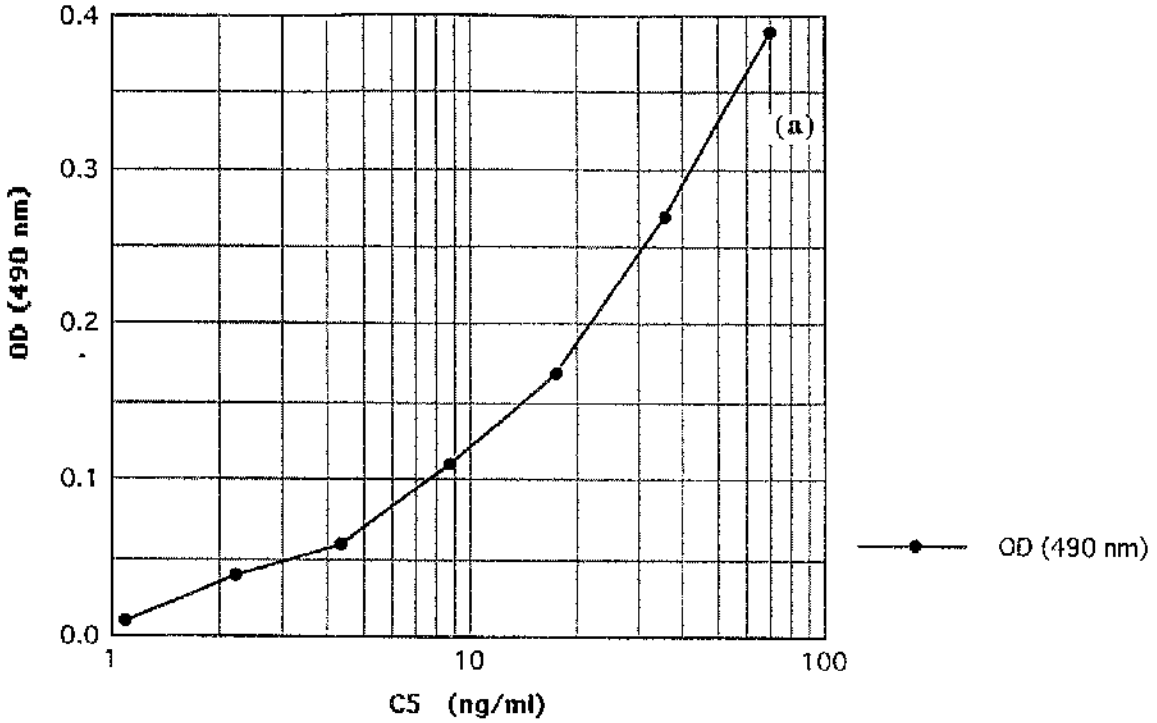


Figure 5-4: Standard curves for measuring C1s:C1INH (a) and C3b-P (b).



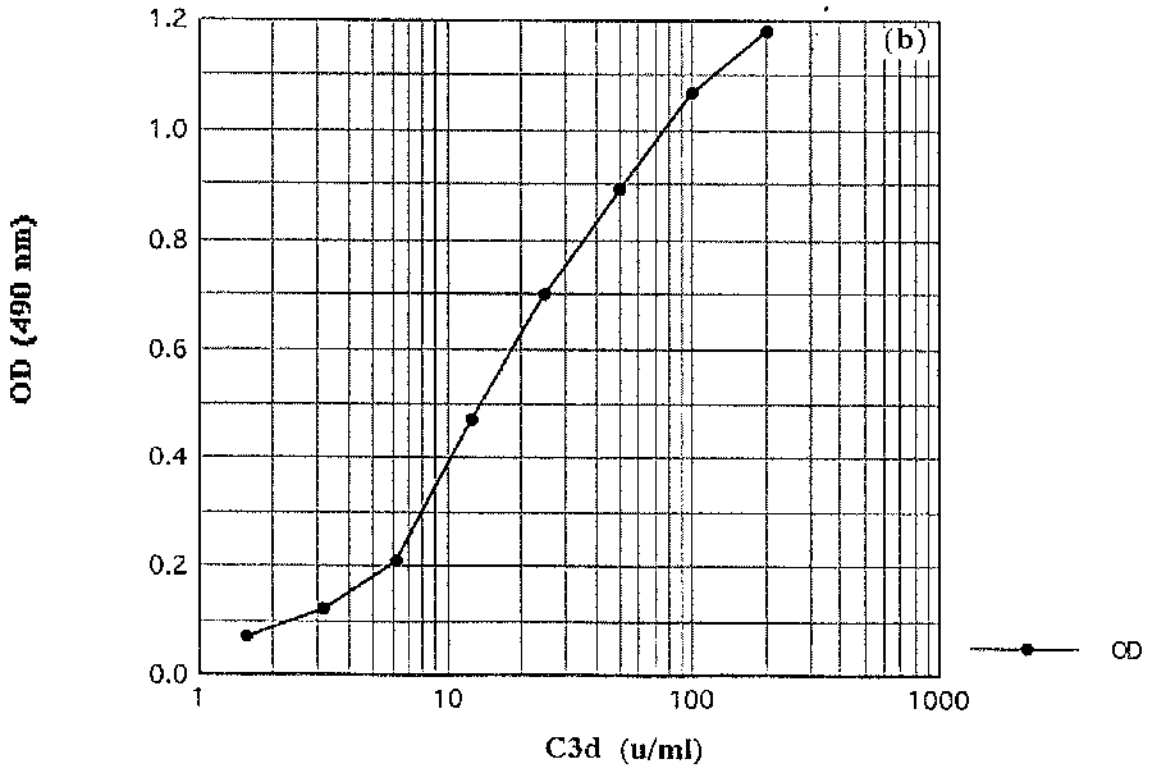


Figure 5-5: Standard curves for measuring C5b-9 (a) and C3d (b).

Measurement of serum C3d levels. An indirect ELISA's for measuring serum C3d levels was developed to assess complement activation by ascribing levels of C3 breakdown (5-4, Table 5-1, Figure 5-5). The intra-assay and inter-assay variations for this assay were 11% and 20% respectively (Table 5-2).

To evaluate whether the presence of intact C3 in serum samples interferes with the C3d assay, the conditions of the antibody for measuring C3d were changed (5-5). A larger difference between the optical densities (OD) of activated NHS (Contained C3d and C3) and the naive NHS (untreated, contained C3) was observed when both trapping and detecting antibodies were anti-human C3d (47.5%) (Figure 5-6) and this difference was reduced when either trapping or detecting antibodies were changed to anti-human C3 (Figure 5-6).

To further overcome the interference of C3 with the C3d measurement, the samples were diluted (5-5) and C3d levels was measured as described (5-4). By diluting the samples the OD of activated NHS (Contained C3d and C3) did not change, however, the OD of the untreated NHS (Contained C3) at the higher dilution of 1/600 showed a 48% reduction compared to the dilution 1/50 (Figure 5-7a). When these ODs were read off the standard curve and multiplied by the dilution factors, a significant increase in the levels of detectable C3d in the activated NHS at the dilution 1/600 compared to the dilution 1/50 was observed (87%). By diluting the untreated NHS (Contained C3) no significant change in the levels of detectable C3d was observed (Figure 5-7b).

Kinetics of C3d formation during complement activation (By immune complexes) over a period of 30 minutes at 37°C was studied and compared to changes in C3 levels (5-5). The levels of detectable C3d in NHS that contained ICs increased by 5 minutes and at 15 minutes reached levels 90% higher than the time. The levels of C3d in the untreated NHS did not significantly change during the incubation at 37°C (Figure 5-8a).

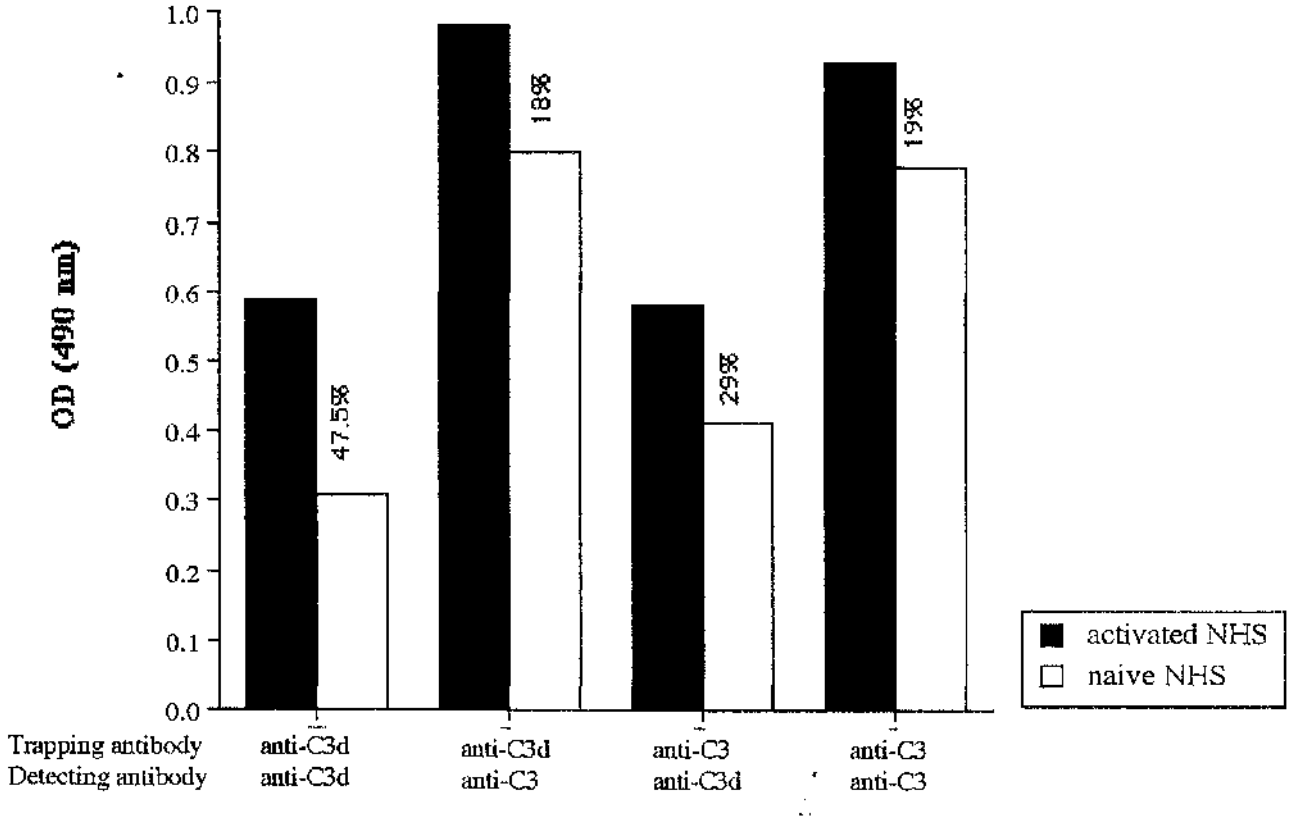


Figure 5-6: Effects of changes in antibody conditions on measurement of free C3d. Free C3d (closed bars) trapped with anti-C3d or anti-C3 and detected with biotin conjugated anti-C3d or anti-C3.
(Horizontal values are percentage of differences between sample and control.)

Intra-assay

Assay	No.	Mean	Median	S.D.	C.V.
C1s:C1-INH	9	129	132	16	12%
C3b-P	9	42	43	2.6	6%
Free C3d	4	13200	13200	1469	11%
Bound C3d	6	0.233	0.22	0.04	17%

Inter-assay

Assay	No.	Mean	Median	S.D.	C.V.
C1s:C1-INH	5	144.8	140	13.4	9%
C3b-P	5	75.2	80	15	19%
Free C3d	5	14160	13200	3134	22%
Bound C3d	5	0.234	0.23	0.05	23%

Table 5-2: Intra-assay and inter-assay variations of methods employed for measuring complement activation products. C1s:C1-INH, C3b-P value are expressed in ng/ml, free C3d in units/ml and erythrocytes bound C3d values are the OD of lysed cell at 405 nm.

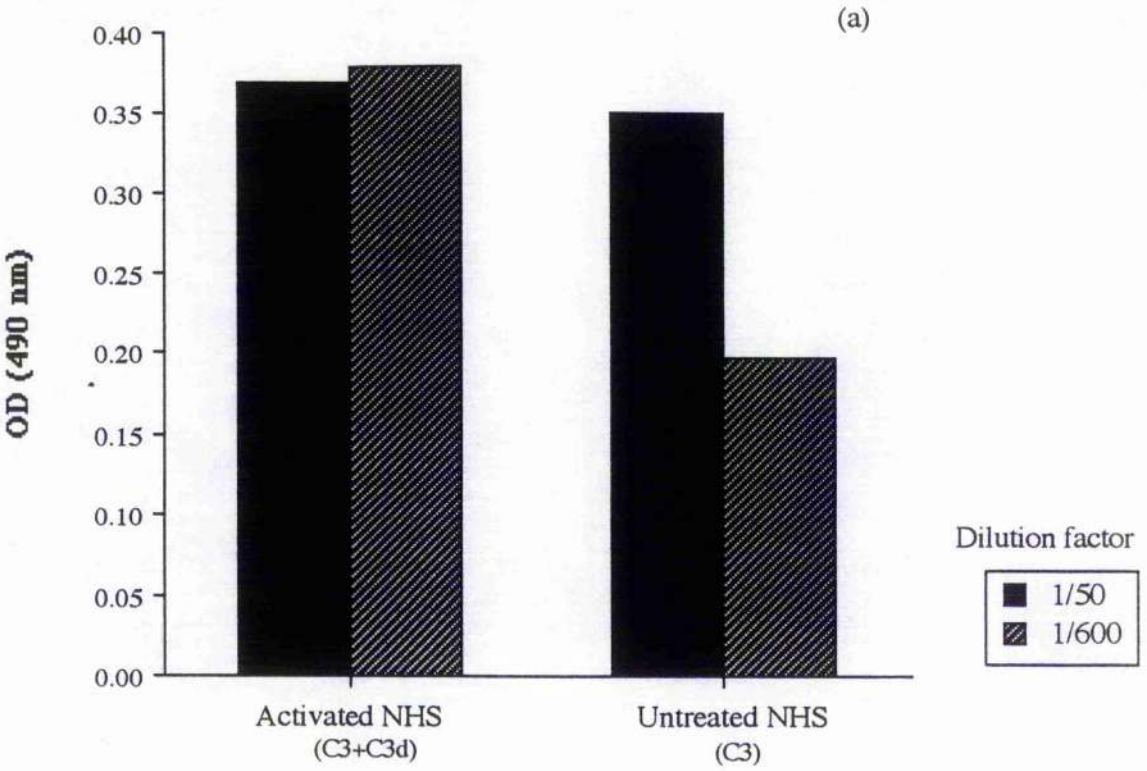


Figure 5-7a: The effect of dilution on the levels of free C3d detected by ELISA.

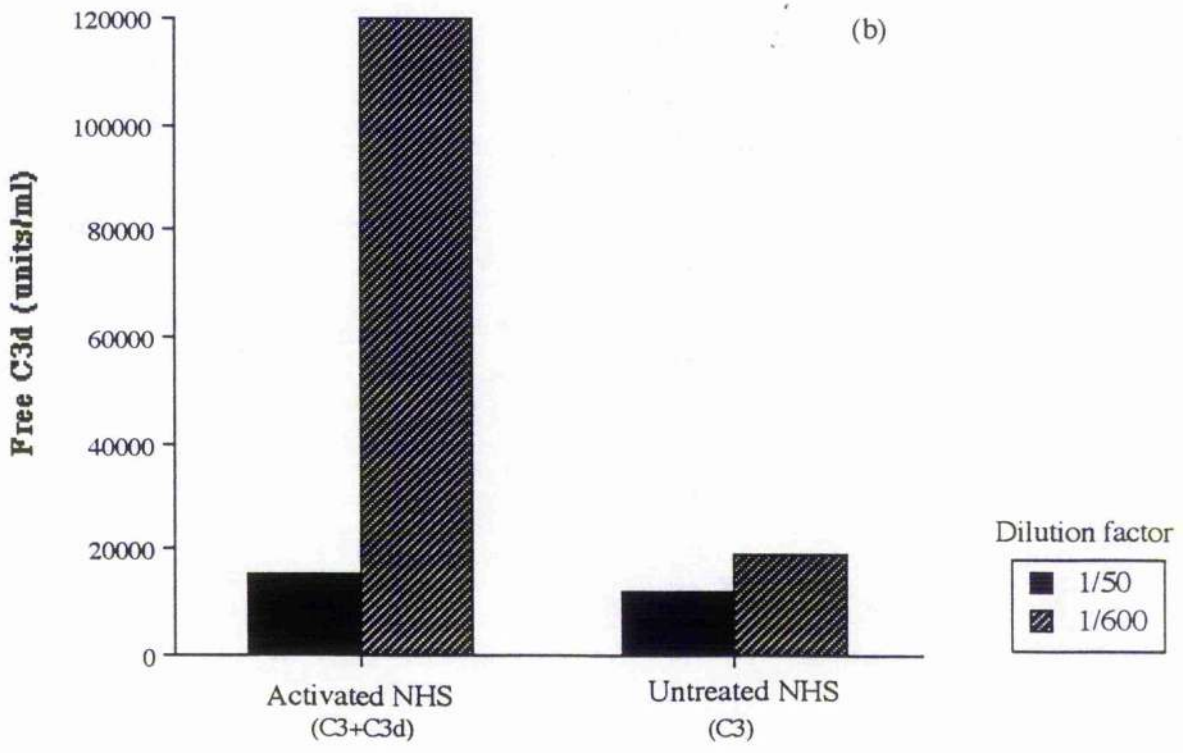
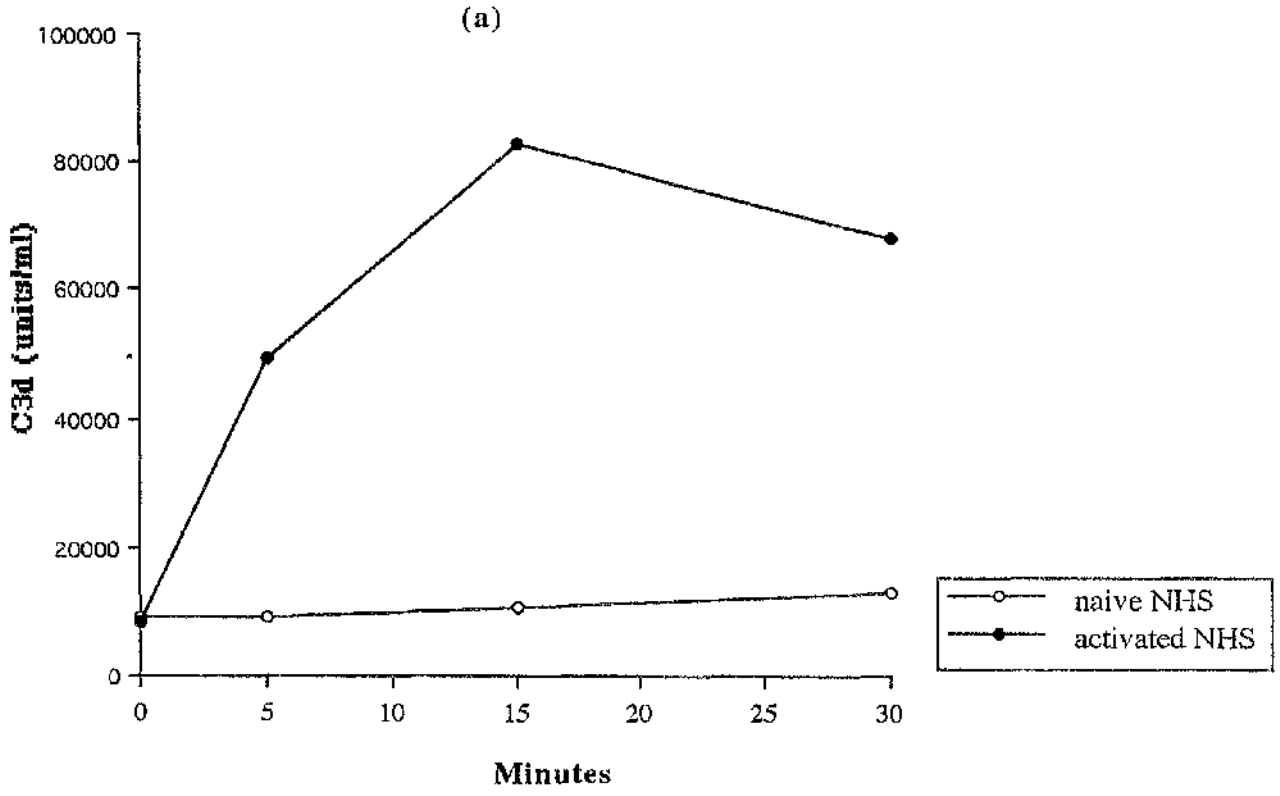


Figure 5-7b: The optical density of samples (a) were read off the standard curve, the values multiplied by the dilution factors and expressed in units/ml.



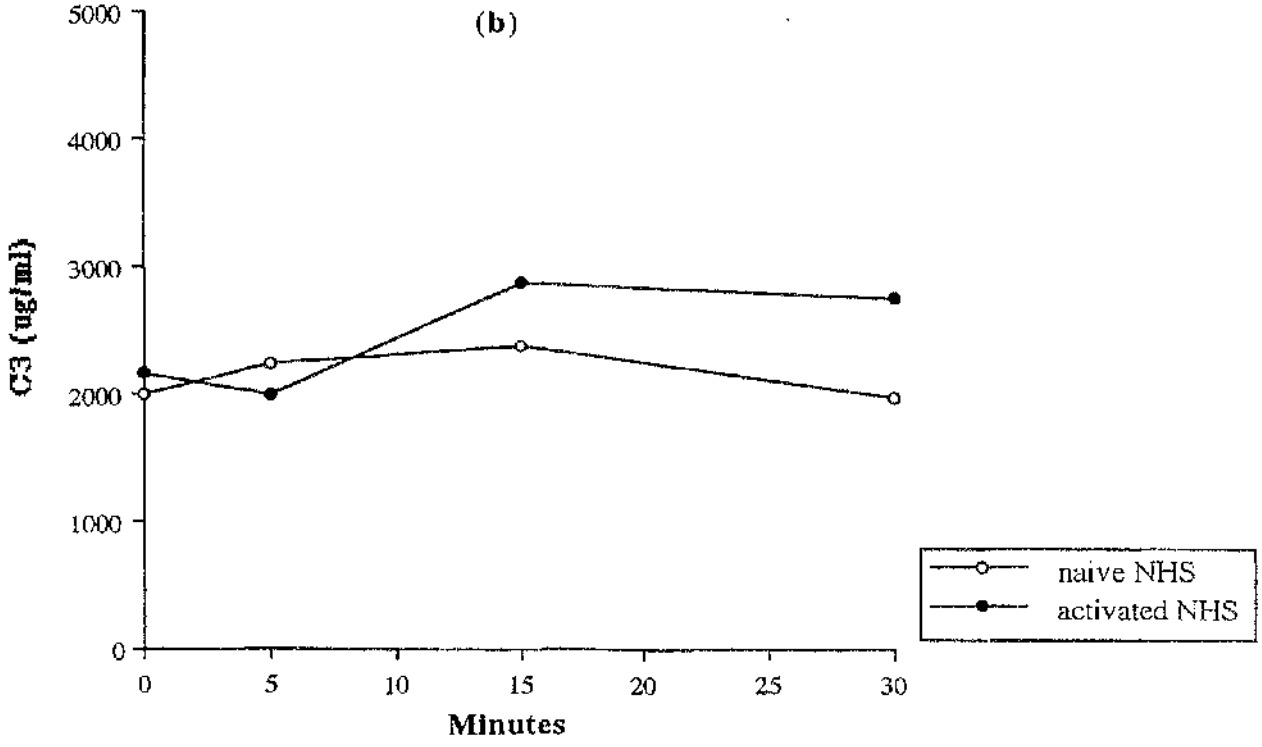


Figure 5-8: Kinetics of changes in the levels of C3d (Figure 4-8a) and C3 (Figure 4-8b) during complement activation. The levels of C3d increased by 90% at 15 minutes (a) and C3 levels did not change significantly (b).

On the other hand the levels of C3 in activated NHS and untreated NHS did not significantly change during the incubation at 37°C (Figure 5-8b). The increase in the C3d levels and constant levels of C3 during the period of incubation indicate that the ELISA system used (5-4) specifically detected C3d and the interference of intact C3 was minimised by the assay.

Detection of the bound complement components on Erythrocytes by modified ELISA. As described during complement activation the degradation product C3d is deposited on the erythrocytes (E's). To detect complement components, C3, properdin, Factor B and C3d, bound to erythrocytes a modified ELISA technique was developed (5-6).

Erythrocyte-bound C3, properdin and Factor B were trapped in ELISA plate by goat anti-human C3, anti-properdin and anti-Factor B and the bound E's were lysed with water. The optical densities of lysed cells were measured at 405 nm. All these components were detectable on erythrocytes when the levels were compared to controls (Figure 5-9). C3 bound to erythrocyte tohigher extent (81% higher than control) followed by Factor B (43% higher than control) then properdin (20% higher than control) (Figure 5-9). The limited use of these detections systems did not warrant determination of intra- and inter-assay variations.

Intra-assay and inter-assay variations of erythrocyte-bound C3d by the modified ELISA technique were 17% and 23% respectively (Table 5-2). Erythrocytes bearing C3d during complement activation were trapped by different concentrations of rabbit anti-human C3d (from 0.625 μg to 80μg/ml) and the ODs of bound and lysed E's was determined (5-6). E's incubated with naive NHS (Absence of ICs) showed little binding to anti-C3d on ELISA plate (Figure 5-10). E's incubated with NHS and ICs (Activated NHS) showed significant binding to anti-C3d on ELISA and the ODs of

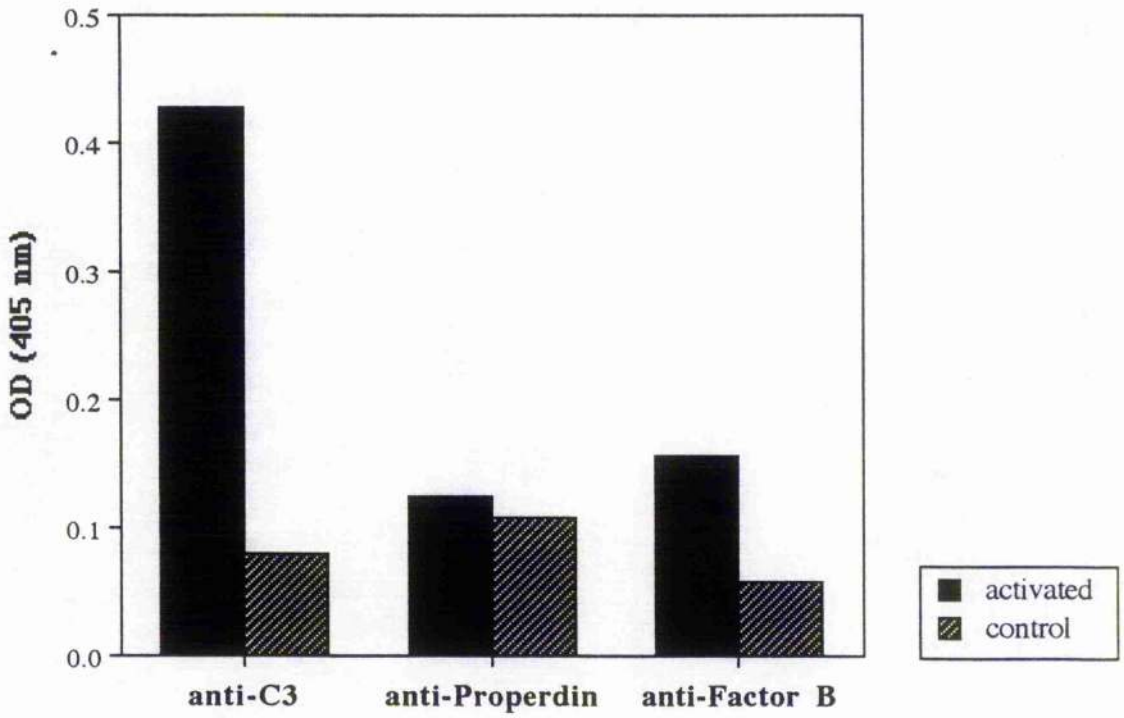


Figure 5-9: Erythrocytes bound C3, Properdin and Factor B were detected by the modified ELISA.

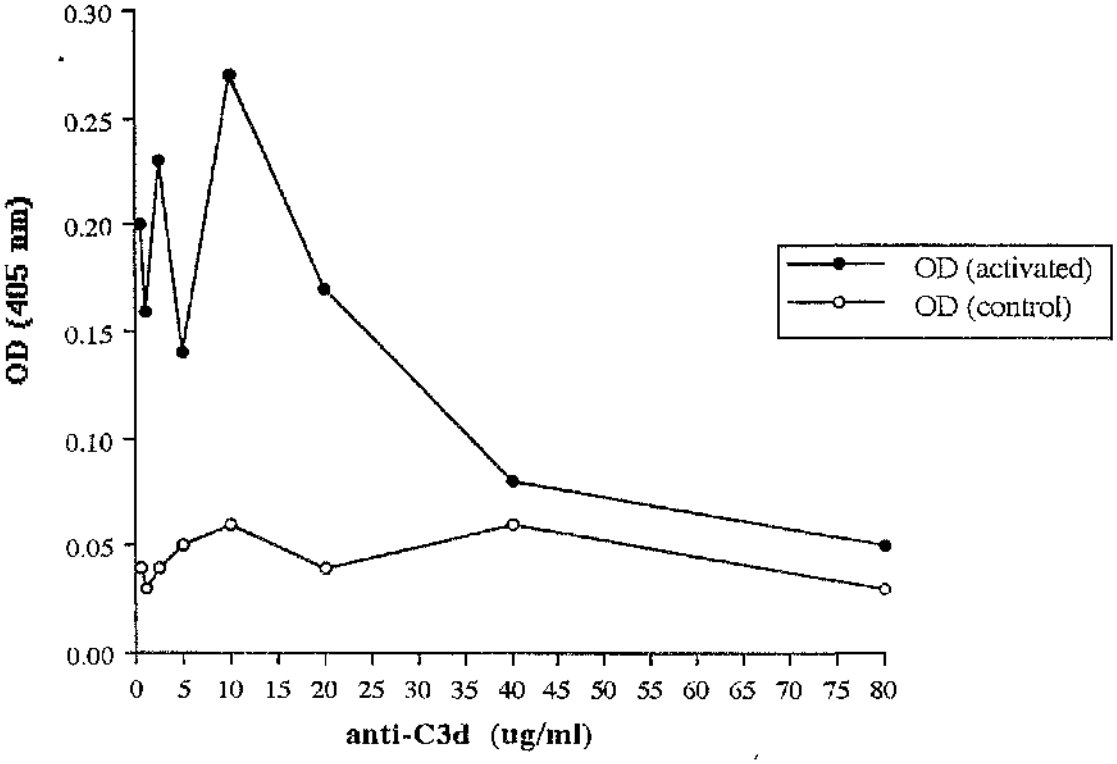


Figure 5-10: Detecting of erythrocyte bound C3d by modified ELISA with increasing anti-C3d coating concentrarions.

bound and lysed E's was significantly higher than naive E's ($p<0.05$) (Figure 5-10). This peaked with coating concentration of 10μg/ml of rabbit anti-human C3d (78% of higher than control) (Figure 5-10). The OD values of lysed cells (Activated NHS) correlated with the concentration of coating anti-C3d antibody ($r=0.72$, $p<0.02$) (Figure 5-10).

Complement activation products during administration of thrombolytic therapy. In the group of the 10 patients administered r-tPA there was no statistically significant difference in values of C1s:C1-INH, C3b-P or C5b-9 between pre- and post- r-tPA infusion (Time points 5, 30 minutes, then daily until discharge). One patient showed an elevation in C5b-9 only at days 2 and 4. At days 3 and 5 the levels dropped back to baseline value. All the ten patients administered streptokinase, showed an elevation in levels of C1s:C1-INH post-therapy compared with the pre-therapy levels (Time points 15, 30 and 60 minutes, days 1 and 2, $p<0.01$) (Figure 5-11). After day 1 the levels of C1s:C1-INH returned to pre-therapy levels and remained low until discharged (Figure 5-11). Analysis of the individual responses of the three patients F.M., C.D., E.M. with elevated levels of anti-streptokinase antibodies, showed that they had particularly high levels of generated C1s-C1-INH post-administration of streptokinase (Figures 5-11).

The levels of C1s:C1-INH in the 10 patients given streptokinase correlated inversely with the levels of anti-streptokinase antibodies in particular at 2 hours after thrombolytic treatment and the day of discharge from the hospital (Table 5-3, Figure 5-12). 60%, 20%, 10% and 60% of the patients showed a negative correlation with the levels of IgG, IgA, IgM and neutralising anti-streptokinase at 2 hours respectively, this was 90%, 20%, 30% and 80% at day of discharge (Table 5-3, Figure 5-12).

Only one patient (F.M.) had levels of C3b-P and C5b-9 which increased substantially post-administration of streptokinase (Figure 5-13). The pattern of increased followed C1s:C1-INH and returned to base-line levels at day 1 (Figure 5-13). The other 9

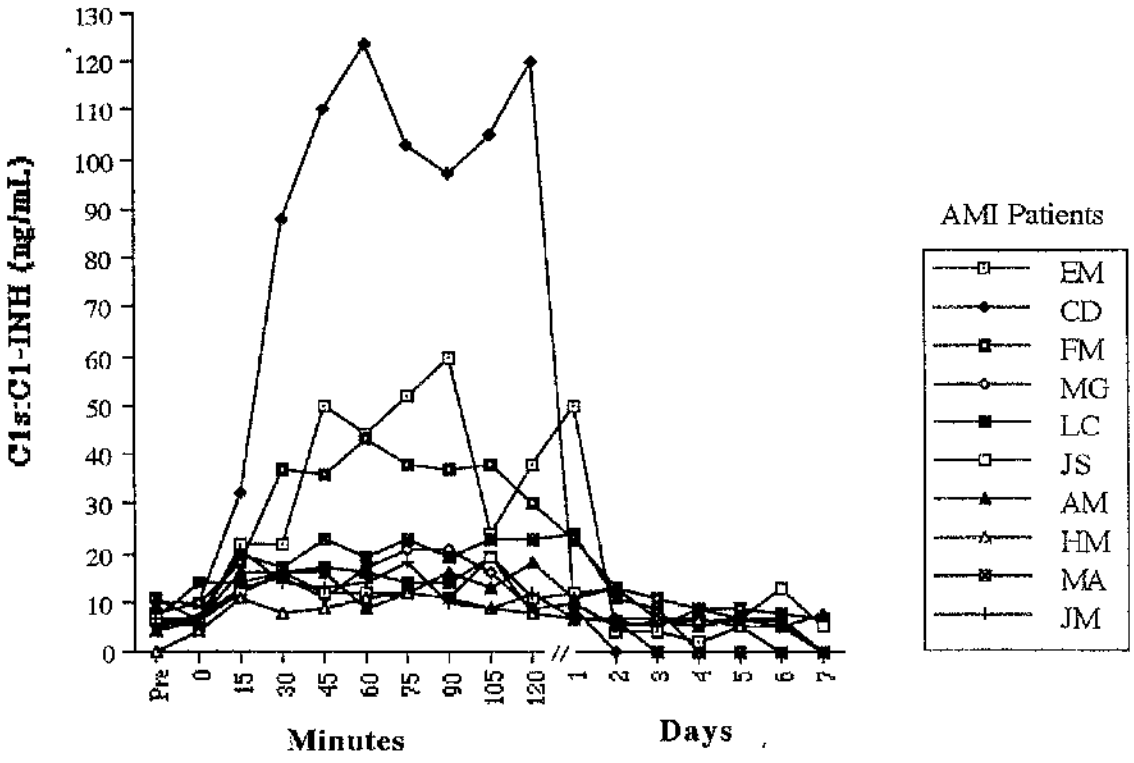


Figure 5-11: Fluctuations of C1s:C1-INH in 10 patients with AMI treated with streptokinase.

	IgG		IgA		IgM		ASK	
Patient	30 min	2 hours	30 min	2 hours	30 min	2 hours	30 min	2 hours
FM	-0.7 (p<0.004)	- 0.34 (N.S)	-0.73 (p<0.002)	- 0.64 (p<0.04)	-0.08 (N.S.)	0.35 (N.S.)	-0.88 (p<0.001)	- 0.7 (p<0.02)
EM	-0.5 (p<0.08)	- 0.66 (p<0.04)	-0.46 (N.S.)	- 0.42 (NS)	0.12 (N.S.)	-0.4 (NS)	- 0.65 (p<0.01)	- 0.50 (NS)
CD	- 0.71 (p<0.02)	- 0.71 (p<0.02)	- 0.77 (p<0.01)	- 0.77 (p<0.01)	-0.66 (N.S.)	-0.66 (N.S.)	- 0.80 (p<0.006)	- 0.80 (p<0.006)
MG	- 0.79 (p<0.001)	- 0.65 (p<0.04)	0.28 (N.S.	- 0.35 (N.S.)	0.001 (N.S.	-0.17 (N.S.)	-0.38 (N.S.)	- 0.52 (N.S.)
LC	- 0.70 (p<0.01)	- 0.61 (p<0.06)	-0.30 (N.S.)	- 0.1 (N.S.)	- 0.65 (p<0.03)	- 0.57 (p<0.08)	- 0.85 (p<0.001)	- 0.85 (p<0.001)
JM	- 0.56 (p<0.03)	- 0.12 (N.S.)	0.13 (N.S.)	- 0.01 (N.S.)	0.53 (N.S.)	0.2 (N.S.)	- 0.24 (N.S.	- 0.47 (N.S.)
JS	- 0.55 (P<0.04)	- 0.52 (N.S.)	0.001 (N.S.)	0.06 (N.S.)	- 0.15 (N.S.)	- 0.14 (N.S.)	- 0.84 (p<0.001)	- 0.84 (p<0.001)
AM	- 0.6 (P<0.008)	- 0.40 (N.S.)	0.31 (N.S.)	0.33 (N.S.)	0.12 (N.S.)	- 0.03 (N.S.)	- 0.48 (p<0.06)	- 0.54 (N.S.)
HM	- 0.78 (p<0.005)	- 0.70 (p<0.02)	0.03 (N.S.)	- 0.18 (N.S.)	- 0.11 (N.S.)	- 0.18 (N.S.)	- 0.84 (p<0.001)	- 0.84 (p<0.001)
MA	- 0.68 (p<0.02)	- 0.59 (p<0.07)	- 0.45 (N.S.)	- 0.32 (N.S.)	0.44 (N.S.)	0.36 (N.S.)	- 0.86 (p<0.001	- 0.86 (p<0.001)

Table 5-3: Correlation between the levels of anti-streptokinase antibodies and generated levels of C1s:C1-INH in 10 AMI patients treated with streptokinase (during the first two hours of treatment).

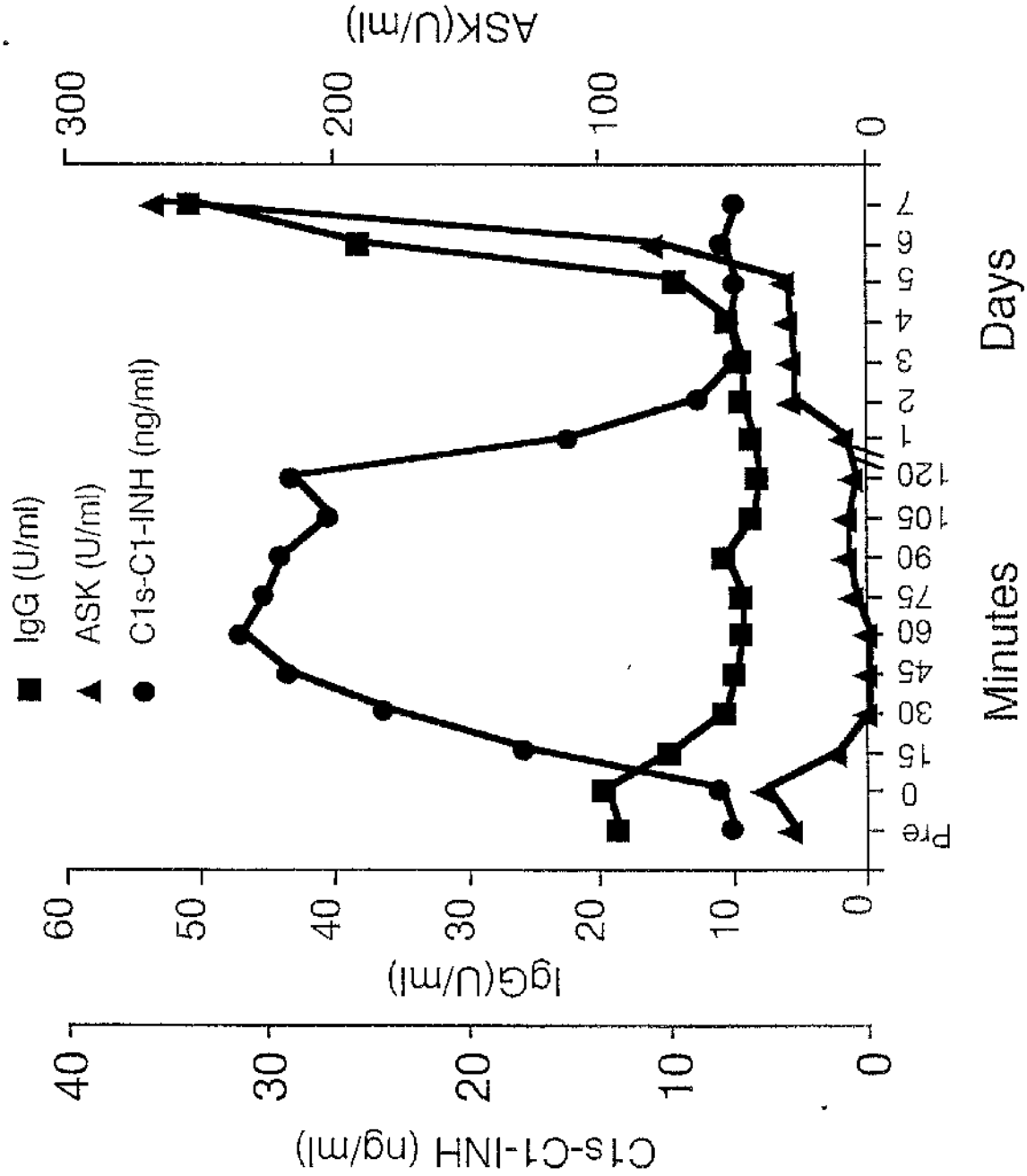


Figure 5-12: Mean values fluctuations of C1s:C1-INH and anti-streptokinase antibodies in 10 AMI patients treated with streptokinase.

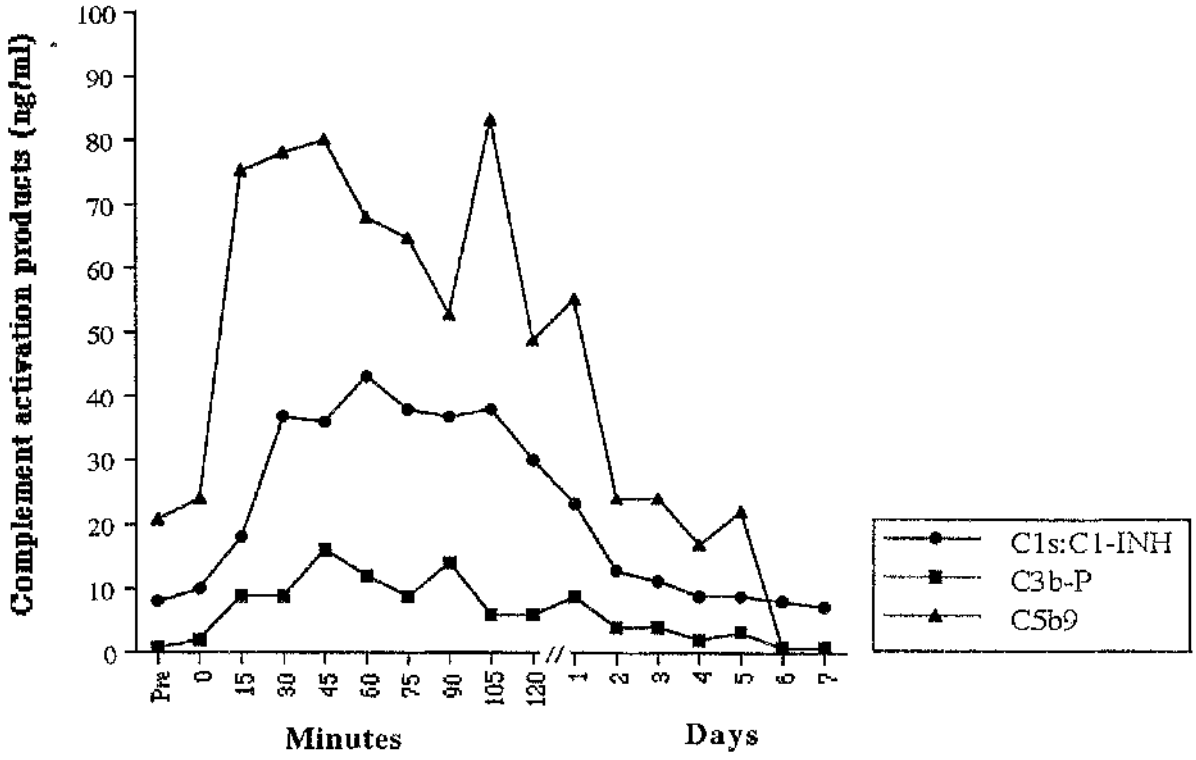


Figure 5-13: Complement activation products (ng/ml) during streptokinase treatment (Patient F.M.).

patients had very low or undetectable levels of C3b-P and C5b-9, and these were not significantly different from pre-therapy values.

As described of 10 patients treated with streptokinase, 5 had early reperfusion, 3 had late reperfusion and 2 did not reperfused (Chapter 3, Table 3-4). The levels of generated C3b-P during streptokinase administration in ten patients treated with streptokinase did not show any difference in early, late and non reperfusion groups (mean values at two hour, 2.2 ng/ml, 2.66 ng/ml and 2 ng/ml respectively). However, the levels of generated C1s:C1-INH were higher in late (mean value=49.66 ng/ml) and non-reperfused groups (mean value=28.5 ng/ml) compared with early reperfused group (mean value=16.6 ng/ml).

Dose and kinetics of streptokinase on the complement activation. NHS was prepared from ten normal individuals to study the effect of different doses and the kinetics of streptokinase alone on the complement activation (5-8). All of these normal individuals had levels of anti-streptokinase antibodies which fell within the normal ranges (Table 3-2), the mean values for IgG, IgA, IgM and neutralising anti-streptokinase antibodies in this group being 8.1 units/ml, 4.5 units/ml, 3.25 units/ml and 16.25 units/ml respectively. The effects observed could be considered due only to streptokinase and not ICs.

Streptokinase was added at concentrations of 66 μg/ml, 133 μg/ml and 333 μg/ml to NHS and incubated at 37°C (Therapeutic dose = 100 μg/ml). Streptokinase at concentration 66 μg/ml did not generate significant levels of C1s:C1-INH at any time points when compared with the controls (Table 5-4, Figure 5-14a). However, streptokinase at concentrations 133 μg/ml and 333 μg/ml generated significant levels of C1s:C1-INH ($p<0.05$, time points 15 and 30 minutes, Table 5-4, Figure 5-14a).

a

Minutes	C1s:C1-INH	C3b-P
0	79 (NS)	67 (NS)
5	51 (NS)	54 (NS)
15	31 (NS)	26 (NS)
30	29 (NS)	8.5 (P<0.05)

b

Minutes	C1s:C1-INH	C3b-P
0	83 (NS)	64 (NS)
5	59 (NS)	52 (NS)
15	15 (P<0.05)	21 (P<0.05)
30	14 (P<0.05)	8 (P<0.05)

c

Minutes	C1s:C1-INH	C3b-P
0	80 (NS)	60 (NS)
5	29 (NS)	25 (NS)
15	7 (P<0.05)	20 (P<0.05)
30	7.5 (P<0.05)	8 (P<0.05)

Table 5-4: The significance of generated levels of C1s:C1-INH and C3b-P by 66 μg/ml (a), 133 μg/ml (b) and 333 μg/ml (c) streptokinase compared to controls at different time points incubation at 37°C in 10 normal individuals (Therapeutic dose =100 μg/ml). (Mann-Whitney test, the first values are U values, tabulated U value for a group of 10 samples for the probability of 5% is 3-23).

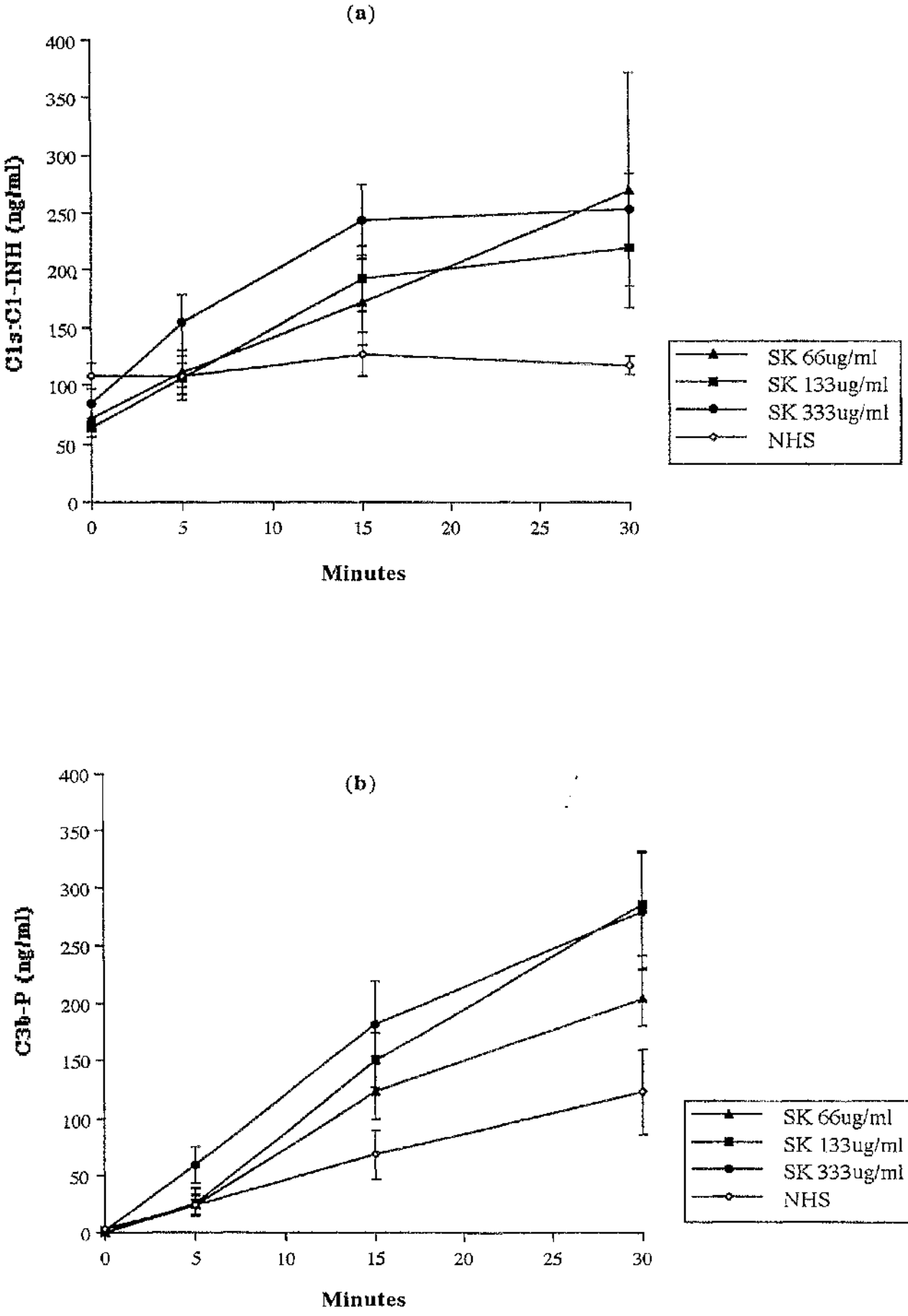


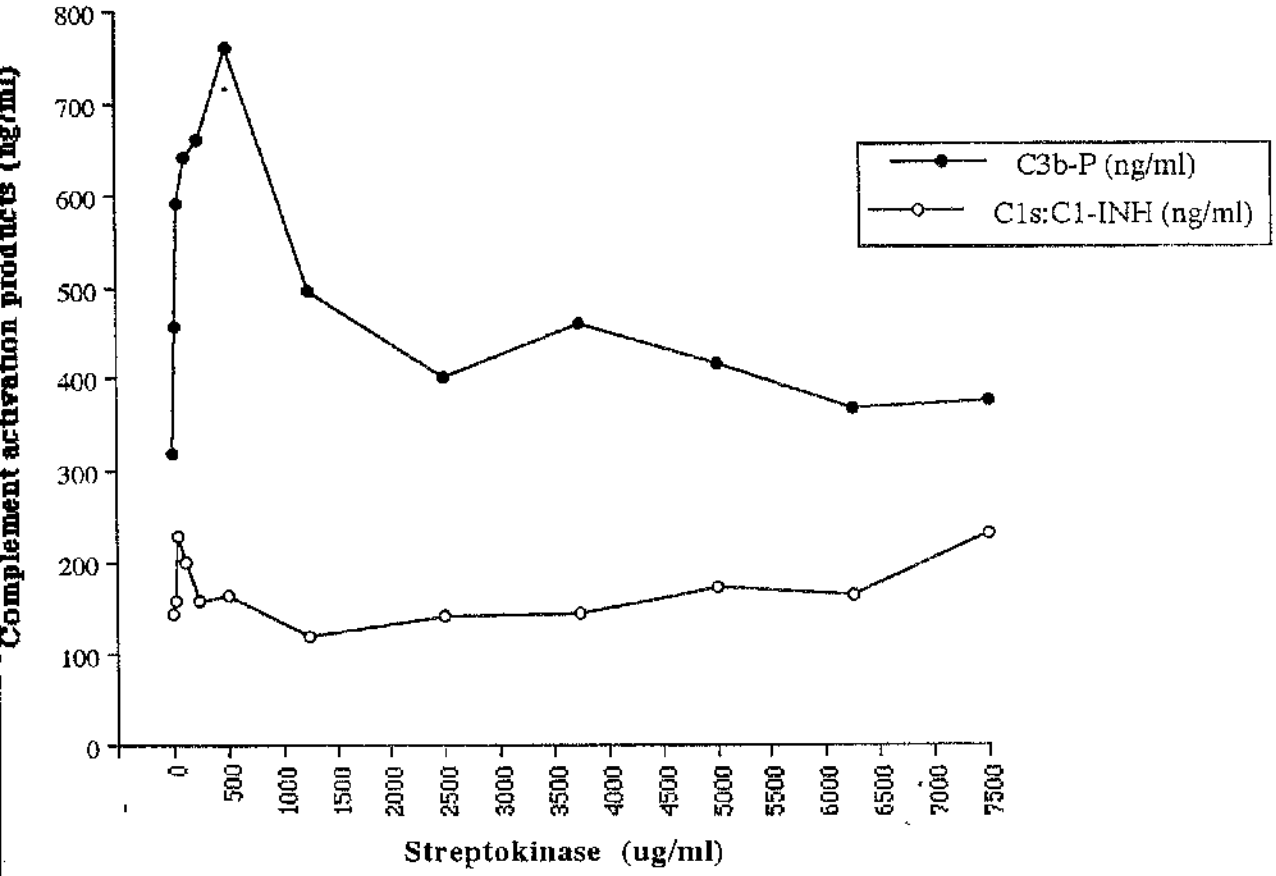
Figure 5-14: Study the effects of different doses and the kinetics of streptokinase on C1s:C1-INH (a) and C3b-P (b) in 10 normal individuals. Therapeutic dose= 100ug/ml.

Streptokinase at concentration 66 μg/ml generate significant levels of C3b-P at 30 minutes when compared with controls (Table 5-4, Figure 5-14b). Streptokinase at concentrations 133 μg/ml and 333 μg/ml generated significant levels of C3b-P ($p<0.05$, time points 15 and 30 minutes, Table 5-4, Figure 5-14b).

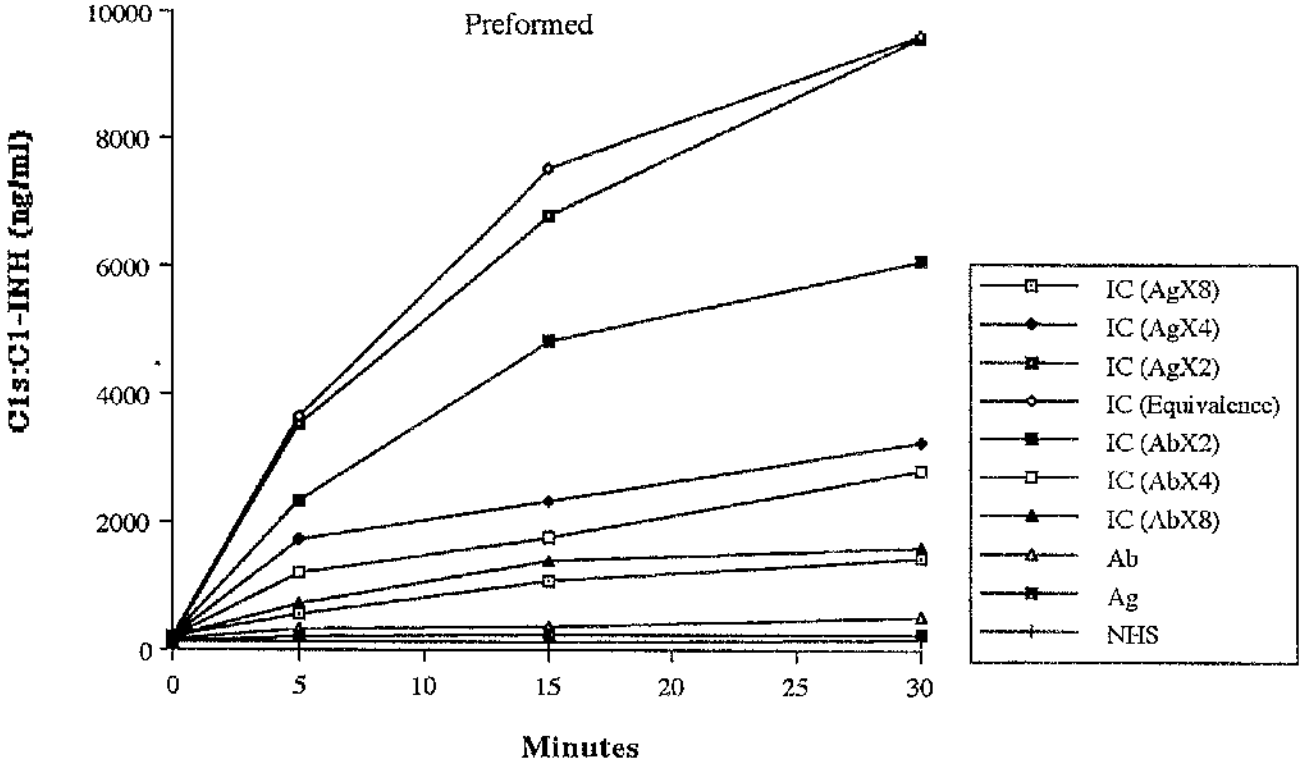
There was no significant difference in the levels of complement activation products generated by different concentrations of streptokinase (Figure 5-14). The levels of C1s:C1-INH at time points 0 in samples contained streptokinase were lower than the control levels (Figure 4-14a).

The extent of C1s:C1-INH and C3b-P generation in the ten individuals did not correlate with the levels of anti-streptokinase antibodies (Table 5-5), however, using streptokinase at concentrations below the therapeutic dose (66 μg/ml) generated levels of complement activation products showed a weak correlation with the levels of IgG anti-streptokinase antibodies (Time points 5, 15 and 30 minutes) but did not correlate with the levels of IgA, IgM and neutralising anti-streptokinase antibodies (Table 5-5). With streptokinase concentrations beyond the therapeutic levels (25 μg/ml to 7500 μg/ml streptokinase, therapeutic dose = 100μg/ml, 5-8) the levels of C1s:C1-INH were maximum with streptokinase at 62.5μg/ml (37% higher than control) and beyond that concentration the levels of C1s:C1-INH did not change significantly (Figure 5-15). C3b-P levels were maximum at concentration of 500 μg/ml of streptokinase (58% higher than control) and then decrease to values that were 14-35% higher than control level (Figure 5-15).

The effect of streptokinase and thyroglobulin immune complexes on complement activation. Preformed and nascent immune complexes were prepared to study the in vitro effects of immune complexes on the complement activation (5-2 and 5-9).



gure 5-15: The levels of C1s:C1-INH and C3b-P after incubation with streptokinase at concentrations eyond the therapeutic doses for 30 minutes at 37oC. Therapeutic doses = 100 ug/ml)



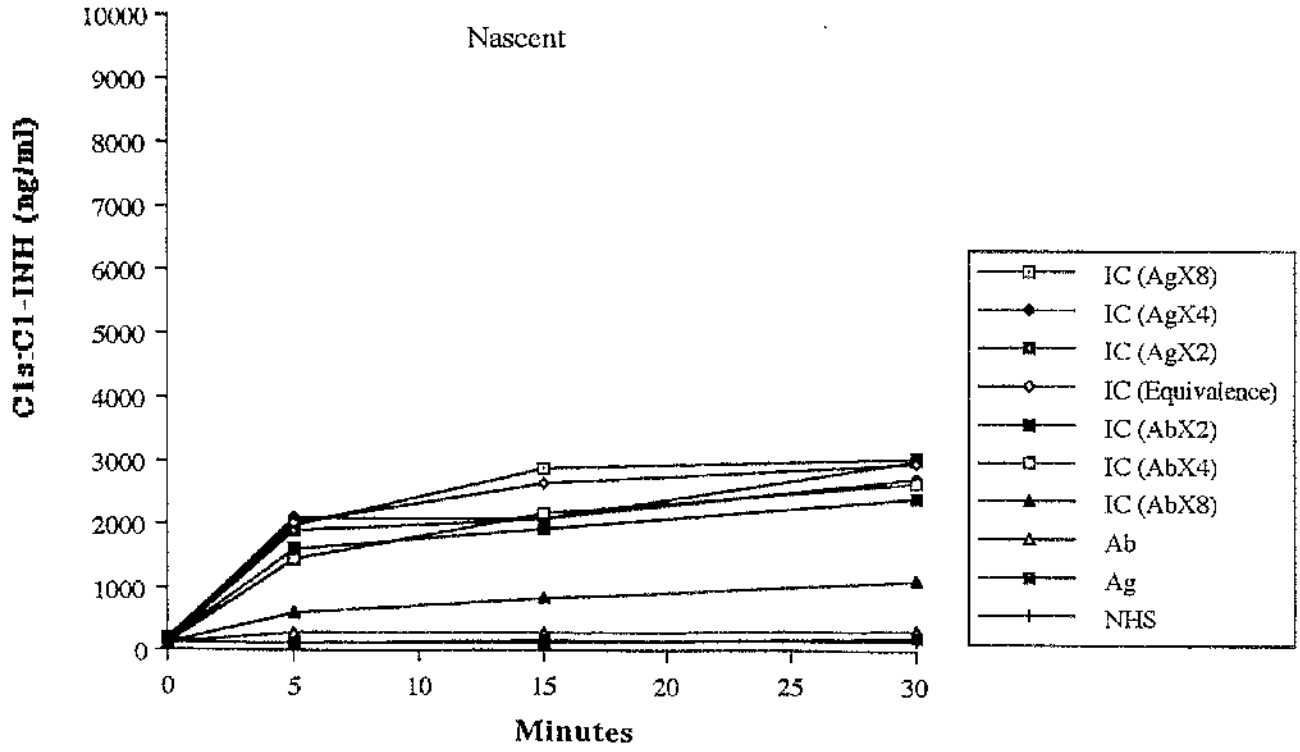
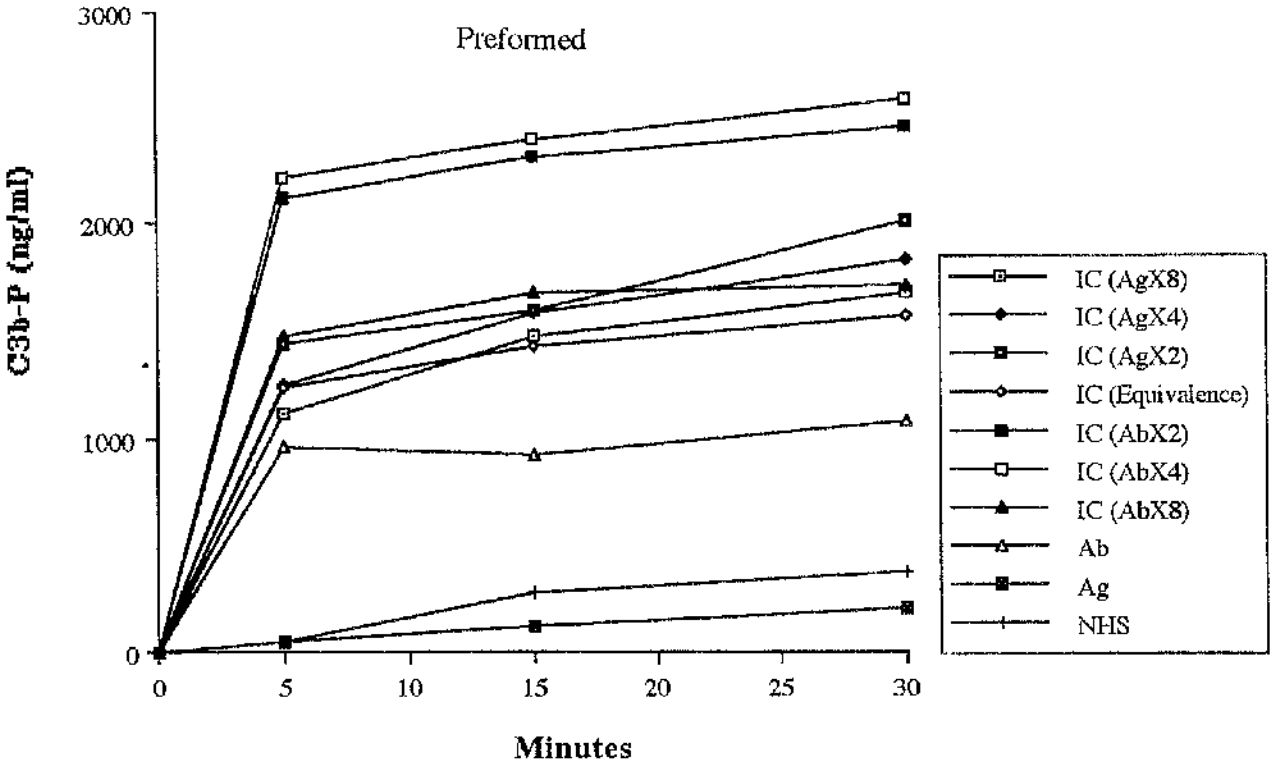


Figure 5-16: Classical pathway activation by preformed and nascent rabbit streptokinase immune complexes at antigen excess, equivalence and antibody excess.



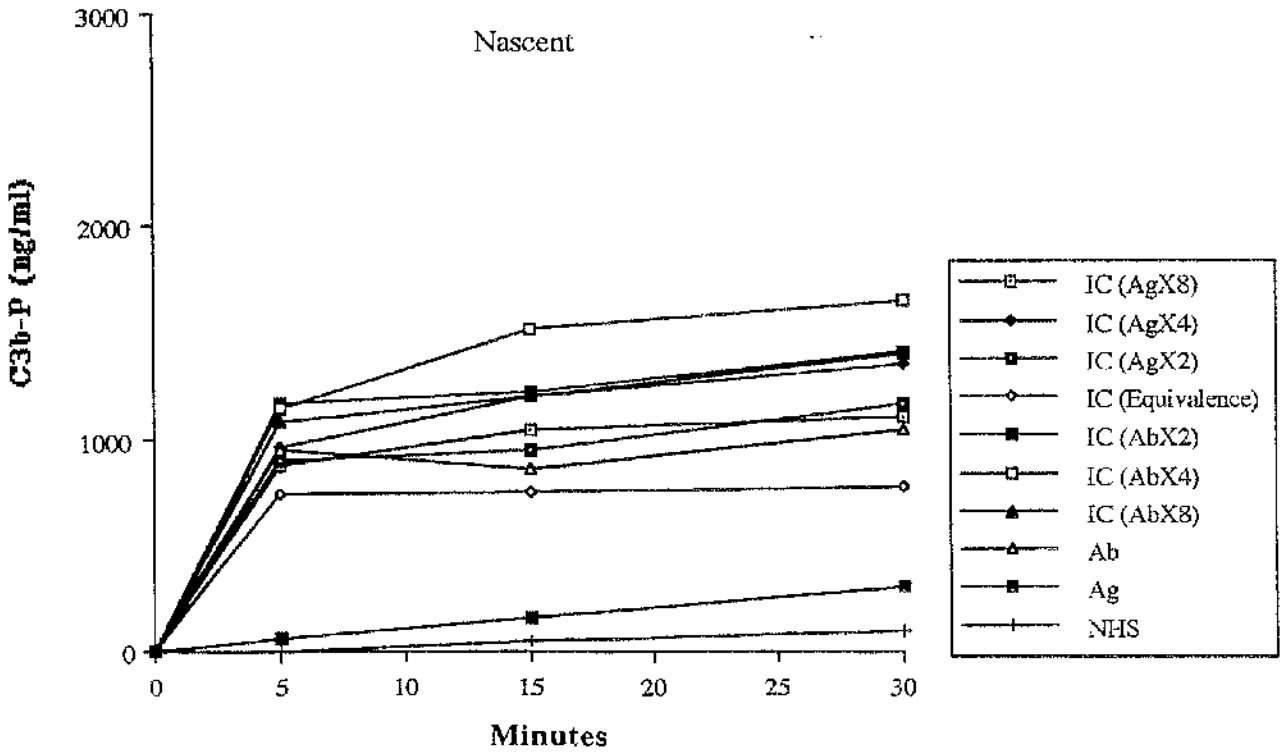


Figure 5-17: Alternative pathway activation by preformed and nascent rabbit anti-streptokinase immune complexes at antigen excess, equivalence and antibody excess.

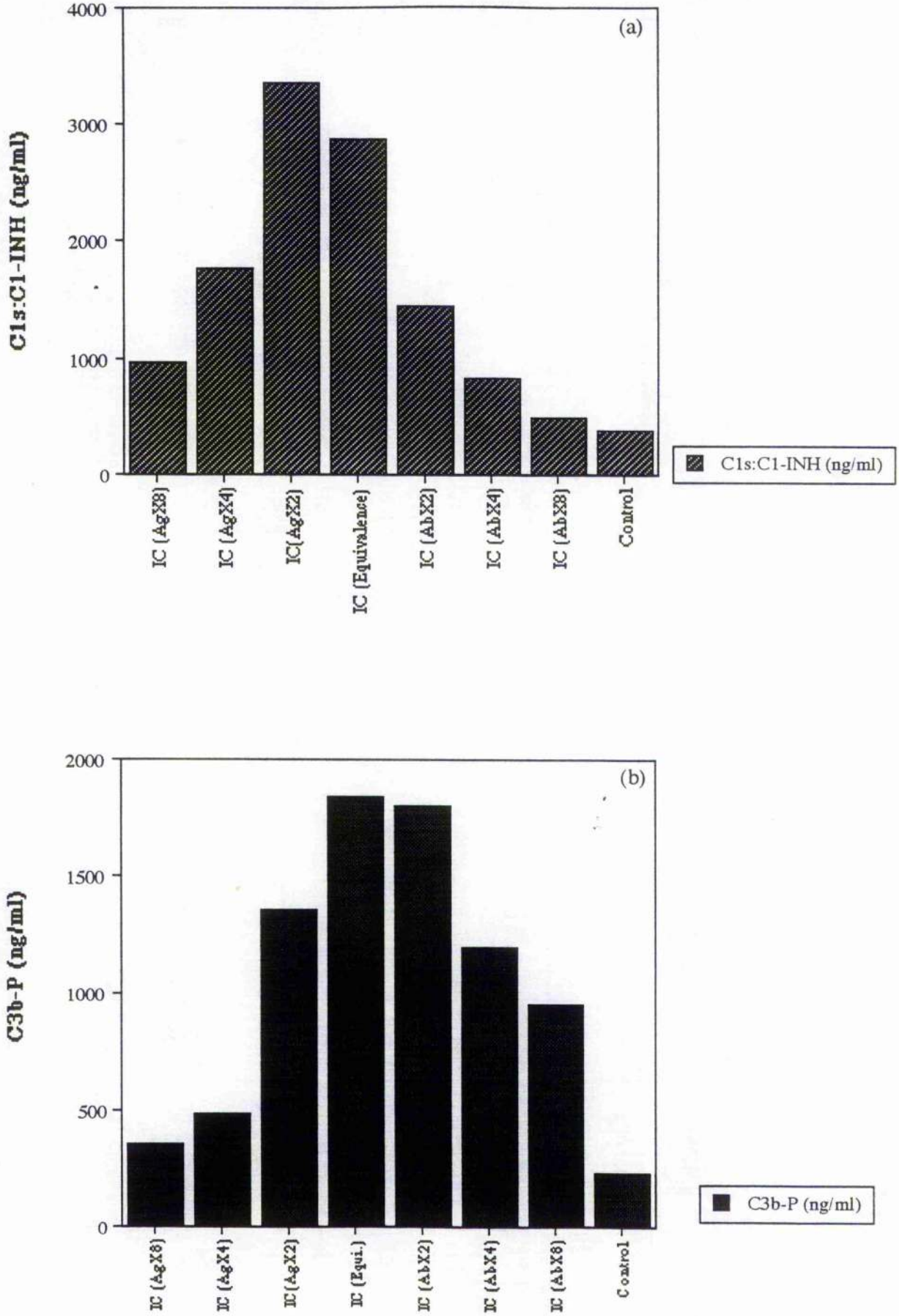


Figure 5-18: The effect of preformed human streptokinase immune complexes on the classical (a) and alternative (b) pathways at 5 minutes incubation at 37oC.

The effect of rabbit thyroglobulin immune complexes (preformed and nascent) at equivalence, antigen excess or antibody excess on complement activation were studied. Rabbit thyroglobulin (preformed and nascent) immune complexes at equivalence, antigen excess and antibody excess caused detectable levels of C1s:C1-INH and C3b-P by 5 minutes when compared to the controls (Figures 5-19 and 5-20).

The levels and the rate of C1s:C1-INH generation was higher with nascent immune complexes than preformed immune complexes (Figures 5-19). On the other hand, the levels and the rate of C3b-P generation was higher with preformed immune complexes compared to nascent immune complexes (Figures 5-20).

Both preformed and nascent thyroglobulin immune complexes at antigen excess generated higher levels of C1s:C1-INH and C3b-P than antibody excess (Figures 5-19 and 5-20).

The levels of complement activation products in absence of activating factors (Antigens, antibodies or ICs) at 37 °C were used as control throughout the experiments and did not increase significantly during the incubation periods.

NHS treated with EDTA or Mg^{++}, EGTA was incubated with immune complexes at 37°C and complement activation products were measured (5-9). EDTA caused a significant reduction in the levels of generated C1s:C1-INH and C3b-P by preformed human streptokinase ICs at equivalence (Figure 5-21). On the other hand, EGTA which also decreased significantly the levels of C1s:C1-INH, had no significant effect on the levels of C3b-P generated by the ICs (Figure 5-21).

The effect of EDTA and EGTA on inhibition of complement activation products generated by preformed rabbit streptokinase immune complexes and rabbit thyroglobulin at equivalence were comparable with the human streptokinase immune complexes (Figure 5-22). EDTA inhibited the generation of C1s:C1-INH and C3b-P in all the immune complexes (Figure 5-22). EGTA inhibited the generation of C1s:C1-

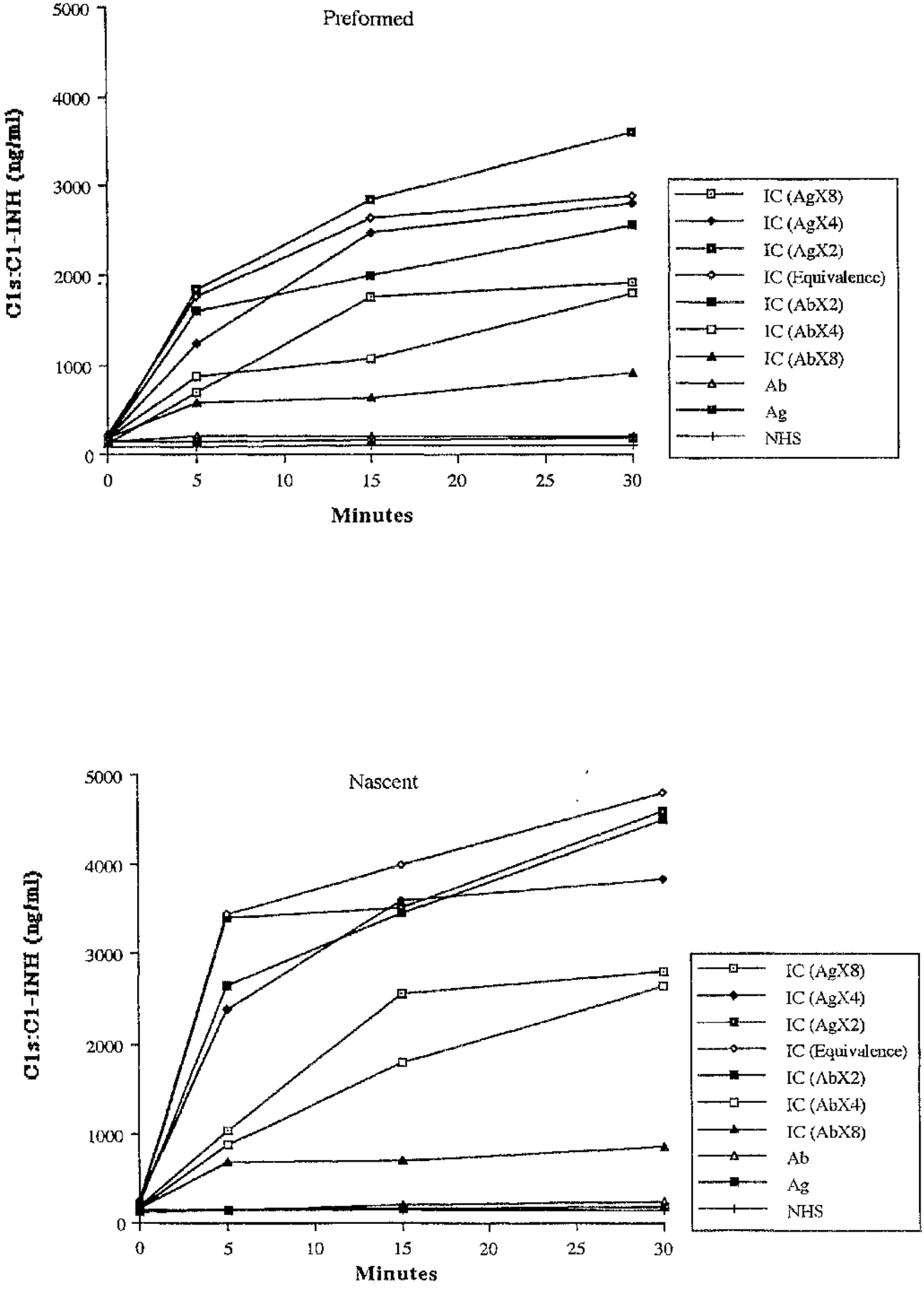


Figure 5-19: The effect of preformed and nascent thyroglobulin immune complexes on the classical pathway activation, at antigen excess, equivalence and antibody excess.

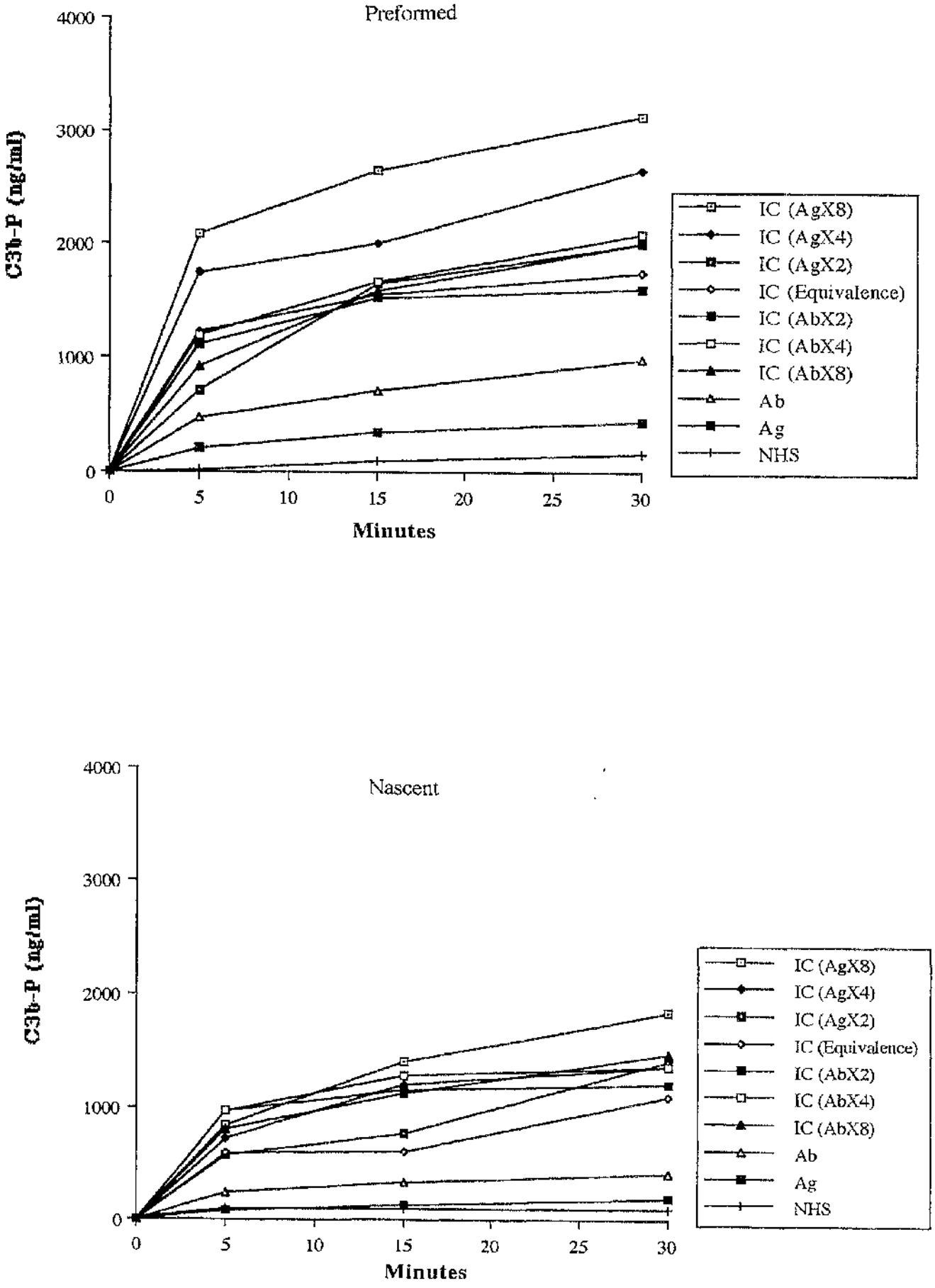


Figure 5-20: The effect of preformed and nascent thyroglobulin immune complexes on the alternative pathway activation, at antigen excess, equivalence and antibody excess.

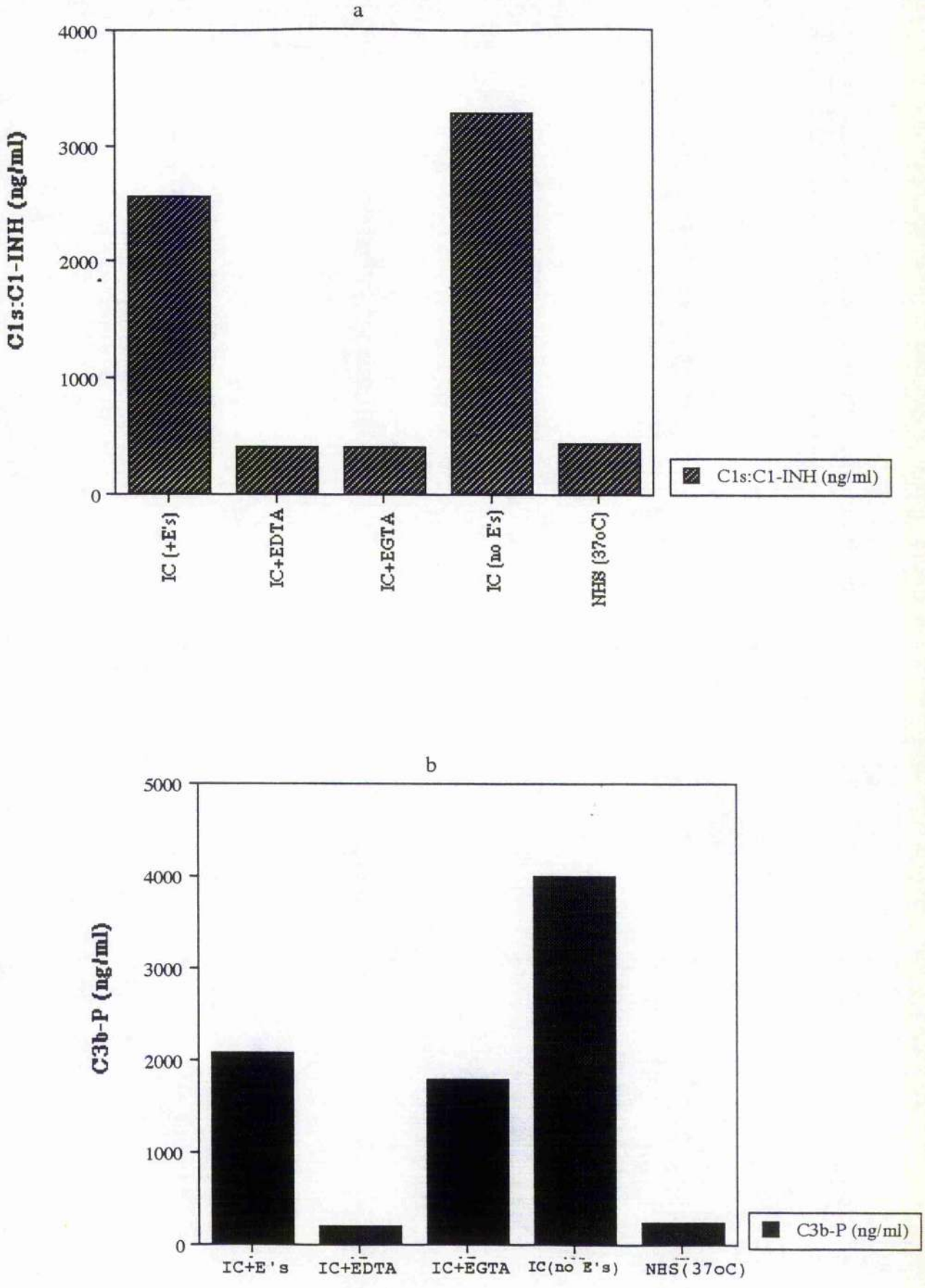


Figure 5-21: The effect of EDTA and EGTA on the levels of C1s:C1-INH (a) and C3b-P (b) generated by human streptokinase immune complexes.

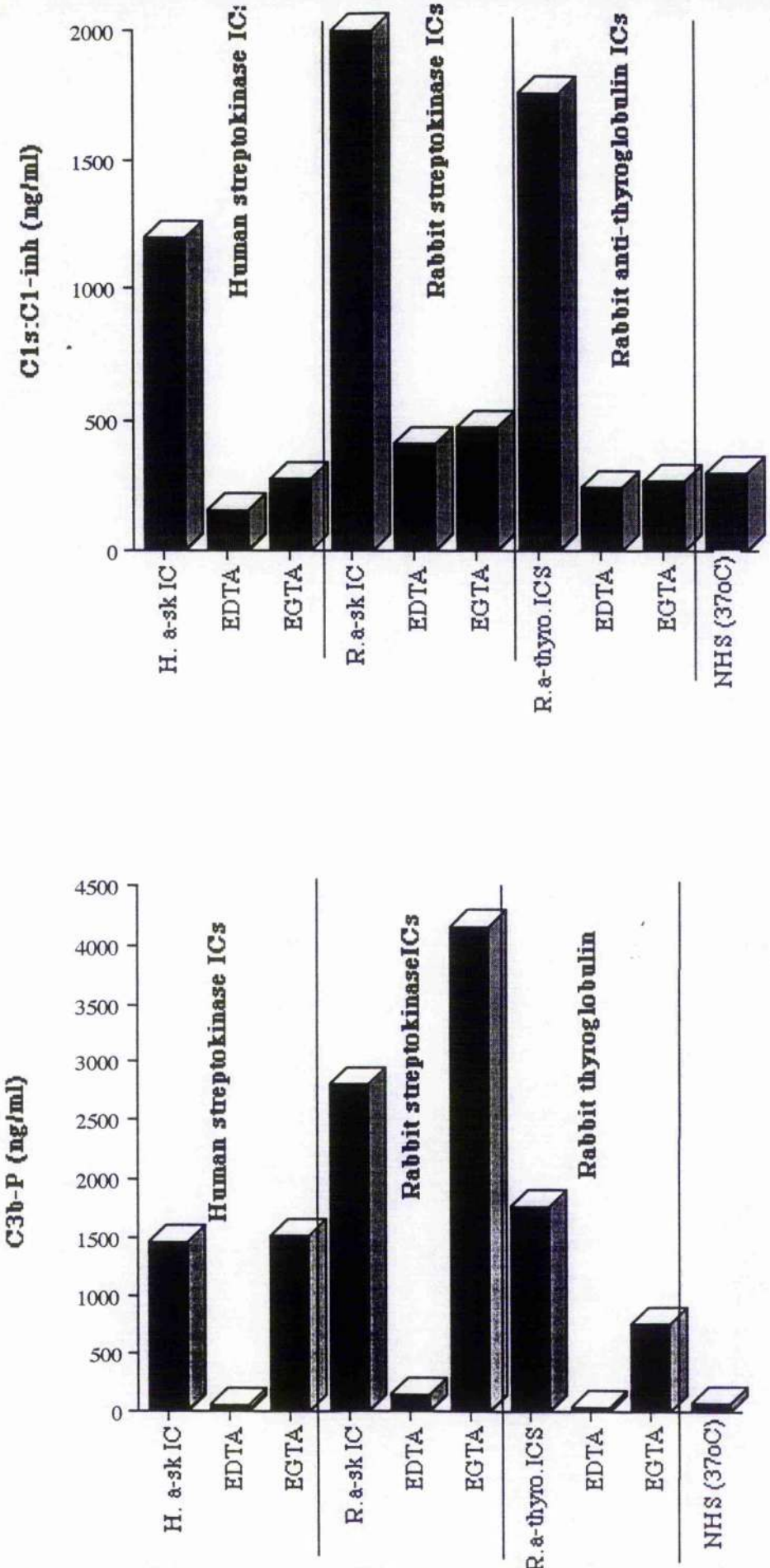


Figure 5-22: The effect of EDTA and EGTA on the complement activation products generated by streptokinase immune complexes (human and rabbit) and thyroglobulin immune complexes.

INH in all the immune complexes (Figure 5-22a) however, it only inhibited the generation of C3b-P by thyroglobulin immune complexes (Figure 5-22b) and had no effect on the generated levels of C3b-P by human or rabbit streptokinase immune complexes (Figure 5-22b).

The effect of erythrocytes on complement activation. The effects of erythrocytes were studied as they may influence the levels of generated complement activation products, through the action of CR1 or acting as a surface to which these products are deposited on them.

a) The effect of erythrocytes: The levels of C1s:C1-INH and C3b-P generated by nascent thyroglobulin immune complexes (ICs) and preformed human streptokinase ICs at equivalence, in presence and absence of erythrocytes (E's) were studied (5-10). Only preformed streptokinase ICs were used due to limitation of anti-streptokinase anti-sera from human source. The levels of C1s:C1-INH, C3b-P and free C3d generated by preformed rabbit streptokinase ICs at equivalence were also studied in presence and absence of E's (5-10).

In the absence of E's C1s:C1-INH and C3b-P were generated by all these ICs and free C3d by rabbit streptokinase ICs (Figures 5-21, 5-23, 5-24 and 5-25).

Erythrocytes had no significant effect on the levels of C1s:C1-INH generated by all these immune complexes when compared with the levels in absence of E's (Figures 5-21a, 5-23a and 5-25a).

Erythrocytes had no significant effect on the levels of free C3d generated by rabbit streptokinase immune complexes (Figure 5-24).

In the presence of erythrocytes, there was a substantially reduction in rate and extent of C3b-P generation by rabbit streptokinase ICs (79% reduction at 5 minutes compared to ICs in absence of E's) (Figure 5-23b). The presence of erythrocytes also caused a

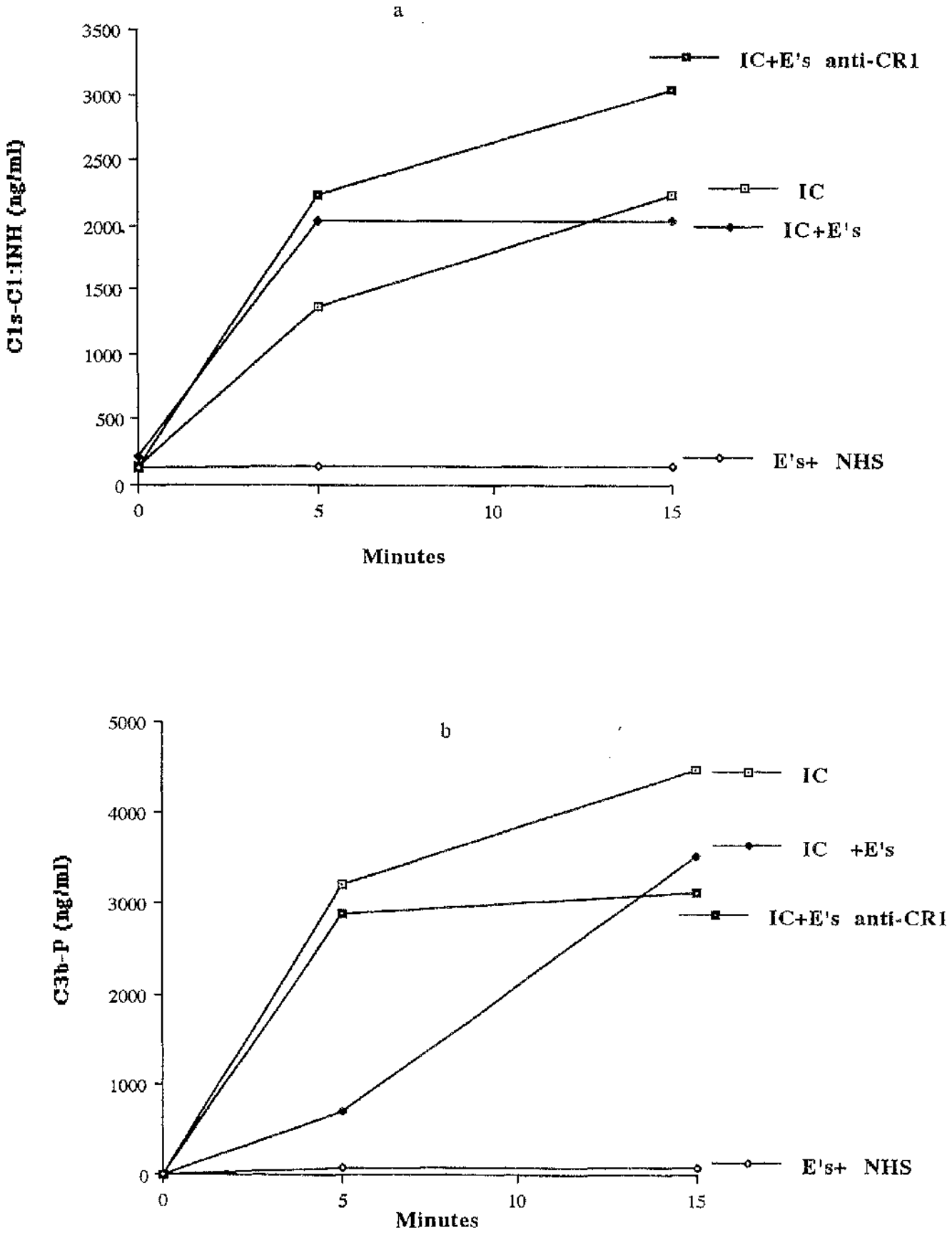


Figure 5-23: The effect erytrocytes (E's) on the levels of C1s:C1-INH (a) and C3b-P (b) generated by preformed rabbit streptokinase immune complexes at equivalence.

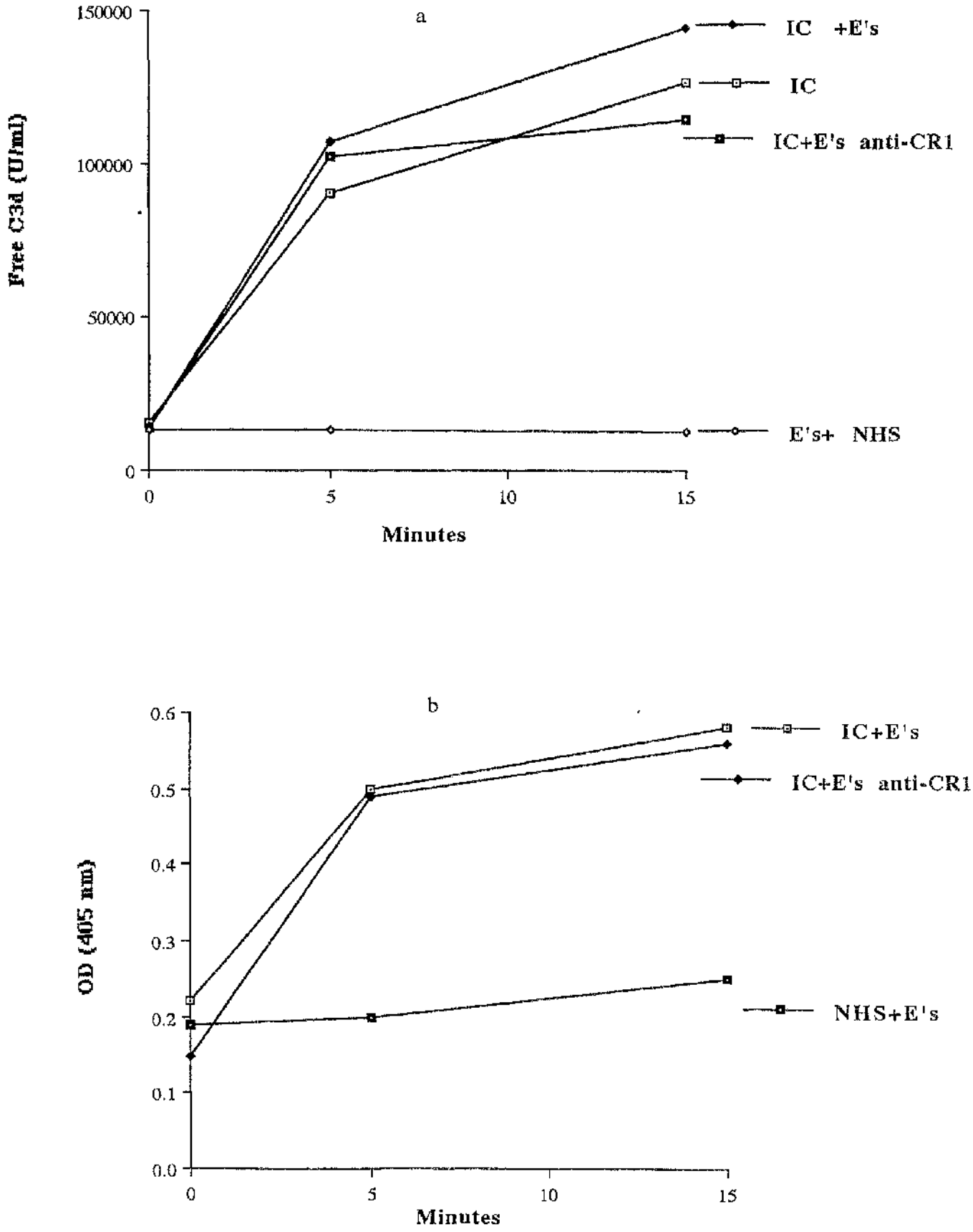


Figure 5-24: The effect of erythrocytes (E's) on free (a) C3d, the effect of anti-CR1 on bound C3d (b) generated by preformed rabbit streptokinase immune complexes at equivalence.

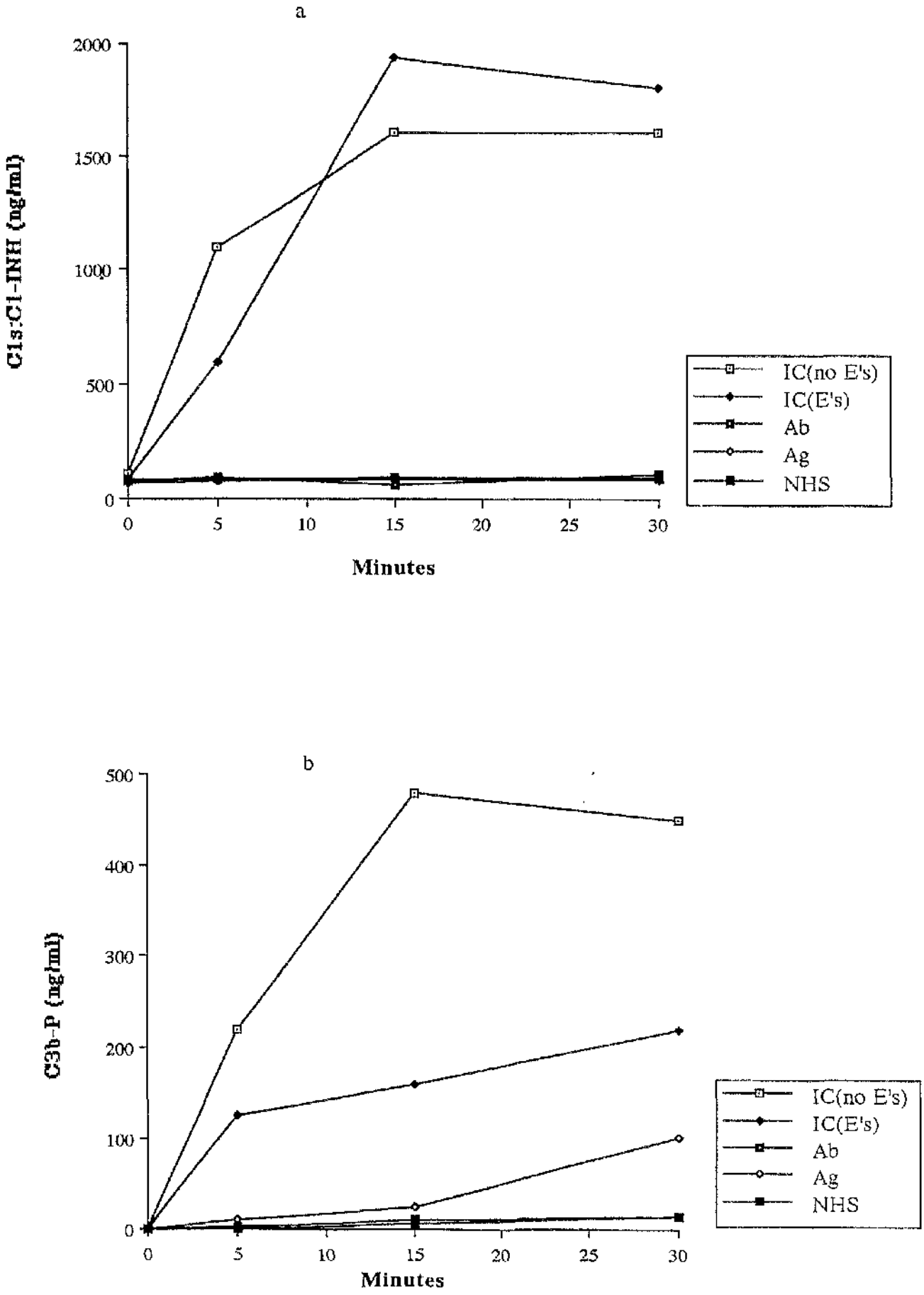


Figure 5-25: The effect of erythrocytes (E's) on the levels of C1s:C1-INH (a) and C3b-P (b) generated by nascent thyroglobulin immune complexes at equivalence.

reduction (48%) in the levels of C3b-P generated by human streptokinase ICs at 5 minutes (Figure 5-21b). The levels of C3b-P generated by thyroglobulin ICs in presence of E's was also decreased at all time points of the incubation period (Figure 5-25b). When comparing the response of the two types of ICs, thyroglobulin ICs showed substantial reduction at all time points (Figure 5-25b), however, when streptokinase ICs were studied the reduction of C3b-P in presence of E's was at 5 minutes after which levels approach those of ICs alone (Figure 5-23b). The lower amount of C3b-P generated by nascent thyroglobulin compared to preformed streptokinase ICs may account for the observed differences.

b) The effect of CR1 receptors: To study the effect of the CR1 receptors on the levels of complement activation products generated by rabbit streptokinase ICs, levels were measured in the presence of erythrocytes treated with monoclonal anti-CR1 (5-10).At 5 minutes there was no different in the levels of C1s:C1-INH in presence of E's or E's treated with anti-CR1 (E-CR1) (Figure 5-23a). E-CR1 at 15 minutes increased the levels of C1s:C1-INH by 33% compared to the levels in the presence or absence of E's (Figure 5-23a).

Use of preformed rabbit streptokinase ICs has already shown that E's reduced substantially C3b-P levels at earlier time points compared to the levels in the absence of E's (Figure 5-23b). At 5 minutes the effect of E's was reversed by E-CR1 and the levels of C3b-P increased to the levels equal to the levels of C3b-P observed in absence of E's (Figure 5-23b).

The presence of anti-CR1 did not influence the levels of free or erythrocyte bound C3d (Figure 5-24).

The effect of plasmin on the levels of complement activation products.

Streptokinase can initiate complement activation by two mechanisms, through plasminogen activation and generation of plasmin and by immune complexes formation

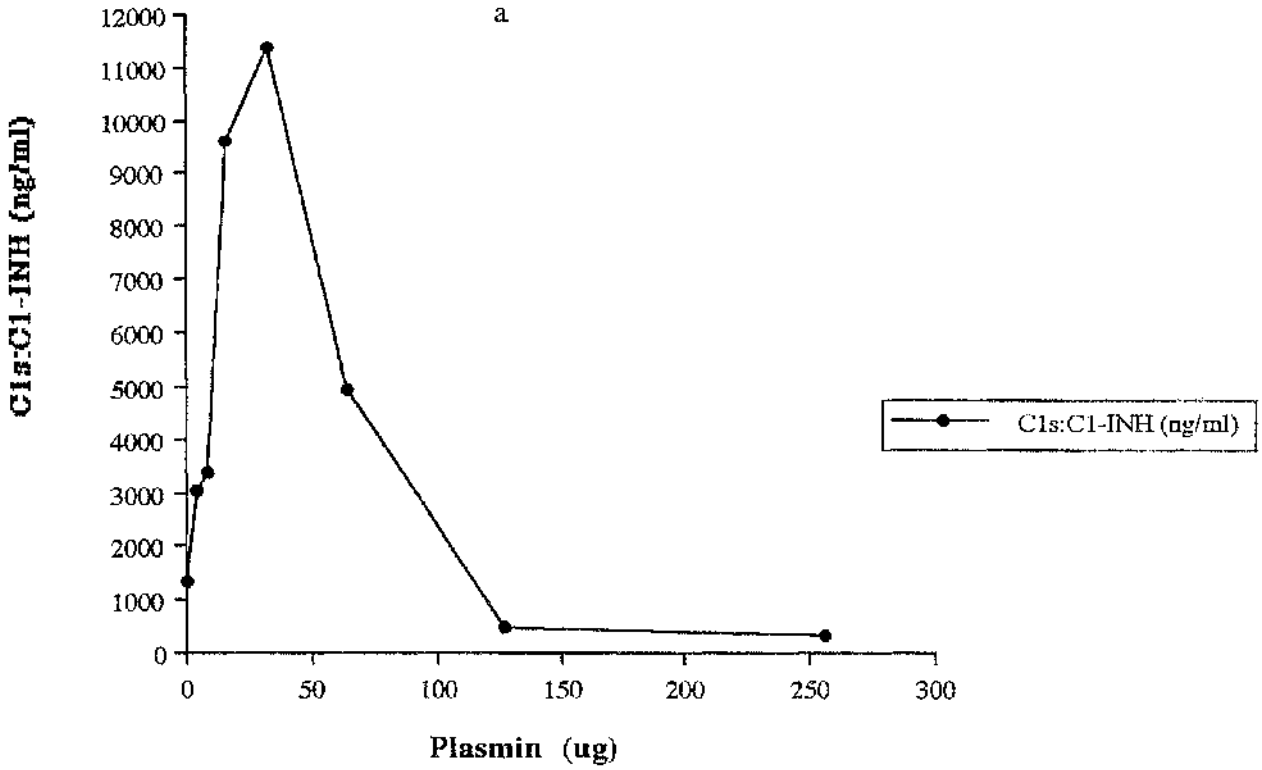
(1-10). Plasmin can also interact with complement components (See introduction) and therefore may influence the detectable levels of complement activation products. These effects were studied (5-11).

The direct effect of plasmin on generated levels of complement activation products was studied using nascent rabbit thyroglobulin immune complexes at equivalence (5-11). Streptokinase ICs were not used as streptokinase has potential effects on serum. Serial amounts of plasmin (from 4μg to 256μg) were added to a constant amount of thyroglobulin ICs and NHS, after a 30 minutes incubation at 37°C the complement activation products were measured (5-3).

The presence of 4 μg plasmin increased significantly the levels of C1s:C1-INH and C3b-P generated by the ICs. The levels of C1s:C1-INH and C3b-P in presence of 32 μg plasmin were highest, bieng 88% and 45% higher than the levels of ICs alone respectively (Figure 5-26).

The levels of C1s:C1-INH and C3b-P then began to decrease and in the presence of 128 μg plasmin reached to their lowest levels and did not rise again (Figure 5-26).Controls: The mixtures of 256 μg plasmin + NHS, NHS alone and thyroglobulin ICs + NHS at 30 minutes in 37°C for were used as controls. The mixture of 256 μg plasmin and NHS generated levels of C1s:C1INH that were marginally (19%) greater than NHS alone (336 ng/ml generated by plasmin and 272 ng/ml generated by NHS alone). The mixture of 256 μg plasmin and NHS did not generate C3b-P when compared with the levels of NHS alone (3 ng/ml generated by both mixtures).

To study the indirect effect of streptokinase through generation of plasmin on the levels of complement activation products generated by thyroglobulin immune complexes, serial amount of streptokinase from 50 μg/ml to 400 μg/ml (Therapeutic dose = 66-100 μg/ml) were added to a constant amount of the ICs and NHS. The mixtures were incubated at 37°C and complement activation products were measured at 0, 5 15 and 30 minutes.



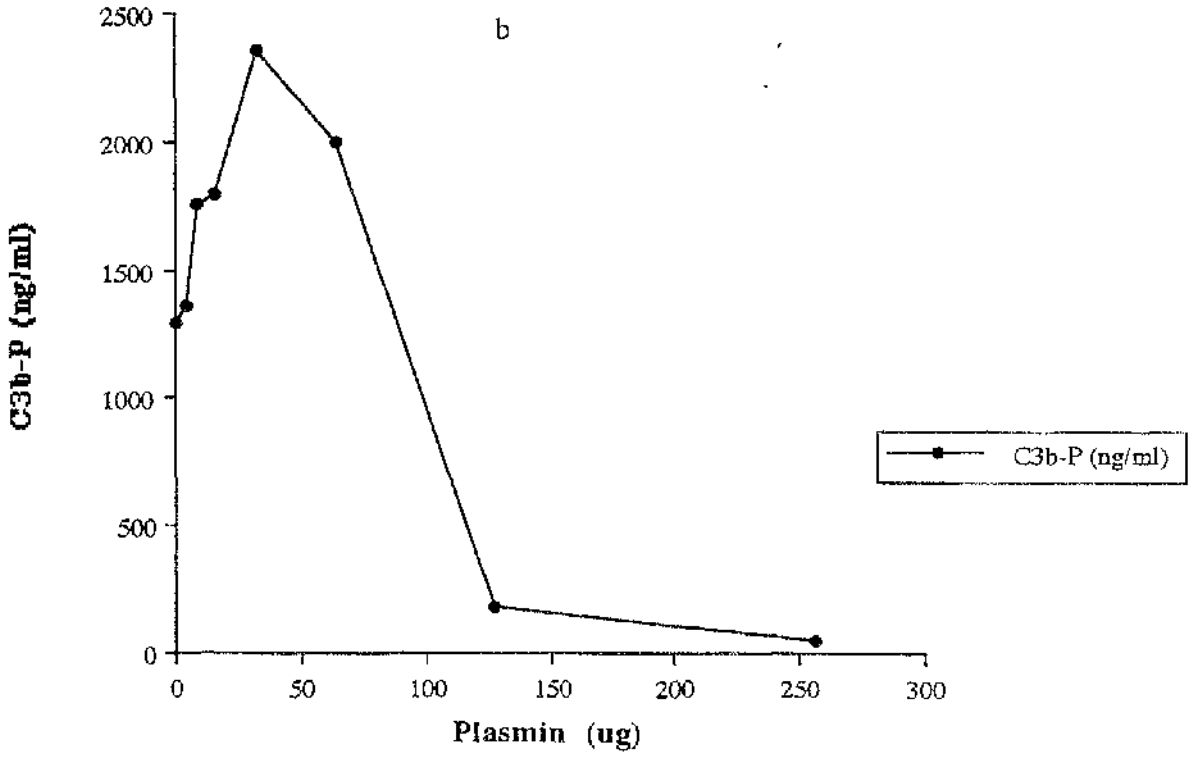


Figure 5-26: The effect of increasing amount of plasmin (From 4ug to 256 ug) on the levels of C1s:C1-INH (a) and C3b-P generated by nascent thyroglobulin ICs at equivalence at 30 minutes incubation at 37oC.

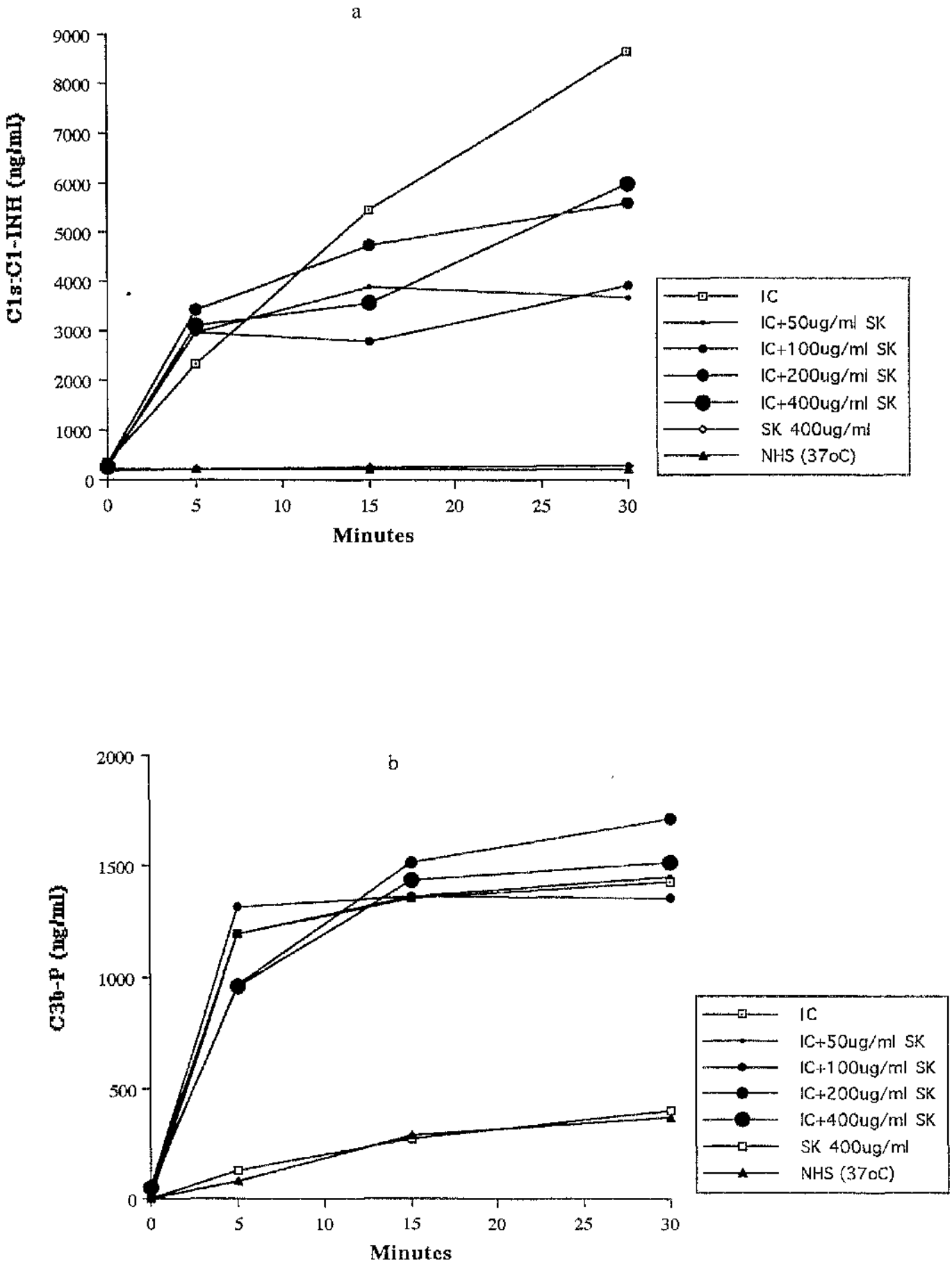


Figure 5-27: The effect streptokinase on the levels of complement activation products generated by nascent thyroglobulin immune complexes at equivalence.

The presence of streptokinase decreased the levels of C1s:C1-INH generated by the ICs at 15 and 30 minutes compared with the ICs alone (Figure 5-27a). Streptokinase at therapeutic doses (50-100 μg/ml) reduced the levels of C1s:C1-INH to a greater extent at 30 minutes when compared with the ICs alone (Figure 4-27a, 50 μg/ml streptokinase caused 57% reduction, 100 μg/ml =54% reduction, 200 μg/ml= 35% reduction and 400 μg/ml =30% reduction). This reduction was optimal with the lowest streptokinase concentrations (Figure 5-27a).

The presence of streptokinase had no effect on the levels of C3b-P generated by the ICs compared with the ICs alone (Figure 5-27b).

Discussion

In chapter 3, one patient with elevated levels of pre-existing anti-streptokinase antibodies who received streptokinase, developed an adverse immunological reaction. There was also a relationship between poor reperfusion and elevated levels of anti-streptokinase antibodies. However, from that study it was not clear why elevated levels of anti-streptokinase antibodies was not always accompanied by such difficulties. Since complement system has a central role in processing immune complexes and preventing tissue damage a study of the interactions of the complement system and immune complexes was important to approach this question (1-8). The first study to mention the effect of streptokinase on the complement system was in the early 1970's (Spottl 7& Mosuni 1974), and despite the increasing use of this thrombolytic agent in particular in patients with AMI, there are only few studies about complement recruitment in streptokinase therapy or r-t-PA

The levels of individual complement components i.e. C3 and C4 may not provide a precise evaluation of ongoing complement activation since these components are over-produced to balance the components used during illness and the levels are often normal

or even elevated (Auda et al 1990). Measurement of complement activation products therefore provides a better judgement of ongoing complement activation and hence the technique introduced by Auda et al (1990) with some modifications was used to measure the levels of C1s:C1-INH, C3b-P and C5b-9 complexes. In this technique by employing two antibodies that recognise different components of the complexes the intact complement components are not recognised in the assays (5-3).

The C3b-P assay used showed a better sensitivity than C1s:C1-INH assay (Table 5-2) which was in agreement with the results of Auda et al 1990. On the other hand, C1s:C1-INH assay showed a better reproducibility (Table 5-2), there is no report for inter-assay variations of C1s:C1-INH and C3b-P by Auda et al 1990.

Serum C3d levels, the final degradation product of C3b by Factor I, reflects ongoing complement activation. Rocket immunoassay and ELISA techniques have been employed to measure the serum C3d levels (Mollnes 1985, Freysdottir et al 1993). In this chapter an indirect ELISA for measuring serum C3d levels was developed (5-4). Preliminary laboratory investigations have indicated that ELISA's are more sensitive than agar techniques (data not shown).

Intact C3 present in serum samples cross-reacts with the rabbit anti-human C3d (Figure 5-6) used in ELISA system, however, this interference was minimised by diluting the samples to 1/600. The kinetics of C3 and C3d during complement activation at this dilution showed that the levels of C3d increased significantly during complement activation but the levels of C3 did not change (Figure 5-8). The increase of the C3d levels and constant levels of C3 in the period of complement activation validated that the ELISA system used specifically detects C3d and the interference of intact C3 was minimised. The intra-assay and inter-assay variations for this technique were 11% and 20% respectively (Table 5-2).

During complement activation degradation products are deposited on the erythrocytes. To detect erythrocyte-bound C3d a modified ELISA technique was developed to evaluate the relationship of bound C3d and the levels of free complement components (5-6).

The modified ELISA during complement activation by ICs showed that C3, properdin, Factor B and C3d were deposited on erythrocytes (Figures 5-9 and 5-10), the deposition of these components can be explained by direct binding to the surfaces that erythrocytes provide and also by indirect binding through incorporation into the ICs which bind to E-CR1 via IC bound C3b.

The modified ELISA for detecting the levels of bound C3d on erythrocytes showed a high C.V. for intra- and inter-assay variations (Table 5-2). This can be explained by the ease, with which erythrocytes easily precipitate in test tubes and therefore the number of cells during transferring from test tubes to ELISA plate is not highly reproducible.

The levels of complement activation products in 20 patients treated with thrombolytic agents were studied in conjunction with Dr.E.Holme University Department of Immunology, Western Infirmary, Glasgow. Patients treated with r-tPA did not generate C1s:C1-INH, C3b-P or C5b-9. The activation of complement system after r-tPA administration has been previously reported, Agostoni et al 1994, measuring anaphylatoxins C4a and C3a by RIA showed that the extent of this activation is substantially lower than streptokinase. Complement activation in AMI patients following r-tPA administration is thought to be the effect of conversion of plasminogen to plasmin (Munkuvad 1993).

All the ten patients administered streptokinase generated C1s:C1-INH within the 2 hours of treatment ($p<0.01$) and only one patient (F.M.) had increased levels of C3b-P and C5b-9. The significant correlation between C1s:C1-INH, IgG, IgA levels and neutralising antibodies clearly indicates the activation of the classical pathway by

streptokinase immune complexes formed during treatment (Figures 5-11 and 5-12). It was not surprising that the levels of generated C1s:C1-INH were higher in the group of patients who poorly responded to streptokinase treatment (Late reperfusion or non reperfusion, 5-7) since the levels of anti-streptokinase antibodies in these patients were higher than patients who reperfused within 2 hours of treatment. In ten patients treated with streptokinase, one patient (CD with elevated levels of IgG, IgA and C1s:C1-INH) had an adverse reaction to streptokinase. Patient EM also had both elevated IgG anti-streptokinase and C1s:C1-INH but did not respond adversely to streptokinase administration.

Freydottir et al (1993), analysed the extent of complement activation in patients administered streptokinase. As seen in our patients administered streptokinase, highest levels of complement activation as assessed by production of C3d occurred in those individuals with highest levels of antibodies to the administered streptokinase. The results of Freydottir et al (1993) and this study suggest that the presence of pre-existing antibodies to streptokinase results in the formation of immune complexes (ICs) and significant turnover of the classical pathway occurs. Despite this however, very little if any recruitment of alternative pathway (C3bP formation) and membrane attack complex (MAC) occurred in vivo. This observation is supported by similar results observed by in vitro experiments performed with rabbit streptokinase immune complexes (Figures 5-16, 5-17, 5-18, 5-21, 5-22 and 5-23).

The effects of the antigen alone (Streptokinase) was investigated. In vitro studies with streptokinase at a range of concentrations covering the therapeutic dose, showed that streptokinase at therapeutic dose (100 μg/ml) could generate significant levels of C1s:C1-INH and C3b-P (Figure 5-14). The extent of C1s:C1-INH and C3b-P generation in all the ten normal individuals studied did not correlate with the levels of anti-streptokinase antibodies, indicating that the effect of streptokinase alone was not due to immune complex formation (Table 5-5).(1-10).These studies also showed that

levels of C1s:C1-INH generated are optimal at therapeutic doses (Figure 5-14), whilst C3b-P generation is optimal beyond therapeutic dose (Figures 5-14 and 5-15) as beyond the 500 μg/ml streptokinase, the levels of C1s:C1-INH remained constant but C3b-P decreased to levels correspond to control levels (Figure 5-15) this can be account for the effect of generation of plasmin and dissociation of C3b-P by proteolytic effect of plasmin.

Plasmin is thought to activate the complement system in two ways, firstly, plasmin like trypsin and kallikrein can activate C1s in absence of immune complexes and secondly it can activate the alternative pathway by acting as Factor D and degrading Factor B (1-0). Generation of plasmin therefore may influence the detectable levels of complement activation products not only by activation of the pathways (1-0) but also by dissociation of complement activation products through the proteolytic effect.

As streptokinase alone does influence C1s:C1-INH and C3b-P formation in vitro, the in vitro effects in the presence of immune complexes was studied. For this study thyroglobulin ICs were chosen as thyroglobulin IC does not interfere with clotting systems. Streptokinase when added to the ICs reduced C1s:C1-INH generation by thyroglobulin ICs, optimal reduction observed with the therapeutic dose (50-100 μg/ml) (Figure 5-27). Streptokinase had no effect on C3b-P generation by thyroglobulin ICs.

On the other hand, the presence of low concentrations of plasmin (From 4μg to 32μg) increased significantly the levels of complement activation products (C1s:C1-INH and C3b-P) generated by rabbit thyroglobulin ICs (Figure 5-26). However, the larger concentrations of plasmin (From 64μg) reduced the levels of generated complement activation products by rabbit thyroglobulin ICs (Figure 5-26). The effects of 256 μg plasmin alone (Used in this experiment as control) confirmed the activation of the classical pathway (336 ng/ml generated by plasmin compared to 272 ng/ml generated

by NHS alone) but not the alternative pathway (5-11), however, a range of plasmin concentrations should be examined to evaluate the in vitro effect of plasmin.

Both streptokinase and plasmin influenced the levels of C1:C1-INH and C3b-P generated by thyroglobulin ICs, although the pattern of changes did not follow each another, this can be explained by the different concentration of plasmin presented in these experiments.

In our patients group r-tPA did not activate the complement system in spite of the fact that this thrombolytic agent activates plasminogen which results in generation of plasmin. However, in patients treated with streptokinase the activation of both pathways occurred and in vitro studies showed that complement activation by immune complexes is enhanced in the presence of plasmin (Figure 5-26), therefore, formation of ICs and generation of plasmin together has amplified the complement response in the patients treated with streptokinase.

The in vitro study of rabbit streptokinase ICs on complement activation showed that preformed immune complexes generate higher levels of complement activation products than nascent ICs (Figures 5-16 and 5-17). Streptokinase immune complexes (Rabbit and human) at antigen excess activated the classical pathway to a higher extent than antibody excess. This was reversed for the alternative pathway activation as determined by the generated levels of C3b-P (Figures 5-16 and 5-18).

In contrast to the streptokinase ICs, nascent thyrogobulin immune complexes generated higher levels of C1s:C1-INH than preformed immune complexes. This can be explained by the huge size of thyroglobulin ICs which precipitated in the test tube during the preparation of preformed immune complexes and decrease the immune complexes available for classical pathway activation (Figure 5-19 and 5-20). The preformed thyroglobulin immune complexes generated higher levels of C3b-P than the nascent immune complexes. In contrast to streptokinase ICs there was no difference for optimal generation of C1s:C1-INH or C3b-P and both preformed and nascent

thyroglobulin ICs at antigen excess generated higher levels than antibody excess (Figures 5-19 and 5-20).

The presence of EGTA had no effect on the levels of C3b-P generated by rabbit and human streptokinase ICs although it inhibited alternative pathway activation by rabbit thyroglobulin ICs (Figure 5-22).

As described erythrocytes may influence the levels of complement activation products (1-7). The presence of erythrocytes did not influence the levels of generated C1s:C1-INH and free C3d generated by rabbit streptokinase ICs. In the presence of erythrocytes the levels of C3b-P decreased and this effect was reversed by blocking CR1, this effect was optimal at 5 minutes, and was only partially reversed at 15 minutes (Figure 5-23). This is probably due to the increased production of C3b-P at 15 minutes together with insufficient concentration or specificity of anti-CR1 to block completely the CR1 receptors, as according to the manufacturer anti-CR1 antibodies are raised against podocytes (Figure 5-23).

The role of CR1 (CD35) on E's in the reduction of the levels C3b-P is through two mechanisms: 1) The clusters of CR1 on E's bind to C3b bound to the ICs and reduce the detectable levels. 2) CR1 participates in the degradation of C3b by serving as a cofactor for factor 1, preventing further C3bBbP formation. The role of CR1 in the reduction of C3b-P may account for the inability to detect elevated levels of C3b-P in patients treated with streptokinase.

The presence of E's treated with anti-CR1 increased the levels of C1s:C1-INH, this increase in the level of classical pathway activation reflects the effect of newly formed ICs (CR1 and anti-CR1) on the complement system.

This study showed that erythrocytes do not modulate significantly free and bound C3d or C1s:C1-INH levels, but exert an influence on C3b-P through the actions of CR1 on erythrocytes. This data suggest that C3b-P levels may not provide a reliable clinical marker of ongoing complement activation.

Chapter 6

Immune complexes and erythrocytes interactions

Introduction

Formation of immune complexes is an important physiological process in humoral defence to eliminate foreign materials. Immune complexes deposited outside the reticuloendothelial system can initiate adverse inflammatory reactions and tissue damage. The destination of immune complexes is determined by several factors such as size of immune complexes, the nature and amount of antigen, the ratio of antigen/antibody and finally the effect of complement on the immune complexes (1-8).

The complement system has a central role in preventing tissue damage by promoting clearance of immune complexes and preventing the precipitation of immune complexes outside the reticuloendothelial system (1-8). Immune complexes coated with C3b can bind to complement receptor CR1, predominant in the circulation, and be cleared from circulation and be disposed in the reticuloendothelial system. The binding of immune complexes to erythrocytes is influenced by the size and nature of immune complexes and the isotype of antibodies which participated in the immune complexes lattice (Schifferli & Peters 1986). This binding also correlates with the number of CR1 present on the erythrocytes which is governed by both environmental and genetic factors (Davies & Walport 1989, Holme et al 1986, Fyfe et al 1987).

By studying the levels of anti-streptokinase antibodies (Chapter 3) and the effects of the streptokinase immune complexes on the activation of the complement system (Chapter 5), it has been demonstrated that pre-existing anti-streptokinase antibodies in patients given streptokinase results in immune complexes formation and complement activation.

However, elevated levels of anti-streptokinase antibodies in these patients did not always provoke hypersensitivity reactions.

In this chapter the binding of streptokinase immune complexes and complement activation products to erythrocytes is investigated. The techniques of flow cytometry and development of modified ELISA allowed investigation of such binding. The aim was to analyse whether differential handling of ICs may allow for variable reactions to streptokinase. Kinetics and dose response of streptokinase and thyroglobulin immune complexes binding to erythrocytes were studied and compared with each other. The relationship between free and bound complement activation products with the levels of bound immune complexes on erythrocytes was studied.

The binding of streptokinase ICs to erythrocytes during streptokinase administration in patients with AMI together with the extent of complement activation and levels of anti-streptokinase antibodies in these patients was examined.

Materials and methods

6-1 Preparation of erythrocytes. Blood was obtained from healthy volunteers in heparin or EDTA and was spun at 1200 rpm, at 4°C for 10 minutes (2-16). The buffy coat layer was completely removed and the cells were washed three times with 1% BSA in PBS and resuspended in the same buffer. The cells were then standardised (2-17).

6-2 Preparation of NHS. Blood was obtained from healthy volunteers. The blood was incubated in glass tubes at 37°C for 30 minutes (to allow clot formation) followed by 30 minutes incubation at 4°C (to allow clot retraction). Aliquots were stored at -70°C and used within ten days.

6-3 Preparation of immune complexes. Preformed streptokinase immune complexes (Rabbit and human) and nascent thyroglobulin immune complexes were prepared at equivalence (5-2, 5-9). The equivalence point for rabbit streptokinase immune complexes was 10 μg streptokinase and 1300 μg IgG, for human streptokinase immune complexes was 10 μg streptokinase and 287.5 μg IgG and for rabbit thyroglobulin immune complexes was 10 μg thyroglobulin and 100 μg IgG (5-2).

6-4 Flow cytometry assays (FACscan, fluorescence-activated cell sorting). Flow cytometry is a technique for rapid analysis of particles or cells using a laser source or arc lamp. Using fluorescence and light scatter of the cells that flow through a flow stream, multiple cellular parameters i.e. cell markers can be measured and subpopulations of the cells can be distinguished. The cells that pass through a detection point (Illuminating beam) are illuminated by the light source and their fluorescent dyes excited. The scattered and fluorescent light generated by the cells is collected by photodetectors and is converted to electronic signals.

A forward lens collects forward scattered light in a forward optical path and conducts it towards a forward detector. By using a set of mirrors and absorption filters that are placed at 45degrees to the illuminating beam, different wavelengths are separated. The first mirror in the side angle optical path to the laser beam reflects wavelengths shorter than 500 nm towards the side angle scatter detector, a second mirror reflects wavelengths greater than 560 nm towards a 578nm bandpass filter and phycoerythrin (PE) fluorescence detector and finally the wavelengths between 550 and 560 nm, pass through a 530 nm filter and fluorescein isothiocyanate (FITC) fluorescence detector. This allows the specific measuring of the scattered (Forward and side) and fluorescence.

These signals (scatter and fluorescence) are processed in the detectors to a voltage which is then translated into a discrete scale by the analogue-to-digital converter (ADC). Finally the results are presented as graphs and statistical data by computer software package (i.e. Lysis II). Scattered light is usually proportional to the size of the cells (Ormerod 1994).

The common forms for displaying the results are frequency histograms and dual parameter correlated plots (Known as cytograms or dot plots). The frequency histogram is a graph that presents the number of events occurring for each channel of the ADC and counts intensity of fluorescence, forward light scatter or side angle light scatter of the cell population. The dot plot is a two-dimensional display of the frequency histogram of one set of ADC channels against the second set (i.e. forward light scatter channels against side angle light scatter channels or forward light scatter against fluorescence intensity channels, etc.). Using the computer programs, markers and gates (windows) can be set at specific channels of the histograms and selected areas of interest to obtain percentage of total, mean, median and other statistical data of the examined population.

All flow cytometry analyses was performed on a Beckton Dickinson flow cytometer that allowed measurement of both scattered light and fluorescence intensity of the populations. 5000 erythrocytes were analysed using the Lysis II software package. A setting was used to study the homogeneous erythrocyte (E's) population and the homogeneity of the E's was checked by the forward scatter against side scatter dot plots. By this setting erythrocytes were differentiated from debris (very small particles), aggregates (very large particles) and contaminating white cells. As described the fluorescent intensity of the cell population under analysis can be expressed by median fluorescent channel number (MFCN) or mean fluorescence intensity. However, skewed distributions can not to be compared by the parametric values, therefore, an

appropriate control for each experiment was used to assess the accuracy and specificity of each measurement. Then a gate on the control was set on a log scale fluorescence intensity histogram to determine the percentage of erythrocytes in samples bearing fluorescence above this value.

To study and analyse the binding of immune complexes to erythrocytes a flow cytometric assay was developed to detect bound immune complexes on the erythrocytes. Erythrocytes were incubated with normal human serum (NHS) in the presence and absence (Control) of immune complexes at 37°C. The reaction was stopped at different time points by EDTA. The supernatants were removed and studied for the presence free complement activation products and the erythrocytes were studied for the bound ICs and complement activation products using FITC conjugated antibodies. The antibodies used for detecting erythrocyte bound ICs and complement activation products were: FITC conjugated donkey anti-rabbit IgG, FITC conjugated rabbit anti-human C3c, FITC conjugated rabbit anti-human C3d, FITC conjugated sheep anti-mouse IgG, FITC conjugated sheep anti-human IgG, FITC conjugated sheep anti-human IgA, FITC conjugated sheep anti-human IgM.

6-5 Dose response of rabbit streptokinase and thyroglobulin immune complexes binding to erythrocytes. Dose response of rabbit streptokinase and thyroglobulin immune complexes binding to erythrocytes was studied. Free complement activation products C1s:C1-INH and C3b-P in the supernatants were measured to study the relationship between the ICs binding and the extent of the complement activation.

Materials: Sources of materials used here are described in 2-1.

FITC conjugated donkey anti-rabbit IgG

NHS

Human Erythrocytes

Falcon round-bottomed tubes

Antigens: Streptokinase, bovine thyroglobulin

Antibodies: Rabbit IgG anti-streptokinase, rabbit IgG anti-bovine thyroglobulin (5-2)

Buffers

Filtered phosphate buffered saline (PBS)

1% BSA in PBS

20 mM isotonic EDTA (2-2)

ELISA buffers (5-3)

Method. Increasing amount of immune complexes at equivalence (5-2) (for rabbit streptokinase preformed ICs from 81.87μg to 1310 μg ICs and for rabbit thyroglobulin nascent ICs from 0.85μg to 110 μg ICs) were added to 100 μl NHS and 20 μl of packed erythrocytes, the volumes were equalised by 1% BSA in PBS to 310 μL for streptokinase ICs and to 158 μl for thyroglobulin. The mixtures were then incubated at 37°C for 30 minutes. The reaction was stopped by addition of equal volume of ice cold 20 mM isotonic EDTA to the mixtures. The erythrocytes and serum suspension was spun at 4°C for 5 minutes and the supernatants removed and kept at -70°C for later measurement of complement activation products (5-3). Erythrocytes were washed with ice cold 1% BSA in PBS once and resuspended in the same buffer. The cells were standardised to $1X10^8$ cells/ml (6-1) and incubated with an equal volume of donkey FITC conjugated anti-rabbit IgG (diluted 1/20 with PBS) for 30 minutes in an ice bath. The cells were washed with 1% BSA in PBS twice and analysed by a FACscan flowcytometer as described (6-4).

FACscan controls: 1) The binding of ICs to E's in absence of NHS was studied at doses equal to the maximum doses used for ICs (for rabbit streptokinase 1310 μg ICs and for rabbit thyroglobulin ICs 110 μg ICs).

2) The binding and complement activation products in the presence of streptokinase and thyroglobulin at doses equal to the maximum doses used for ICs (10 μg) were studied.

3) The binding and complement activation products in the presence of NHS alone was studied. A mixture of 100 μl NHS and 20 μl of packed erythrocytes was equalised with 1% BSA in PBS to 310 μl and to 158 μl (for streptokinase and thyroglobulin controls respectively) and incubated at 37°C.

4) The binding and complement activation products by rabbit anti-streptokinase IgG to erythrocytes equal to amount used for immune complexes (from 81.25 μg to 1300 μg IgG) was also studied.

6-6 Kinetics of rabbit streptokinase immune complexes binding to erythrocytes. The kinetics of rabbit streptokinase immune complexes binding to erythrocytes was studied at 37°C.

Materials and buffers are described in 6-5.

Method. Increasing amounts of the preformed rabbit streptokinase immune complexes at equivalence (5-2) from 131 μg to 2096μg were added to 200 μL NHS and 200 μL of packed erythrocytes and the volumes were equalised by 1% BSA in PBS to 620 μL. The mixtures were then incubated at 37°C and the assay sampled at 0, 5, 10, 25 and 30 minutes. The reaction was stopped by addition of an equal volume of ice cold 20 mM isotonic EDTA to the mixtures. The erythrocytes were for bound immune complexes and the supernatants for free complement activation products, C1s:C1-INH, C3b-P as described (6-5).

Control: The volume of a mixture of 200 μL NHS and 200 μL of packed erythrocytes was equalised with 1% BSA in PBS to 620 μL incubated at 37°C and used as negative control and the levels of C1s:C1-INH, C3b-P and E's bound ICs was determined at 30 minutes.

6-7 Detecting bound C3c on erythrocytes during complement activation by FACscan. The binding of C3c to erythrocytes generated during complement activation by rabbit streptokinase and thyroglobulin immune complexes was studied by flow cytometry. The relationship between erythrocyte bound C3c and free complement activation products was investigated by studying the supernatants for the presence of the C1s:C1-INH and C3b-P.

Materials: Sources of materials used here are described in 2-1.

FITC conjugated rabbit anti-human C3c

NHS

Human erythrocytes

Falcon round-bottomed tubes

Streptokinase

Rabbit IgG anti-streptokinase (5-2)

Rabbit IgG anti-thyroglobulin

Streptokinase

Thyroglobulin

Buffers

Filtered phosphate buffered saline (PBS)

1% BSA in PBS

20 mM isotonic EDTA (2-2)

ELISA buffers (5-3)

Method. Preformed rabbit streptokinase ICs at equivalence (2620 μg ICs, 6-3) were added to 200 μl NHS and 200 μl of packed erythrocytes, nascent rabbit thyroglobulin ICs at equivalence (440 μg ICs, 6-3) were added to 300 μl NHS and 300 μl of packed erythrocytes, the volumes were equalised by 1% BSA in PBS to 620 μl and 712 μl for the mixtures of streptokinase and thyroglobulin ICs respectively. The mixtures were then incubated at 37°C and the assay sampled at 0, 5, 10, 15 and 30 minutes. The

reaction was stopped by addition of equal volume of ice cold 20 mM EDTA to the mixture. The suspension of erythrocytes and serum were spun at 4°C for 5 minutes and the supernatants kept at -70°C for the later measurement of complement activation products (5-3). Erythrocytes were washed with ice cold 1% BSA in PBS once and resuspended in the same buffer. The cells were standardised to $1X10^8$ cells/ml (6-1) and incubated with equal volume of rabbit FITC conjugated anti-human C3c (diluted 1/20 with PBS) for 30 minutes in a ice bath. The cells were washed with the 1% BSA in PBS twice and analysed by a FACscan flow cytometer as described (6-4).

FACscan control: The volumes of mixtures of 200 μL NHS and 200 μl of packed erythrocytes or 300 μL NHS and 300 μL of packed erythrocytes were equalised with 1% BSA in PBS to 620 μl and 712 μl, incubated at 37°C and used as negative control for the streptokinase and thyroglobulin ICs respectively and the levels of C1s:C1-INH, C3b-P and E's bound ICs was determined at 30 minutes.

6-8 Detecting bound C3d on erythrocytes during complement activation by FACscan and modified ELISA. The binding of complement activation product C3d to erythrocytes generated during complement activation by rabbit streptokinase immune complexes was studied by flow cytometry and modified ELISA (5-5). The relationship between erythrocyte bound C3d and free complement activation products was investigated by studying the supernatants for the presence of the C1s:C1-INH and C3b-P.

Materials: Sources of materials used here are described in 2-1.

Rabbit anti-human C3d

FITC conjugated rabbit anti-human C3d

NHS

Human erythrocytes

Falcon round-bottomed tubes

ELISA plate (Immulon 4)

Streptokinase

Rabbit IgG anti-streptokinase (5-2)

Buffers

Filtered phosphate buffered saline (PBS)

1% BSA in PBS

20 mM isotonic EDTA (2-2)

Isotonic 0.086 M EDTA (2-2)

ELISA buffers (5-3)

Method. Preformed rabbit streptokinase immune complexes at equivalence (6-3, ICs 1310 μg ICs) were incubated with 500 μl NHS and 100 μl of packed erythrocytes and the volumes were equalised by 1% BSA in PBS to 750 μL. The mixtures were then incubated at 37°C and the assay sampled at 0, 5, 15 and 30 minutes. The reaction was stopped by addition of equal volume

of ice cold 20 mM isotonic EDTA to the mixture. Erythrocyte bound C3d and free complement activation products were measured as described (6-7).

Erythrocyte bound C3d was also detected by the modified ELISA (5-5). Erythrocytes (100 μL per well from $1X10^8$ cells/ml) were incubated on a ELISA plate (pre-coated with anti-human C3d, 5-5) for 2 hours at room temperature. The plate was washed with the 1% BSA in PBS and the bound E's were lysed by water, the optical density of lysed cell were read at 405 nm (5-5).

Controls: 1) The volume of a mixture of 500 μL NHS and 100 μL of packed erythrocytes was equalised with 1% BSA in PBS to 750 μL. The mixture was incubated at 37°C and sampled at 0, 5, 15 and 30 minutes and used as negative control for both the FACscan and modified ELISA assays.

2) The effect of EDTA on erythrocyte bound C3d and free C1s:C1-INH, C3b-P and C3d. Preformed rabbit streptokinase ICs at equivalence (6-7, 1310 μg ICs) were added to 500 μl NHS or 500 μl NHS treated with isotonic 0.086M EDTA (End point 10mM EDTA) and 100 μl of packed erythrocytes. The volumes of mixtures were equalised by 1% BSA in PBS to 750 μl. The mixtures were then incubated at 37°C and the assay sampled at 10 minutes. The levels of free and bound complement activation products were measured as described (See above).

3)The effect of rabbit streptokinase IgG on the erythrocyte bound C3d and free C1s:C1-INH, C3b-P and C3d at concentration equal to concentration used for the ICs (1300 μg) and same conditions described (10 minutes) was studied.

6-9 Specificity of monoclonal anti-CR1 for human erythrocytes. Mouse monoclonal anti-CR1 according to manufacturer was raised against podocytes. The specificity of the mouse monoclonal anti-CR1 antibody for erythrocytes was therefore examined.

Materials and Buffer: Sources of materials used here are described in 2-1.

Mouse monoclonal anti-human CR1 (IgG1, kappa)

FITC conjugated sheep anti-mouse IgG

Mouse monoclonal anti-human AFP (alpha foetal protein)

Falcon round-bottomed tubes

Membrane filtered PBS

Human erythrocytes

1% BSA in PBS

Method. Erythrocytes were prepared and standardised to $5X10^8$ cells/ml as described (2-17 and 6-1). Increasing amounts of mouse monoclonal anti-human CR1 from 0.138 μg to 4.4 μg was incubated with a constant amount of erythrocytes ($2X10^8$ E's) for half

an hour at room temperature. The E's were washed and standardised to $1X10^8$ cells/ml and incubated with an equal volume of FITC conjugated sheep anti-mouse IgG (diluted 1/20 with PBS) for half an hour at room temperature. The cells were washed with the 1% BSA in PBS twice and analysed by a FACscan flow cytometer as described (6-4).

Controls: 1) The above procedure was followed, however, mouse monoclonal anti-human CR1 was replaced with 10μg monoclonal mouse anti-human AFP.

2) Non-specific binding of FITC to E's was analysed by incubating $2X10^8$ erythrocytes with equal volume of FITC conjugated sheep anti-mouse IgG used for detecting anti-CR1.

6-10 Kinetics of human streptokinase immune complexes binding to erythrocytes and the effect of monoclonal anti-CR1 on binding and generation of complement activation products. Serum from a patient with extremely elevated levels of anti-streptokinase antibodies was used to study the kinetics of human anti-streptokinase immune complexes binding to erythrocytes. The levels of anti-streptokinase antibodies in this serum were as following; IgG > 90 units/ml, IgA> 1000 units /ml, IgM=34 units/ml, ASK=1280 units/ml and IgE was not detectable. The serum was heat inactivated for half an hour at 56°C to inactivate complement. The volume of the serum was not sufficient for purification of individual isotype antibodies. Using quantitative precipitin test (2-18) the equivalence point of the serum and streptokinase determined which was 50 μg streptokinase and 100 μl serum (Figure 6-1).

Flow cytometry was used to detect erythrocyte bound immune complexes (IgG, IgA and IgM) and C3d. The relationship between erythrocyte bound immune complexes and C3d with the extent of complement activation products was studied by measuring the levels of C1s:C1-INH, C3-b-P and free C3d in the supernatants.

Materials: Sources of materials used here are described in 2-1.

FITC conjugated rabbit anti-human C3d

FITC conjugated sheep anti-human IgG

FITC conjugated sheep anti-human IgA

FITC conjugated sheep anti-human IgM

Mouse monoclonal anti-human CR1 (IgG1, kappa)

NHS

Human erythrocytes

Streptokinase

Falcon round-bottomed tubes

Buffers

Filtered phosphate buffered saline (PBS)

1% BSA in PBS

20 isotonic mM EDTA (2-2)

ELISA buffers (5-3)

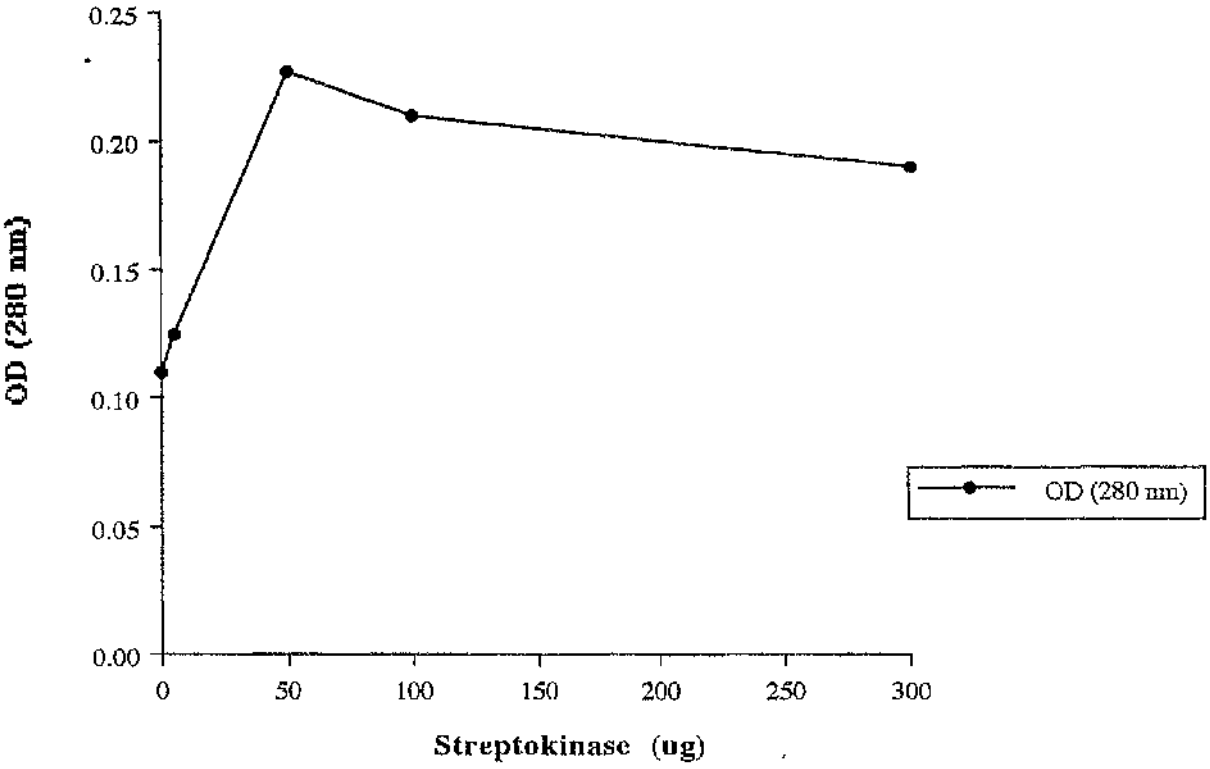


Figure 6-1: Quantitative percipitin test for streptokinase immune complexes, using human anti-streptokinase antibodies derived from positive serum sample.

Method. Preformed immune complexes at equivalence (50 μg streptokinase and 100 μL serum) were added to 500 μl NHS and 100 μL of packed erythrocytes, the volumes were equalised with 1% BSA in PBS to 750 μl and the mixtures incubated at 37°C. The assay sampled at 0, 5, 15 minutes and 24 hours. The reaction was stopped by the addition of an equal volume of ice cold 20 mM isotonic EDTA to the mixture. The mixture was spun at 4°C for 5 minutes and the supernatants removed and kept at -70°C for the later measurement of free complement activation

products, C1s:C1-INH, C3b-P and C3d (5-3). The erythrocytes were washed once with ice cold 1% BSA in PBS and standardised to $1X10^8$ cells/ml (6-1) and further incubated with equal volume of FITC conjugated rabbit anti-human C3d, FITC conjugated sheep anti-human IgG, FITC conjugated sheep anti-human IgA and FITC conjugated sheep anti-human IgM (All diluted 1/20 with PBS) for 30 minutes in an ice bath. After the incubation period the cells were washed twice with the 1% BSA in PBS and analysed by a FACscan flow cytometer as described (6-4).

The effect of monoclonal anti-human CR1: The effect of blocking CR1 receptors on the measurable levels of erythrocyte bound immune complexes, bound C3d and the free complement activation products, C1s:C1-INH, C3b-P and free C3d was studied. The above procedure was followed, however, 100 μl of packed naive E's was replaced with 100 μl of packed E's treated with monoclonal mouse anti-human CR1 as following. Monoclonal mouse anti-human CR1 was added at a concentration 13.8 μg per 100 μl packed erythrocytes and incubated for half an hour at room temperature. The E's were washed and 100 μl of packed E's were incubated with the ICs and NHS as described.

Controls: The following controls were used:

1) The levels of bound immune complexes to erythrocytes in absence of NHS at same conditions described above was studied.

2) The levels of free complement activation products (C1s:C1-INH, C3b-P and free C3d) in absence of erythrocytes at same conditions described above was studied.

3) The levels of erythrocyte bound antibodies, C3d and free complement activation products (C1s:C1-INH, C3b-P and free C3d) in the presence of 100 μl of positive serum (equal to the volume used for the ICs) and absence of streptokinase at same conditions described above was studied.

4) A mixture of 500 μl NHS and 100 μl of packed erythrocytes (Total volume=750 μl) at 37°C was used as negative control.

6-11 Effects of antigen and antibody excess on human streptokinase immune complexes binding to erythrocytes. The binding of human streptokinase immune complexes at equivalence, antigen excess and antibody excess to erythrocytes was studied. Flow cytometry was used to detect bound immune complexes (IgG) and C3d on the erythrocytes. The levels of bound C3d was also studied by the modified ELISA (5-5). The relationship between bound immune complexes, C3d and free complement activation products was studied by measuring C1s:C1-INH, C3b-P and free C3d in the supernatants (5-3).

Materials: Sources of materials used here are described in 2-1.

Rabbit anti-human C3d

FITC conjugated rabbit anti-human C3d

FITC conjugated sheep anti-human IgG

NHS

Human erythrocytes

Streptokinase

Human IgG anti-streptokinase

Falcon round-bottomed tubes

Buffers

Filtered PBS

1% BSA in PBS

20 mM EDTA (2-2)

Isotonic 0.086 M EDTA (2-2)

0.1 M EGTA, $MgCl_2$ (2-2)

ELISA buffers (5-3)

Method. Human IgG anti-streptokinase was purified from the sera of patients with elevated levels of anti-streptokinase antibodies by ion exchange chromatography (5-1) and the equivalence point was determined (5-2). Preformed immune complexes at equivalence (1150 μg IgG+40μg streptokinase), 4 times antibody excess (1150 μg IgG+10μg streptokinase) and 4 times antigen excess (287.5 μg IgG+40μg streptokinase) were added to 250 μl NHS and 50 μl of packed erythrocytes, the volume of the mixtures equalised with 1% BSA in PBS to 440 μl. The mixtures were then incubated at 37°C for 5 minutes. The reaction was stopped by addition of equal volume of ice cold 20 mM isotonic EDTA to the mixtures. The mixture was spun at 4°C for 5 minutes and the supernatants were removed and kept at -70°C for the later measurement of free complement activation products, C1s:C1-INH, C3b-P and C3d (5-3). Erythrocytes were washed once with ice cold 1% BSA in PBS and standardised to $1X10^8$ cells/ml (6-1). The erythrocytes were further incubated with equal volume of sheep FITC conjugated anti-human IgG or FITC conjugated rabbit anti-human C3d (Both diluted 1/20 with PBS) for 30 minutes in a ice bath. The cells were washed twice with the 1% BSA in PBS and analysed by a FACscan flow cytometer (6-4).

Erythrocytes (100 μl per well from the $1X10^8$ cells/ml) were also incubated on a ELISA plate (pre-coated with anti-human C3d, 5-5) for 2 hours at room temperature. The plate

was washed with the 1% BSA in PBS and the bound E's were lysed by water, the optical density of lysed cell were read at 405 nm (5-5).

Controls: The controls for above experiment were: 1) The levels of free complement activation products (C1s:C1-INH, C3b-P and free C3d) in absence of erythrocytes at same conditions described above.

2) To inhibit complement activation, NHS was incubated with isotonic 0.086M EDTA to give a final concentration of 10 mM (2-2). The levels of bound ICs, C3d and free complement activation products (C1s:C1-INH, C3b-P and free C3d) in presence of the NHS treated with EDTA and E's at same conditions described above was studied.

3) To inhibit alternative pathway activation NHS was incubated with 0.1 M $MgCl_2$ EGTA to give a final concentration 6 mM (2-2). The levels of bound ICs, C3d and free complement activation products (C1s:C1-INH, C3b-P and free C3d) in presence of the NHS treated with $MgCl_2$ EGTA and E's at same conditions described above was studied.

4) The levels of bound IgG, C3d and free complement activation products (C1s:C1-INH, C3b-P and free C3d) in presence of antigen or antibody (streptokinase 40μg, IgG 1150μg) at same conditions described above was studied.

5) A mixture of 250 μl NHS and 50 μL of packed erythrocytes (Total volume=440 μl) at 37°C was used as negative control.

6-12 In vivo study of streptokinase immune complexes binding to erythrocytes in patients with AMI. This study was performed in collaboration with Department of Cardiology, Western Infirmary, Glasgow (Dr.Stewart Hood and Dr. David Birnie). The extent of complement activation products (C1s:C1-INH, C3b-P and C5b-9), and its relationship with the levels of pre-existing anti-streptokinase antibodies and the immune complexes clearance in four patients with AMI (2 males, 2 females) treated with streptokinase were studied.

Materials: Sources of materials used here are described in 2-1.

FITC conjugated rabbit anti-human C3c

FITC conjugated rabbit anti-human C3d

FITC conjugated sheep anti-human IgG

FITC conjugated sheep anti-human IgA

FITC conjugated sheep anti-human IgM

Falcon round-bottomed tubes

Buffers

Filtered phosphate buffered saline (PBS)

ELISA buffers (5-3)

Method. The blood samples from 4 AMI patients were used in this set of experiments. The patients received 15 00 000 units of streptokinase over a period of 1 hour. Blood samples were taken prior to administration of streptokinase and at 5 and 30 minutes after treatment.

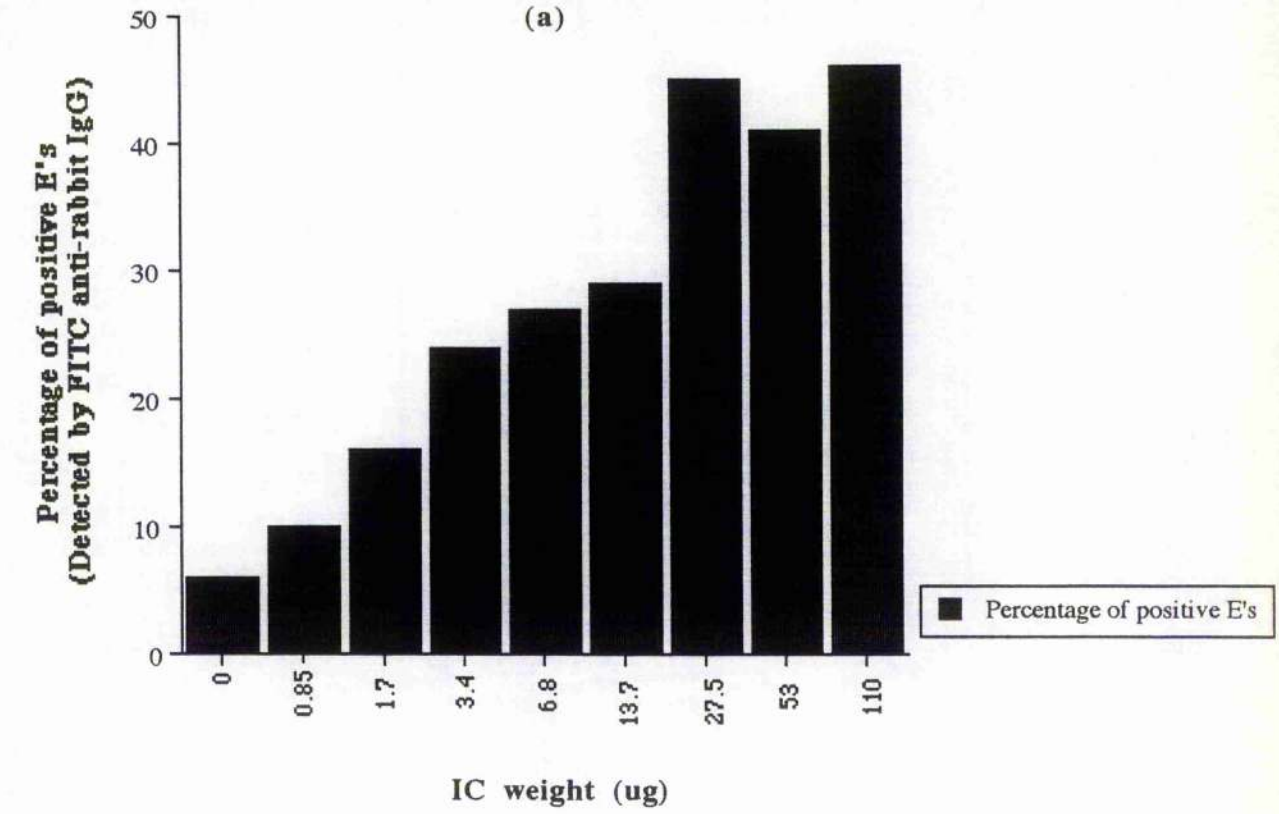
Blood samples were collected in EDTA at time points 0, 5 and 30 minutes after streptokinase administration and spun at 4°C for 5 minutes at 1200 rpm. The plasma was then removed and kept at - 70°C for the later measurement of the levels of IgG, IgA and IgM anti-streptokinase antibodies (3-3), and complement activation products, C1s:C1-INH, C3b-P and C5b-9 (5-3). After removing the buffy coat the erythrocytes were washed once with ice cold PBS and standardised to $1X10^8$ cells/ml (6-1). 100 µl of the erythrocytes were incubated with 100 µl of FITC conjugated rabbit anti-human C3c, FITC conjugated rabbit anti-human C3d, FITC conjugated sheep anti-human IgG, FITC conjugated sheep anti-human IgA and FITC conjugated sheep anti-human IgM (All diluted 1/20 with PBS) in an ice bath for 30 minutes. After incubation the cells were washed twice with PBS and analysed by a FACscan flow cytometer (6-4).

6-13 Statistical analysis. Statistical analysis of data was performed using Spearman rank correlation test, Wilcoxon rank test and Mann-Whitney test.

Results

Dose response of rabbit streptokinase and thyroglobulin immune complexes binding to erythrocytes. A flow cytometric assay was developed successfully to detect bound immune complexes on the erythrocytes. The relationship of E's bound ICs and complement activation products was studied by measuring C1s:C1-INH and C3b-P in the supernatants (6-5).

Using nascent rabbit thyroglobulin immune complexes at equivalence, as the amount of ICs increased (from 0.85 μg to 110 μg) the levels of erythrocyte bound ICs as measured by FACscan (Using FITC conjugated anti-rabbit IgG) and increased generated complement activation products compared with the controls at 30 minutes (Figure 6-2). The levels of C1s:C1-INH increased over the incubation period compared to C3b-P levels which reached to peak of 740 ng/ml at 1.7 μg IC and then reached a plateau (Figure 6-2). The levels of erythrocyte bound ICs correlated significantly with the levels of generated C1s-C1-INH and C3b-P ($r = 0.98$ and 0.91 respectively, $p<0.001$) (Figure 6-2).



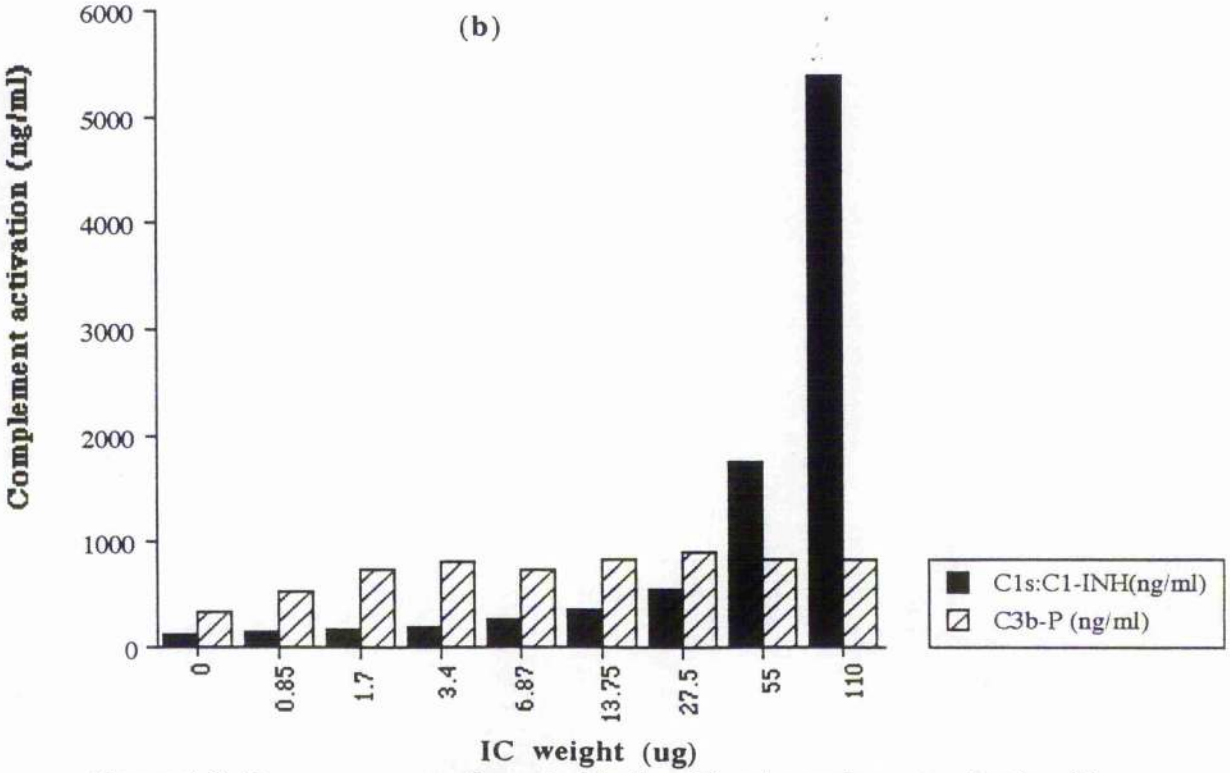


Figure 6-2: Dose response erythrocytes binding (a) and complement activation (b) by nascent thyroglobulin immune complexes at equivalence.

Controls: 1) Rabbit thyroglobulin ICs (110 μg ICs) alone in absence of NHS bound to erythrocytes (32 %) which was 14% lower than the presence of the NHS.

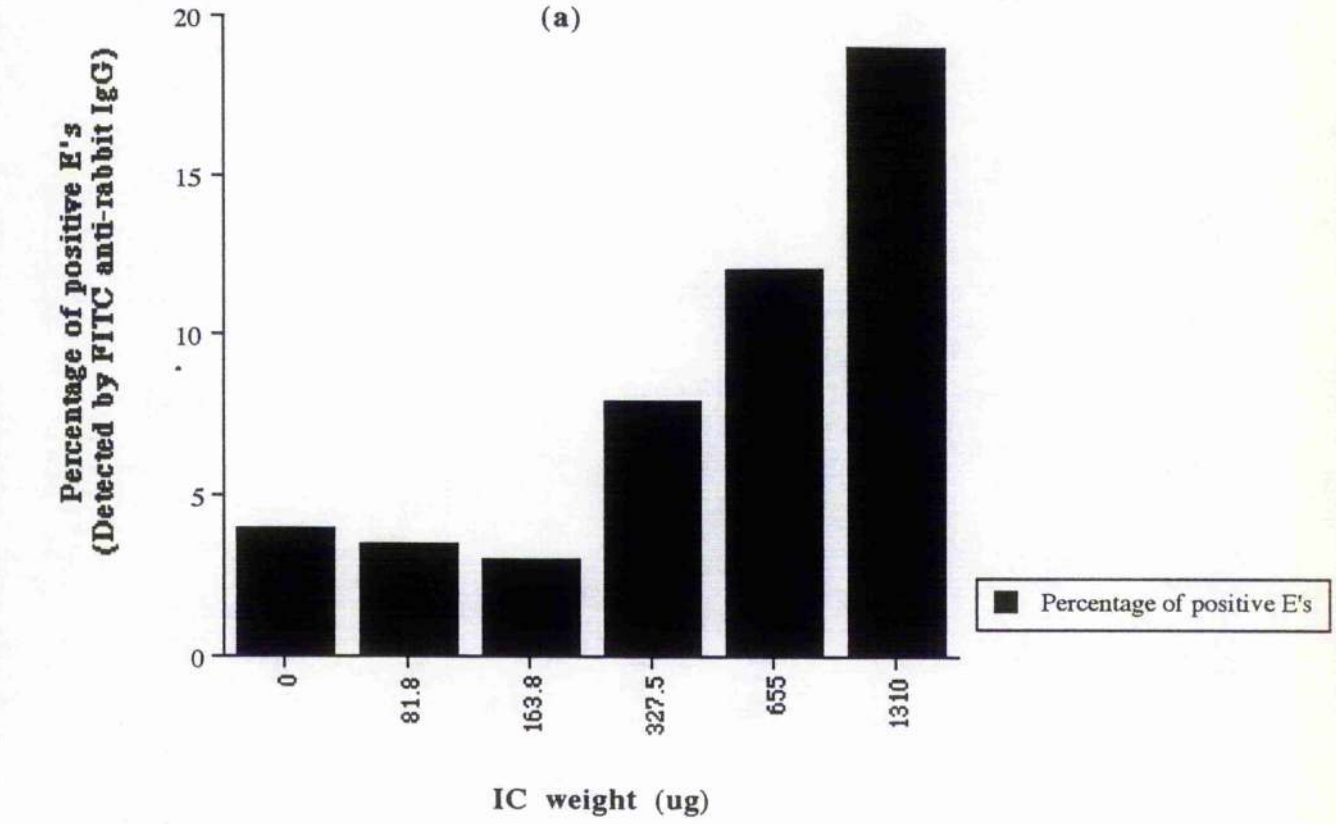
2) Thyroglobulin alone (10 μg) showed a similar percentage of positive E's (7%) and levels of C1s:C1-INH (140 ng/ml) as the NHS+E's alone (6% positive E's and 112 ng/ml C1s:C1-INH). However, it generated 39% higher levels of C3b-P than the values of NHS+E's alone (330 ng/ml), this was 35% lower than the ICs (840ng/ml).

3) IgG alone (100 μg) in absence of thyroglobulin bound to E's (35%) and generated C1s:C1-INH and C3b-P (880 ng/m and 840 ng/ml respectively), this was 6% and 83% lower than the ICs for the binding and C1s:C1-INH respectively and for C3b-P levels did not differ from the ICs.

Using preformed rabbit streptokinase immune complexes at equivalence, increasing the amount of ICs from 81.87μg to 1310 μg resulted in a dose dependent increase in the levels of generated complement activation products compared with the controls at 30 minutes (Figure 6-3). A striking increase in the levels of C1s:C1-INH was observed compared to the C3b-P levels which rose more slowly with increasing concentrations and then reached a plateau as observed with thyroglobulin ICs (Figure 6-3).

The levels of E's bound ICs was detectable at the concentrations of 327.5μg to 1310 μg ICs when compared with the controls and as demonstrated by FACscan using FITC conjugated anti-rabbit IgG at 30 minutes (Figure 6-3). The levels of erythrocyte bound ICs correlated with the levels of generated C1s-C1-INH and C3b-P ($r = 0.77$, $p<0.07$).

Controls: 1) Rabbit streptokinase ICs (1310 μg ICs) in absence of NHS bound to erythrocytes (68 %) which was 49% higher than the presence NHS.



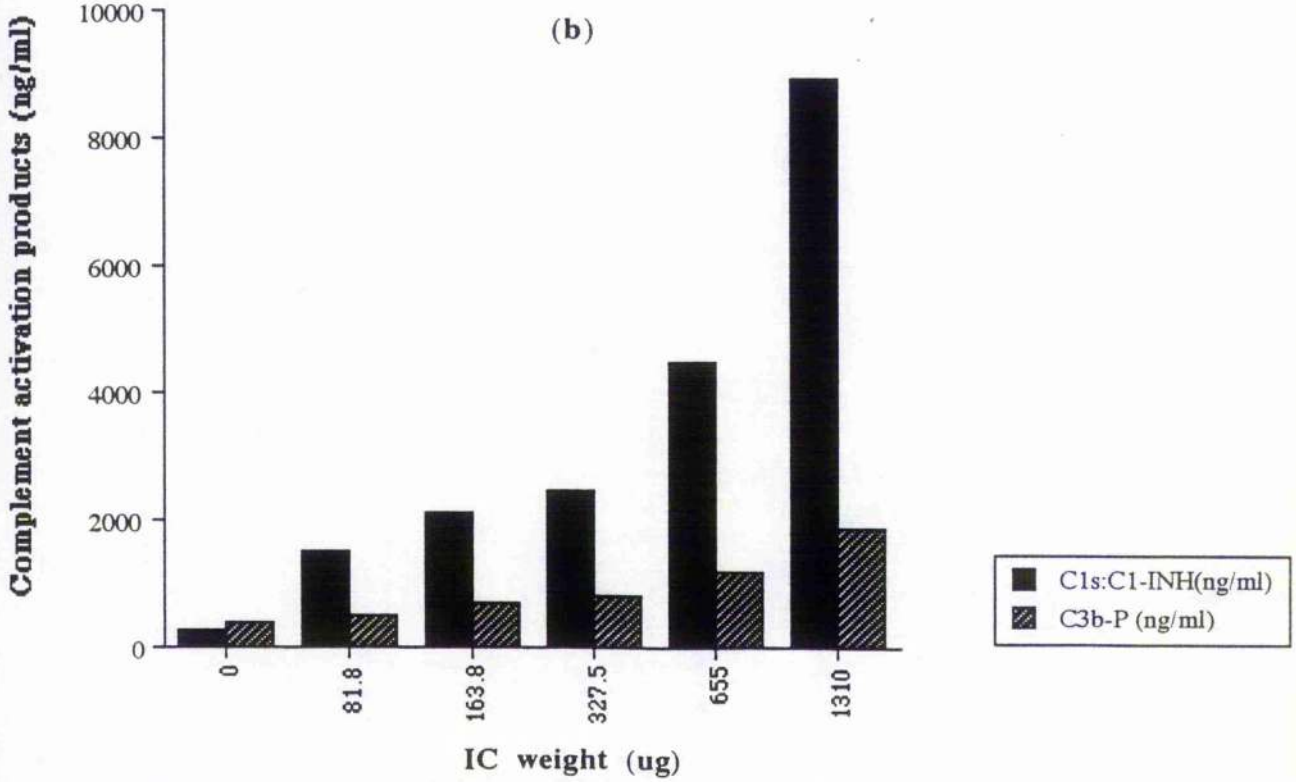


Figure 6-3: Dose response erythrocytes binding (a) and complement activation (b) by preformed rabbit streptokinase immune complexes at equivalence.

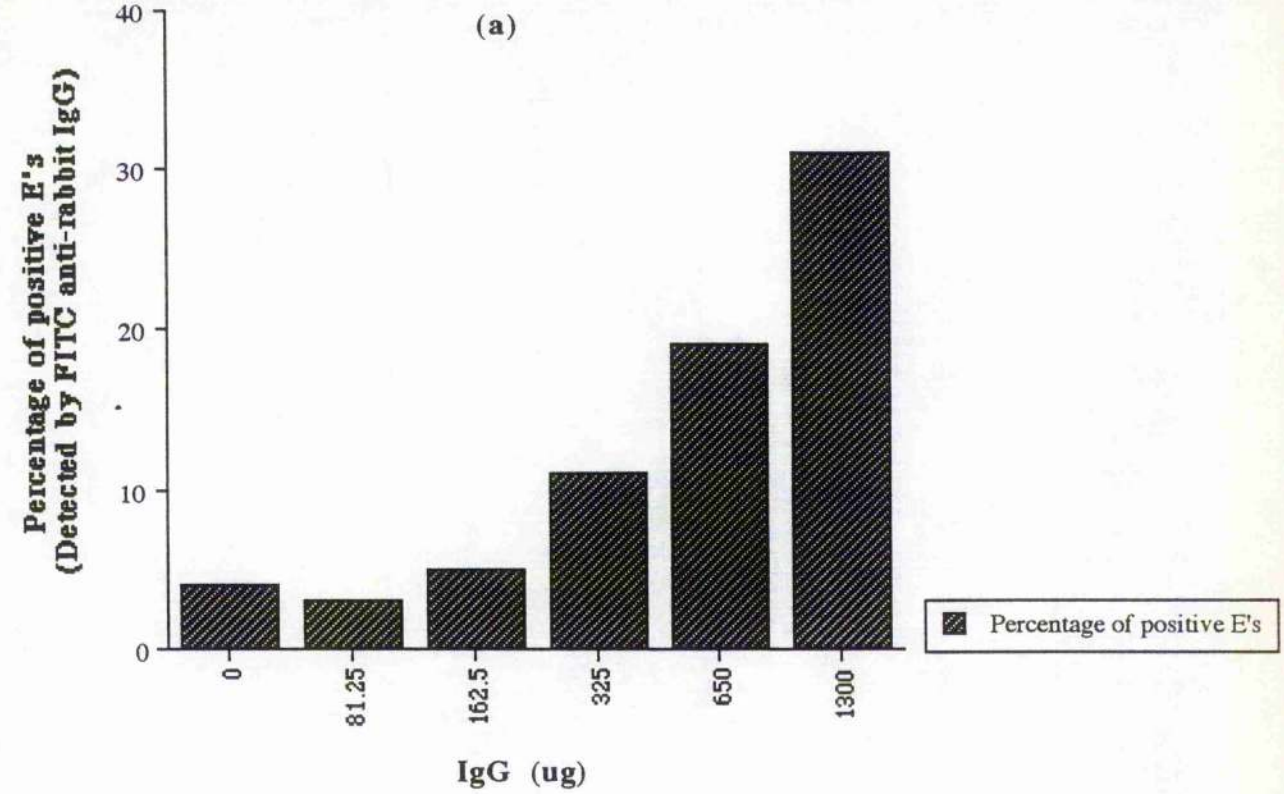
2) Streptokinase alone (10 μg) showed a 4% E's and generated 268 ng/ml C1s:C1-INH and 346 ng/ml C3b-P which did not differ significantly from the values of the NHS+E's alone (4% and 289 ng/ml and 411 ng/ml respectively).

3) Increasing amounts of rabbit anti-streptokinase IgG alone (from 81.25 μg to 1300 μg IgG) resulted in detectable levels of E's bound IgG and C1s:C1-INH and C3b-P (Figure 6-4). The concentrations of IgG added correlated with the levels of E's bound IgG, C1s:C1-INH and C3b-P ($r=0.94$ $p<0.005$, $r=0.98$ $p<0.001$ and $r=0.98$ $p<0.01$ respectively). The levels of E's bound IgG correlated with the levels of C1s:C1-INH and C3b-P ($r= 0.94$ $p<0.005$ and $r=0.75$ $p<0.08$ respectively). The levels of E's bound IgG alone (from 81.25 μg to 1300 μg IgG) were higher than the levels of E's bound ICs (1.5-12%) (Figures 6-3 and 6-4). However, IgG alone generated lower levels of C1s:C1-INH (79-84%) and C3b-P (3-11%) compared to ICs (Figures 6-3 and 6-4).

Kinetics of rabbit streptokinase immune complexes binding to erythrocytes. The kinetics and dose response of preformed rabbit streptokinase at equivalence for binding to erythrocytes and its relationship with the complement activation products C1s:C1-INH and C3b-P was studied (6-6).

Increasing amount of the preformed rabbit streptokinase immune complexes at equivalence (5-2) from 131 μg to 2096μg were incubated with NHS and erythrocytes at 37°C and the assay sampled at 0, 5, 10, 25 and 30 minutes. Over a period of 30 minutes the immune complexes bound to erythrocytes as demonstrated by FACscan using FITC conjugated anti-rabbit IgG (Figure 6-5a). The highest binding was observed with greater dose of ICs, peak of the binding was at 5 minutes for doses of 524 μg, 1024μg and 2096μg ICs and at 15 minutes for 131μg and 262 μg ICs (Figure 6-5a). The levels of E's bound immune complexes then decreased and reached to lowest levels at 30 minutes (Figure 6-5a).

The levels of generated C1s:C1-INH again were higher with greater dose of ICs, 1048 μg and 2096 μg ICs, caused a rapid increase of C1s:C1-INH peaking by 5 and 10 minutes respectively then reached a plateau, a peak for the levels of C1s:C1-INH generated by the lower amount of ICs was not observed (Figure 6-5b).



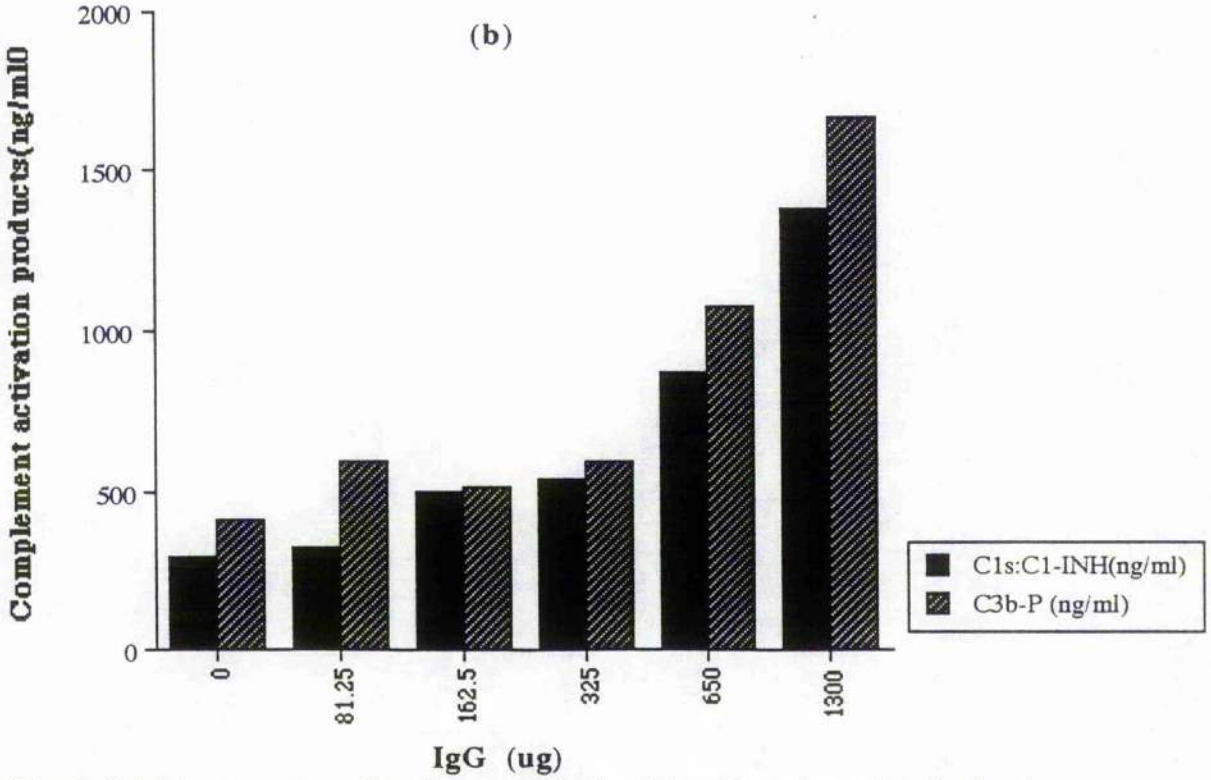


Figure 6-4: Dose response of erythrocytes binding (a) and complement activation by rabbit IgG anti-streptokinase at doses equal to doses used for the ICs.

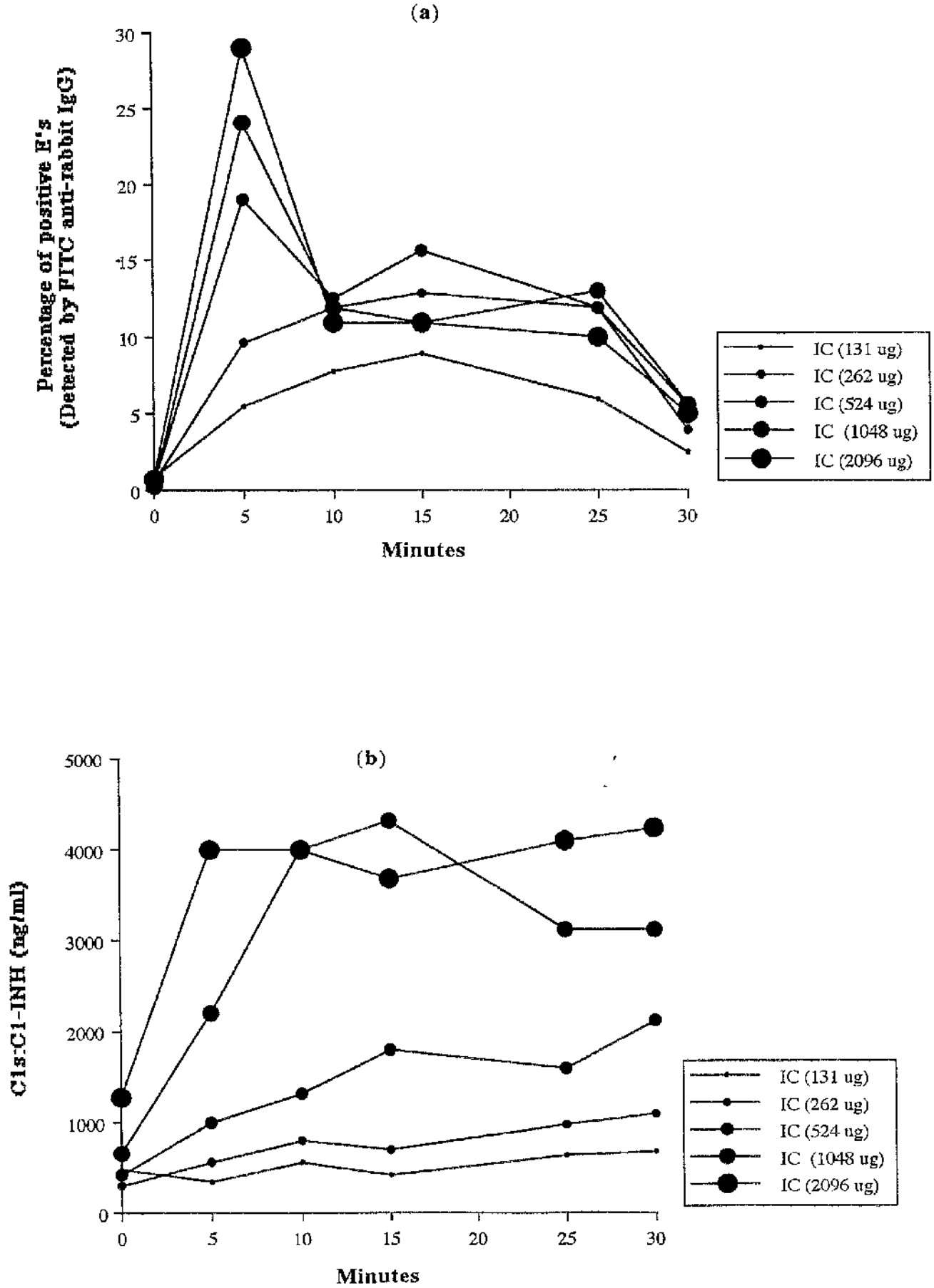


Figure 6-5: Kinetics and dose response of E's binding (a) and C1s:C1-INH (b) generation by the preformed rabbit streptokinase ICs at equivalence.The levels of E's bound IgG and C1s:C1-INH in the control (E's+NHS) were 0.6% and 196 ng/ml respectively.

The levels of generated C3b-P by 1048 μg and 2096 μg ICs increased and reached a peak by 10 minutes, a peak for the levels of C3b-P generated by the lower amount of ICs was not observed (Figure 6-6).

C1s:C1-INH and C3b-P generation mirrored one another closely. Whilst binding peaked at 5 minutes and then dropped quickly for higher IC doses. A lower more substantial binding and release was observed for lower IC doses.

The levels of E's bound ICs correlated significantly with the levels of generated C1s:C1-INH and C3b-P at 5 minutes ($r=0.97$ $p<0.001$ and $r= 0.9$ $p<0.07$ respectively). The peak of E's bound ICs (5 minutes) occurred earlier than the peak of C3b-P (10 minutes) (Figures 6-5 and 6-6).

The levels of E's bound ICs and C1s:C1-INH, C3-P generated by highest amount of ICs (2096 μg) at 5 minutes was significantly higher than levels observed for the lowest amount of ICs (131 μg) ($P< 0.02$, 0.01 and 0.02 respectively).

Detecting bound C3c on erythrocytes during complement activation by FACscan. The binding of complement activation product C3c to erythrocytes generated during complement activation by rabbit streptokinase and thyroglobulin ICs was studied by flow cytometry, using FITC conjugated rabbit anti-human C3. The relationship between erythrocyte bound C3c and free complement activation products was investigated by studying the supernatants for the presence of the C1s:C1-INH and C3b-P.

A constant amount of streptokinase or thyroglobulin were incubated with NHS and erythrocytes at 37°C and the assay sampled at 0, 5, 10, 15 and 30 minutes. Over a period of 30 minutes C3c was detectable on erythrocytes as demonstrated by FACscan (Figure 6-7a). Peak binding for both ICs was at 5 minutes after which detectable C3c began to decrease and by 30 minutes a 34-36 % reduction in the levels of bound E's C3c was observed compared to 5 minutes (Figure 6-7a).

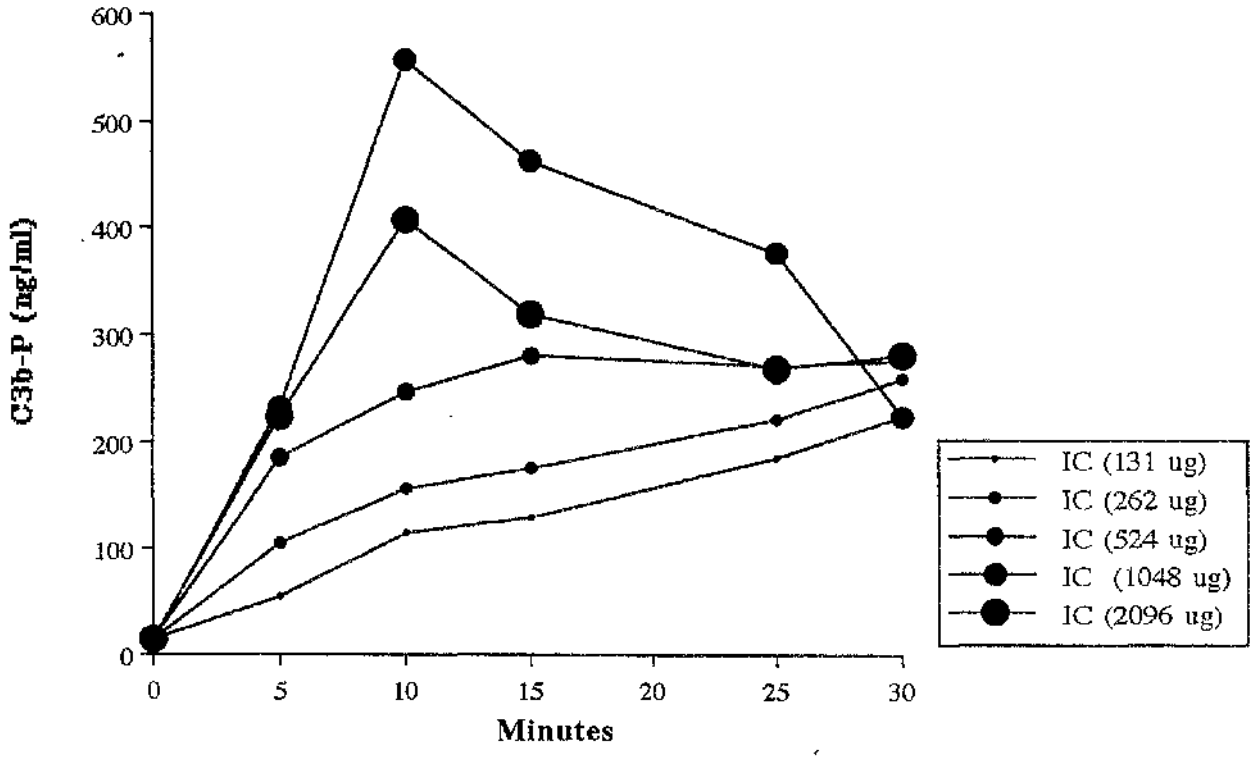


Figure 6-6: Dose response and kinetics of C3b-P generation by preformed rabbit streptokinase immune complexes at equivalence. The levels of C3b-P in the mixture of NHS+E's (Control) was 32ng/ml at 30 minutes.

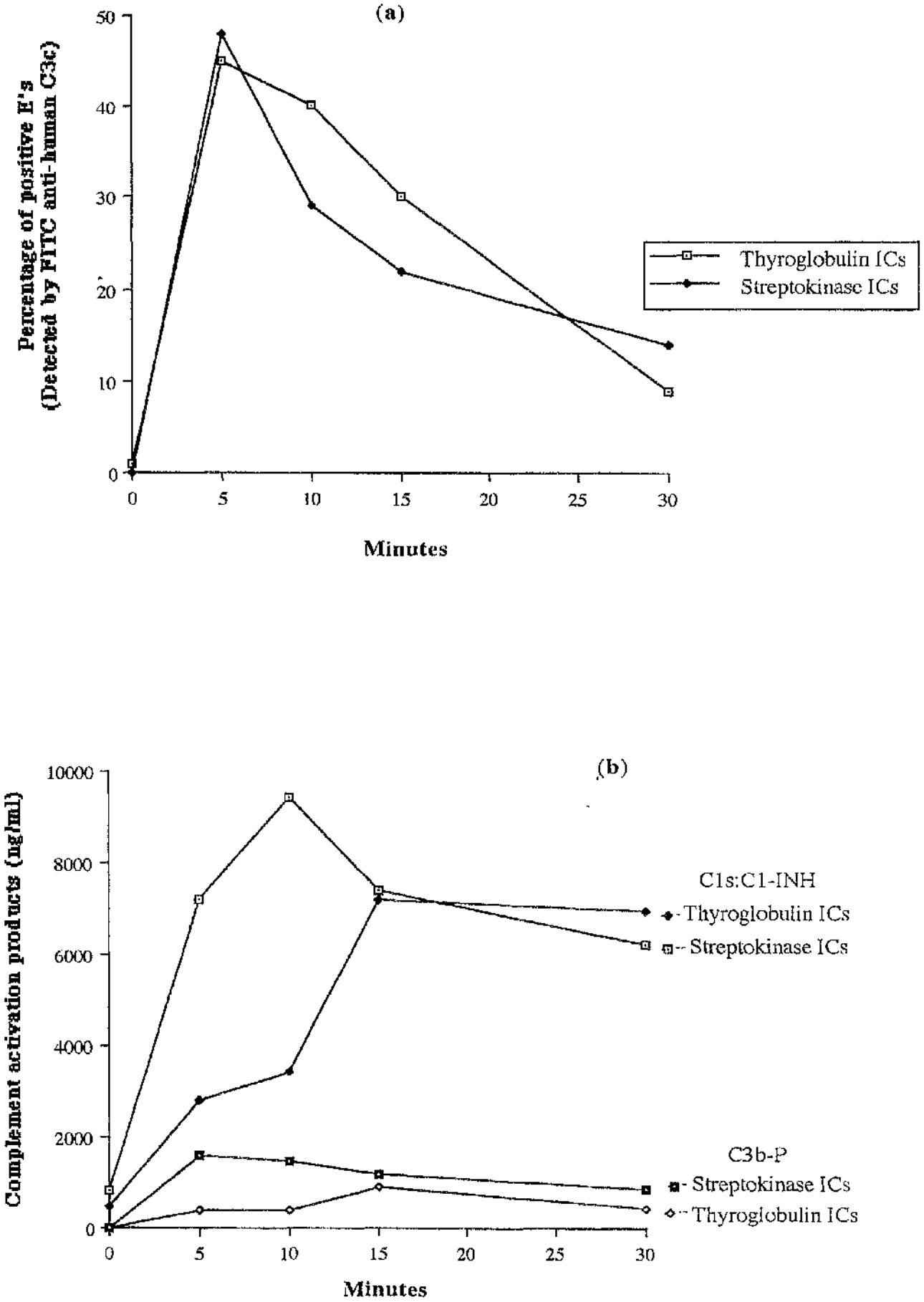


Figure 6-7: Kinetics of E's bound C3c (a) and C1s:C1-INH, C3b-P (b) by rabbit streptokinase and thyroglobulin ICs.

Over the period of 30 minutes the levels of C1s:C1-INH and C3b-P increased with both ICs (Figure 6-7b). The levels of C1s:C1-INH generated by streptokinase ICs reached a peak by 10 minutes and then began to decrease by 30 minutes (34% reduction compared to values at 10 minutes). The levels of C1s:C1-INH generated by thyroglobulin ICs reached a peak by 15 minutes and did not change significantly till 30 minutes (Figure 6-7b).

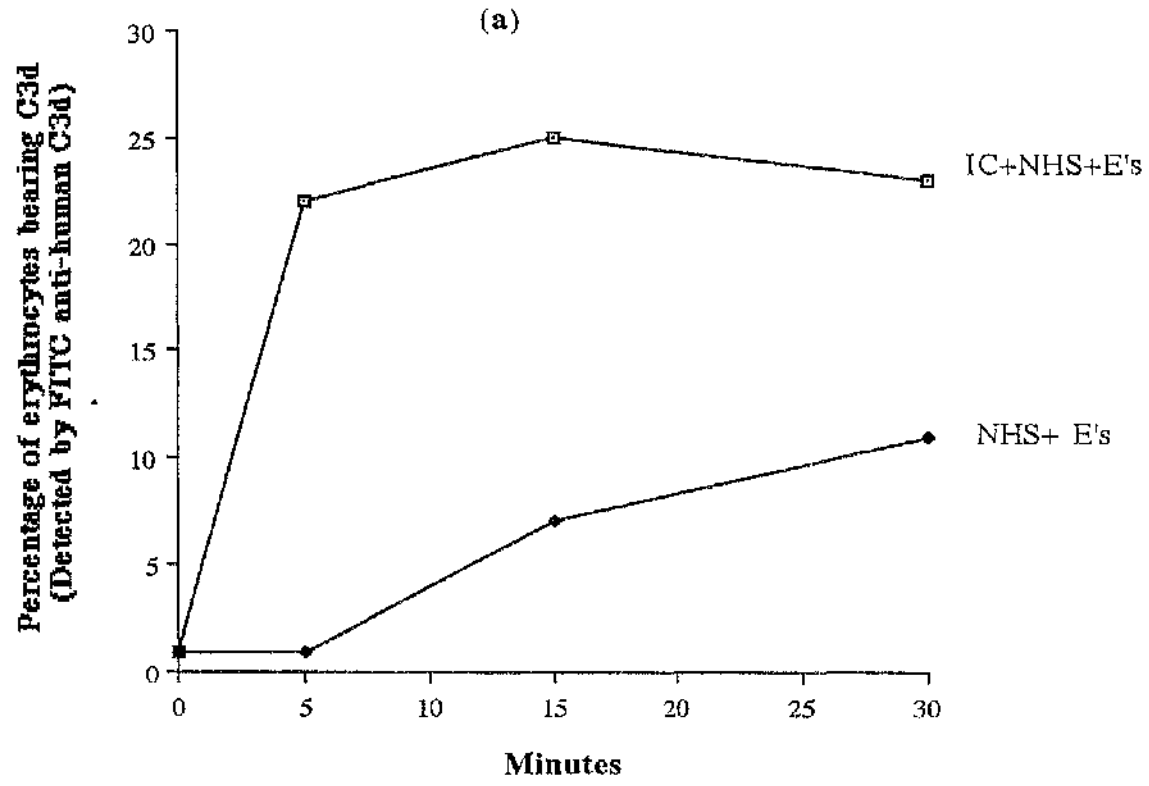
The levels of C3b-P generated by streptokinase ICs reached a peak by 5 minutes and for the thyroglobulin ICs reached to a peak by 15 minutes, the levels of C3b-P did not change significantly after their peaks (Figure 6-7b).

The levels of E's bound C3c correlated with the levels of C3b-P generated by rabbit streptokinase ICs ($r=0.97$, $p<0.001$) but did not correlate with the levels of C1s:C1-INH ($r=0.7$). The levels of E's bound C3c did not correlate with the levels of C1s:C1-INH and C3b-P generated by thyroglobulin ICs.

Detecting bound C3d on erythrocytes during complement activation by FACscan and modified ELISA. The binding of complement activation product C3d to erythrocytes generated during complement activation by preformed rabbit streptokinase immune complexes at equivalence was studied by flow cytometry using FITC conjugated rabbit anti-human C3d and modified ELISA (6-8). The relationship between erythrocyte bound C3d and free complement activation products was investigated by studying the supernatants for the presence of the C1s:C1-INH and C3b-P.

A constant amount of the ICs (1310 μg) was incubated with NHS and erythrocytes at 37°C and the assay sampled at 0, 5, 15 and 30 minutes. Over a period of 30 minutes C3d was detectable on erythrocytes as demonstrated by both FACscan and modified ELISA techniques compared with the controls (E's+NHS at 37°C) (Figure 6-8). Peak

C3d binding for both methods was at 15 minutes, in contrast to E's bound C3c (Figure 6-7a) the levels did not change significantly after reaching the peak (Figure 6-8). The levels of E bound C3d detected by the two methods correlated with one another significantly ($r=0.98$, $p<0.001$).Over the period of 30 minutes the levels of C1s:C1-INH and C3b-P increased in the presence of ICs compared to the controls (Figure 6-9). The levels of C1s:C1-INH and C3b-P increased to peak at 5 minutes and did not change significantly over the 30 minutes (Figure 6-9). The E's did influence the levels of C1s:C1-INH (Figure 6-9a). However, in absence of E's the levels of detectable C3b-P reached to peak at 5 minutes, this was 60% higher than in the presence of E's (Figure 6-9b). Across the time course, levels of C3b-P were greater in absence of E's (34% and 40% higher at 15 minutes and 30 minutes respectively) (Figure 6-9b).



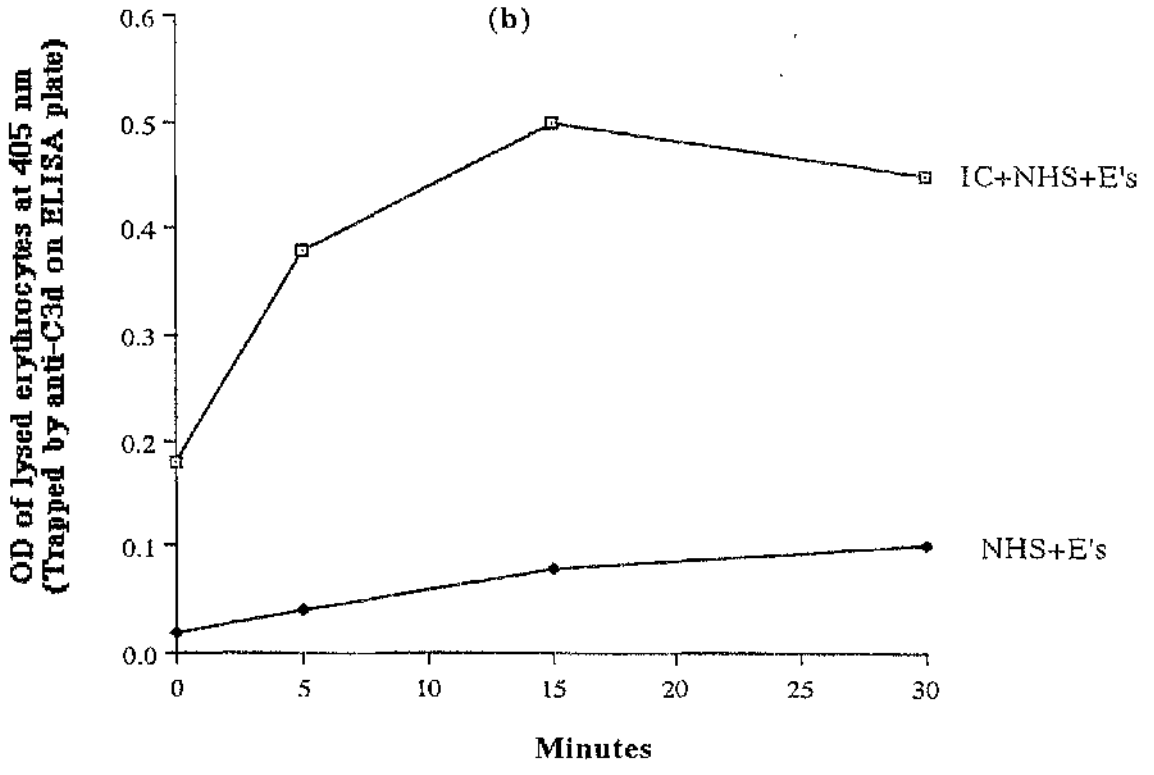


Figure 6-8: Kinetics of erythrocyte bound C3d generated by preformed rabbit streptokinase immune complexes at equivalence. Detected by flow cytometry (a) and modified ELISA (b).

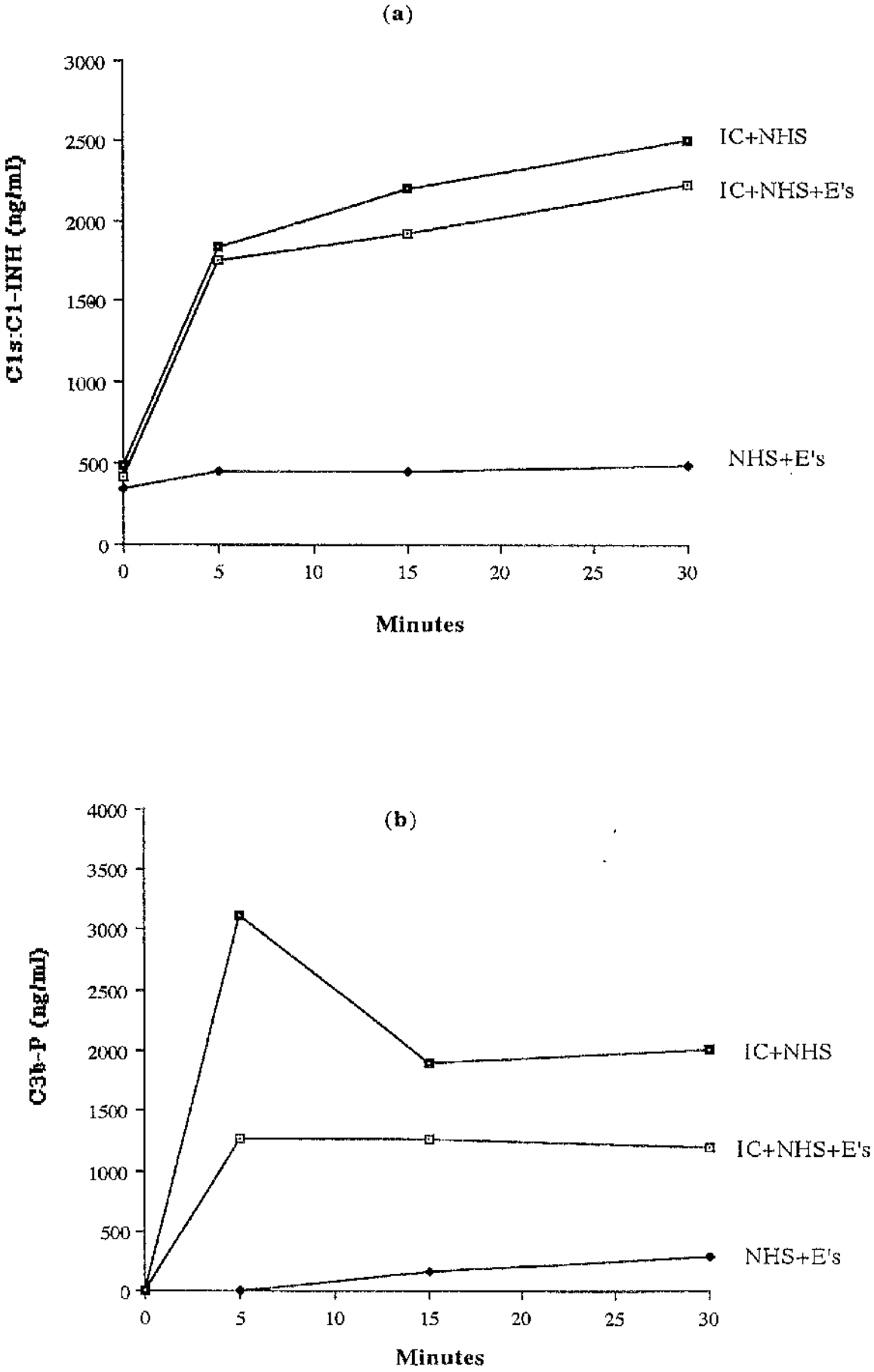


Figure 6-9: Kinetics of C1s:C1-INH (a) and C3b-P (b) by preformed rabbit streptokinase immune complexes at equivalence in presence and absence of erythrocytes (E's).

The levels of E bound C3d as measured by both techniques did not correlate with the levels of C1s:C1-INH and C3b-P generated by the ICs.

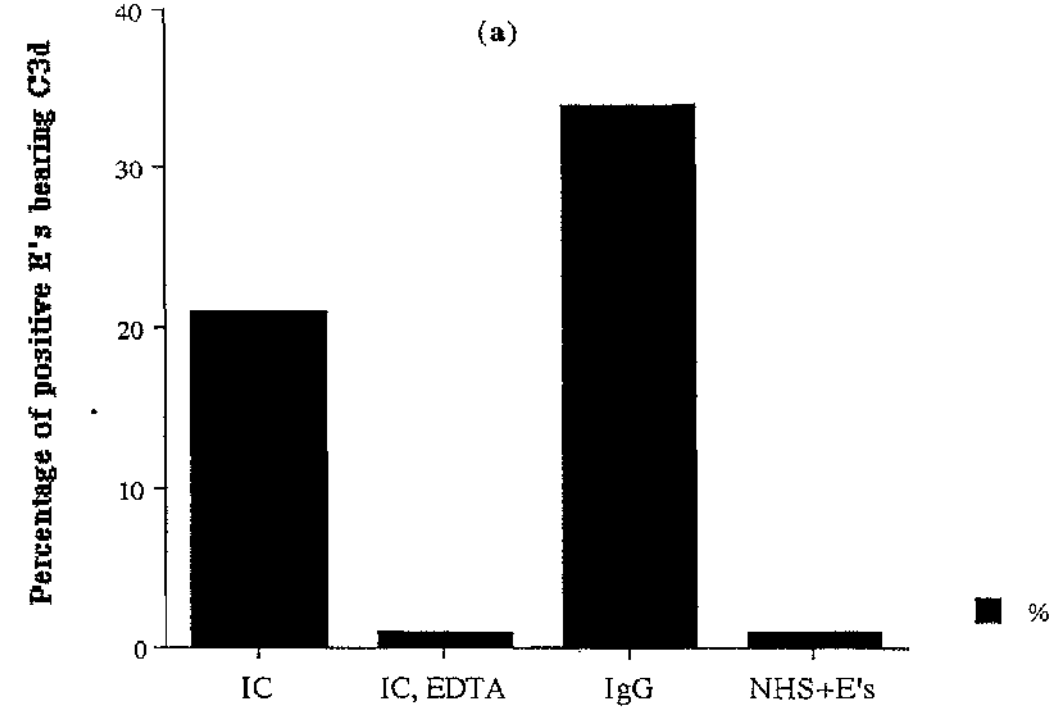
Controls: 1) NHS +E's was (Total volume 750 μL) at 37°C was used as negative control. The low levels of E's bound C3d by both techniques reflected a trace spontaneous complement activation (Figure 6-8).

2) The effect of EDTA on erythrocyte bound C3d and free C1s:C1-INH, C3b-P and C3d was studied by incubating 1310 μg preformed rabbit streptokinase ICs with NHS or NHS treated with isotonic 0.086M EDTA and erythrocytes at 37°C for 10 minutes (6-8). EDTA decreased the levels of E's bound C3d, detected by both techniques, C1s:C1-INH, C3b-P and free C3d (Figures 6-10 and 6-11).

The presence of erythrocytes had no effect on the levels of C1s:C1-INH and free C3d, however a 54 % reduction in the levels of C3b-P was observed at 10 minutes (Figure 6-11).

3) Rabbit IgG anti-streptokinase (1300 μg) and generated E bound C3d and free C1s:C1-INH, C3b-P and C3d at 10 minutes to a lower extent compared to the ICs (Figures 6-10 and 6-11).

Specificity of monoclonal anti-CR1 for human erythrocytes. The specificity of the mouse monoclonal anti-CR1 IgG for erythrocytes was examined by incubating increasing amounts of the antibody from 0.138 μg to 4.4 μg with a constant number of erythrocytes ($2X10^8$ E's) (6-9). Non-specific binding was determined by incubating 10μg monoclonal mouse anti-huma AFP IgG with $2X10^8$ E's or conjugated sheep anti-mouse IgG alone. Thr E's were then analysed by FACscan using FITC conjugated sheep anti-mouse IgG (6-9).



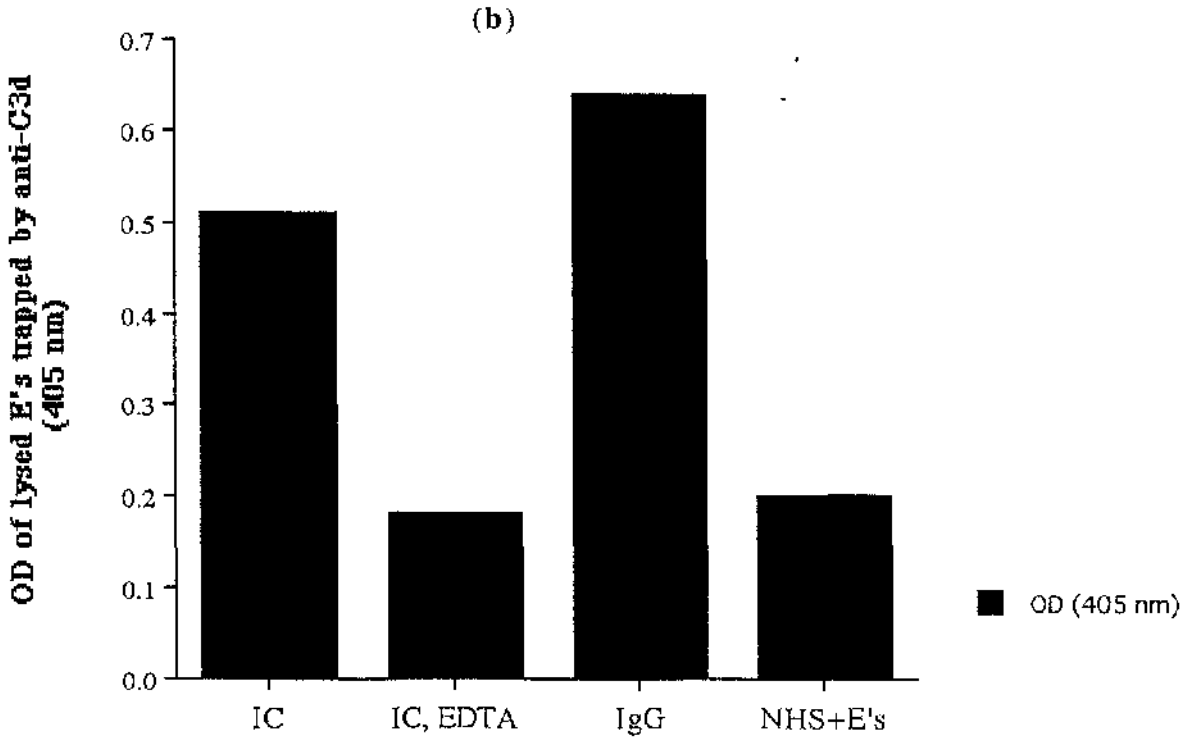
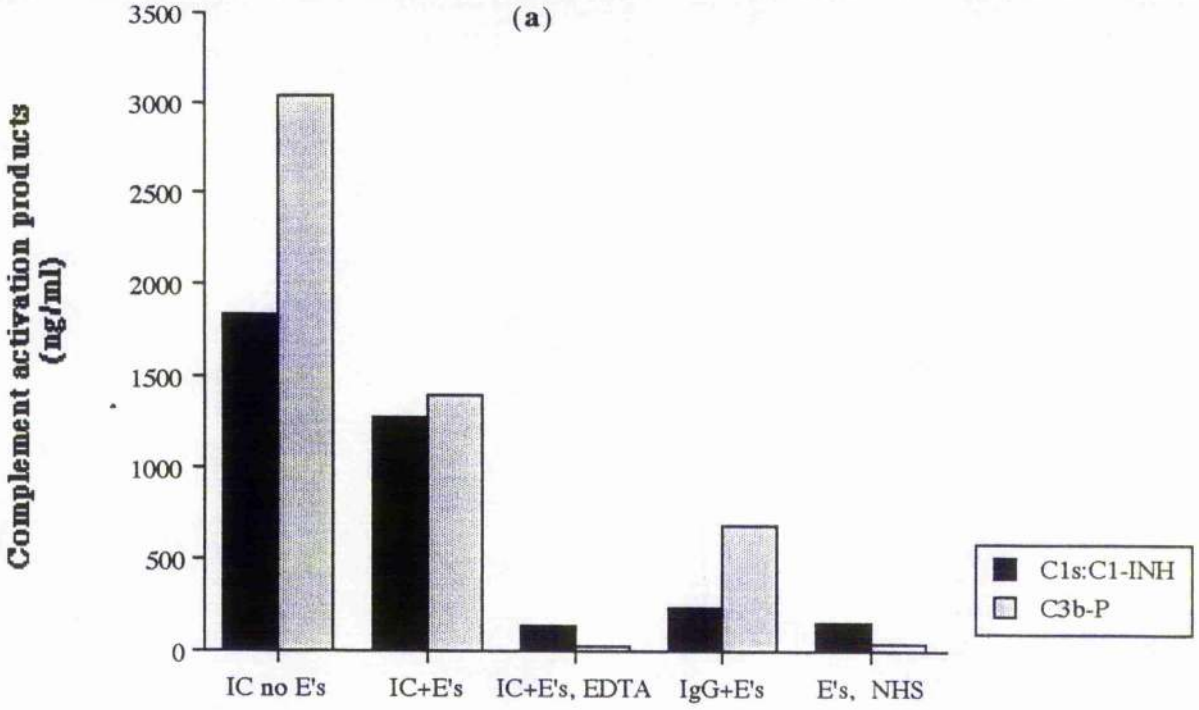


Figure 6-10: The levels of erythrocyte (E's) bound C3d generated by preformed rabbit streptokinase immune complexes at 10 minutes at 37oC. Detected by flow cytometry (a) and modified ELISA (b).



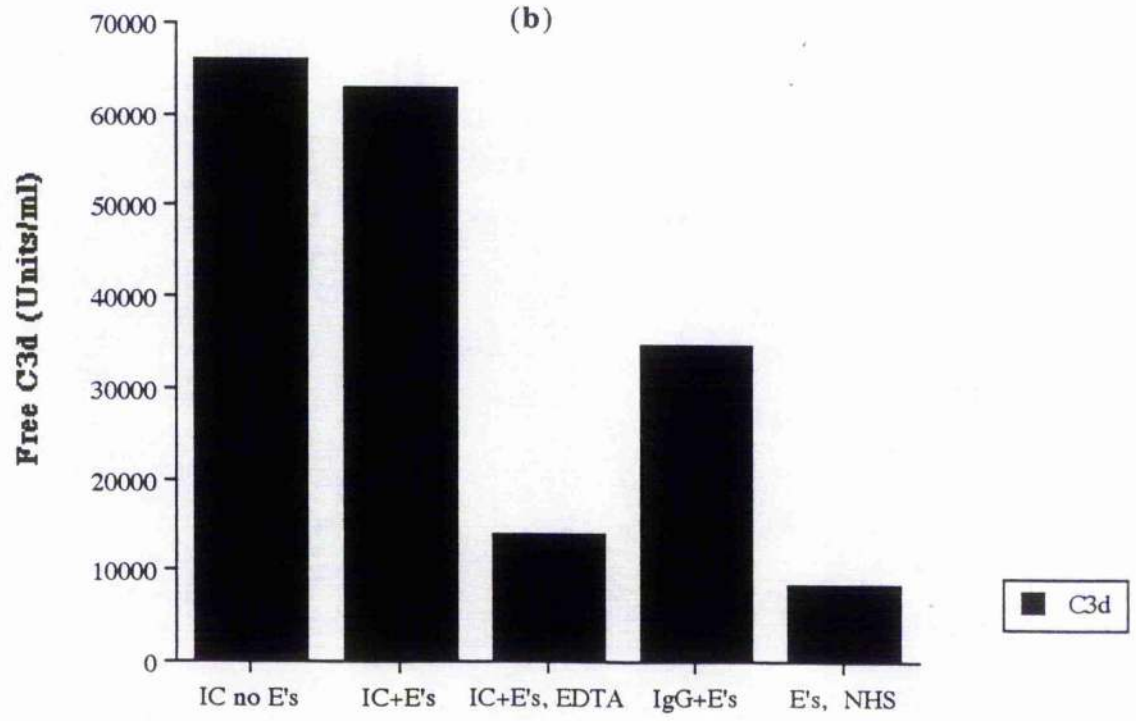


Figure 6-11: The levels of free complement activation products generated by preformed rabbit streptokinase at equivalence by 10 minutes at 37oC.

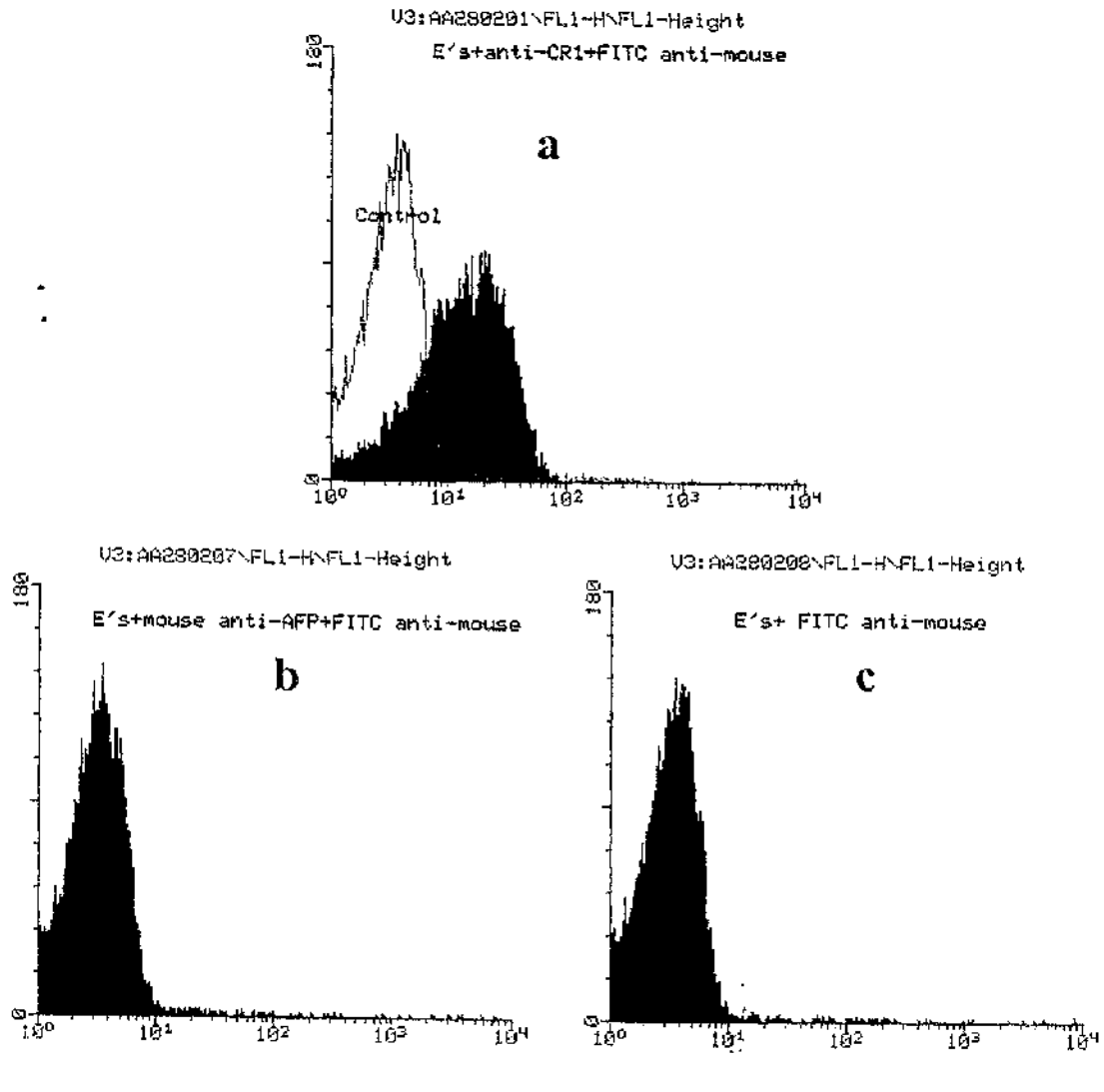


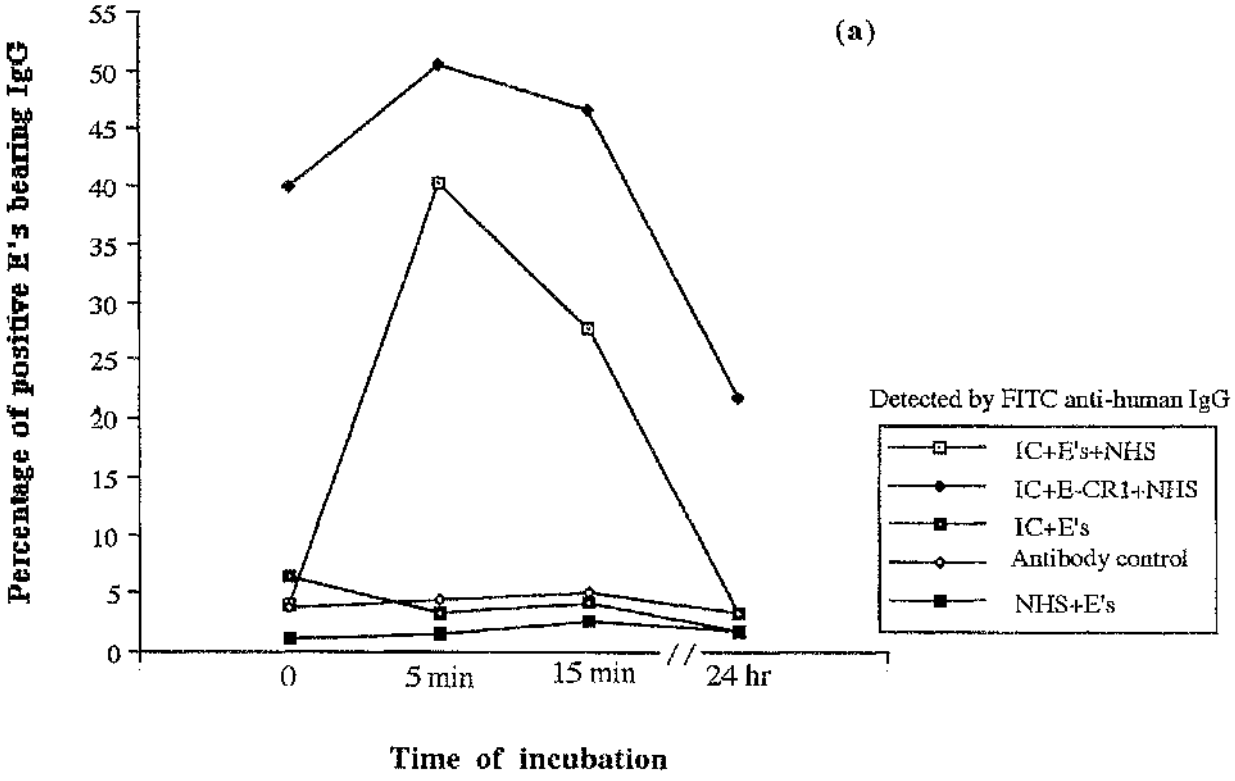
Figure 6-12: Histograms of fluorescence intensity for human erythrocytes incubated with monoclonal mouse anti-human CR1 (a). Mouse monoclonal anti-human AFP (b) and FITC sheep anti-mouse IgG alone (c) were used to analyse non-specific binding. Y axis displays the number of erythrocytes for each channel and X axis is a log scale of fluorescence intensity.

Mouse monoclonal anti-CR1 was detectable on 81% of the erythrocytes compared with the controls (Figure 6-12). Increasing the concentration of the anti-CR1 antibody did not cause any significant change in the levels of detectable anti-CR1 antibodies on the E's. Monoclonal mouse anti-human AFP IgG and FITC sheep anti-mouse alone were not detectable on the erythrocytes (Figure 6-12).

Kinetics of human streptokinase immune complexes binding to erythrocytes and the effect of monoclonal anti-CR1 on binding and generation of complement activation products. Preformed human anti-streptokinase immune complexes prepared from the serum of a patient with elevated levels of anti-streptokinase antibodies were used in this experiment (6-10). As the levels of anti-streptokinase antibodies in this serum were high (IgG > 90 units/ml, IgA> 1000 units /ml, IgM=34 units/ml, ASK=1280 units/ml), it provides a good model of human streptokinase immune complexes when combined with streptokinase. Flow cytometry was used to study the kinetics of human immune complexes binding and C3d to erythrocytes over a period of 15 minutes at 37°C and 24 hours at room temperature (6-10). The relationship between bound immune complexes, C3d and the free generated complement activation products was studied by measuring C1s:C1-INH, C3b-P and free C3d in the supernatants. The effects of anti-CR1 on the out come of this experiment was also studied (6-10).

The levels of bound human streptokinase immune complexes detected by FITC conjugated anti-human IgG and FACscan analysis increased and reached a peak at 5 minutes, after which the levels began to decrease and at 24 hours were not detectable on the erythrocytes (Figure 6-13a). In the presence anti-CR1 the levels of detectable surface IgG as detected by FITC conjugated anti-human IgG showed an 36%, 10% and

19% increase at 0, 5 and 15 minutes compared with the absence of anti-CR1 (Figures 6-13a). At 24 hours the binding of IgG was still elevated at 22% (Figure 6-13a).



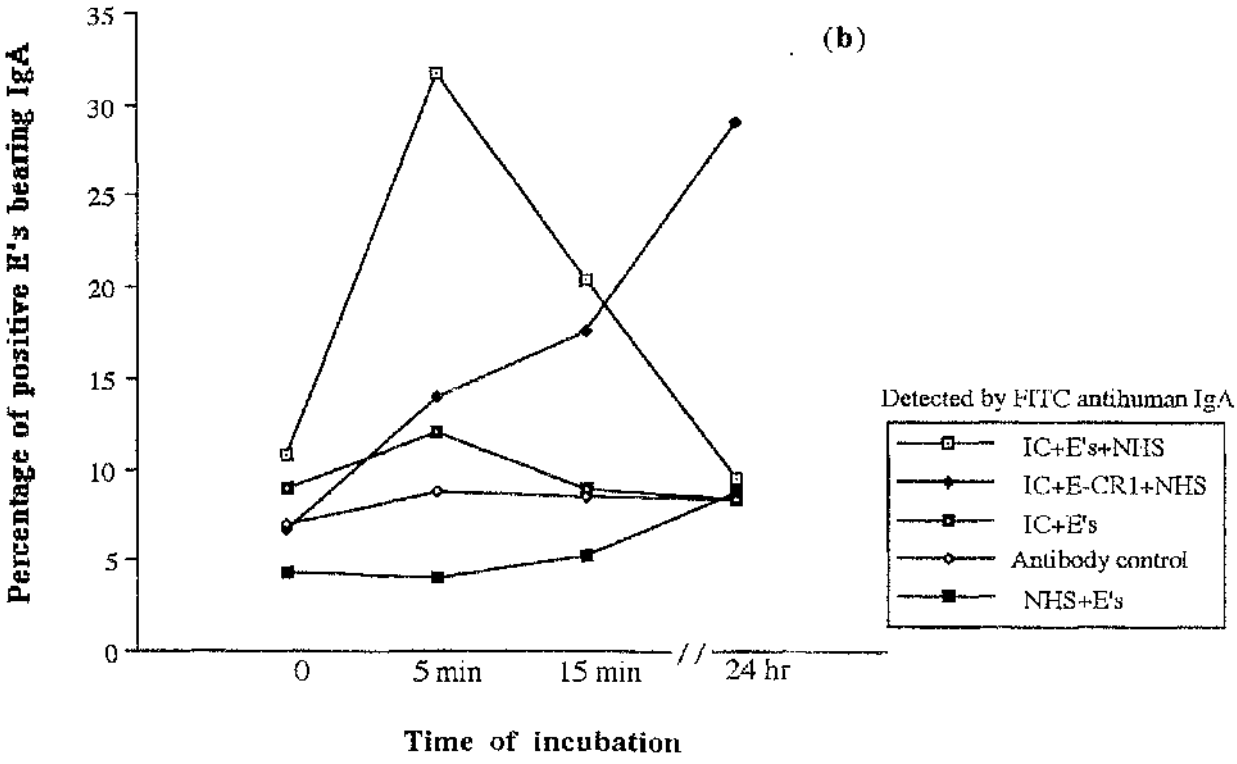
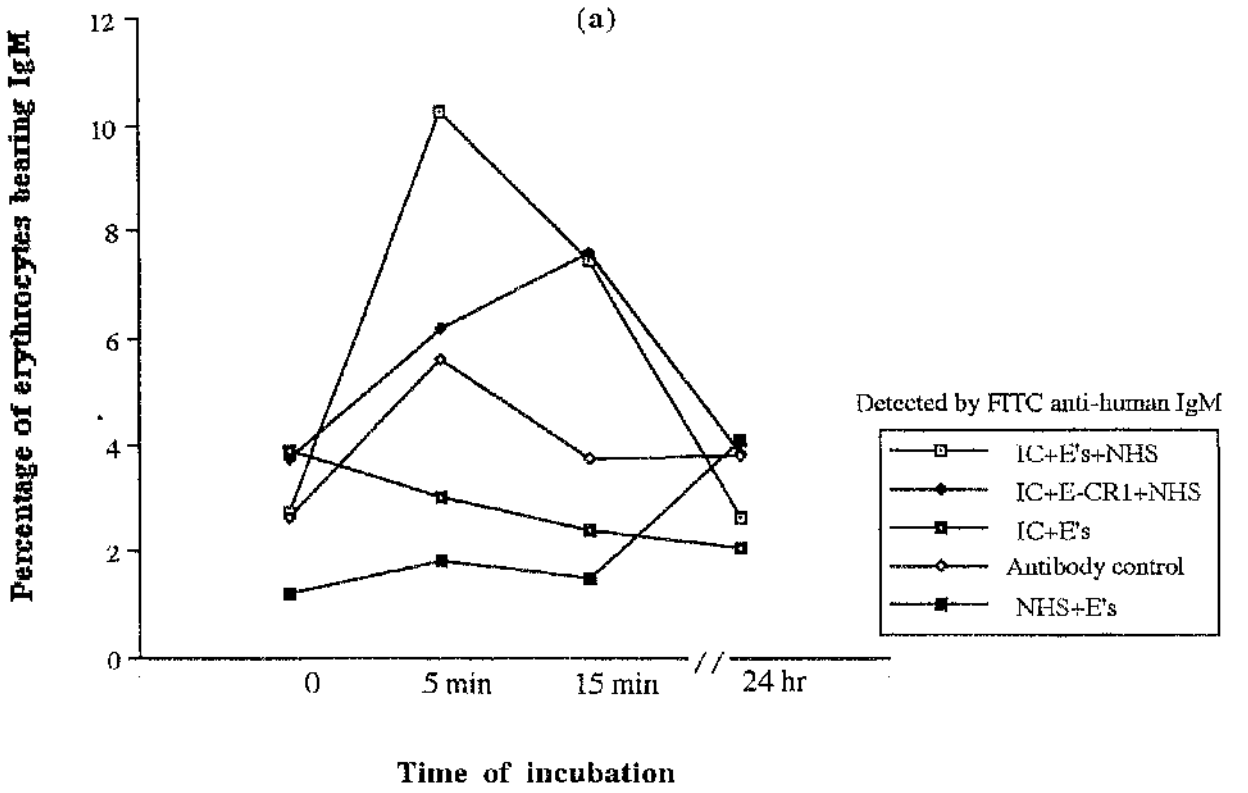


Figure 6-13: Kinetics of erythrocytes (E's) bound human IgG (a) and IgA (b) incorporated in streptokinase immune complexes (ICs) detected by FACscan.



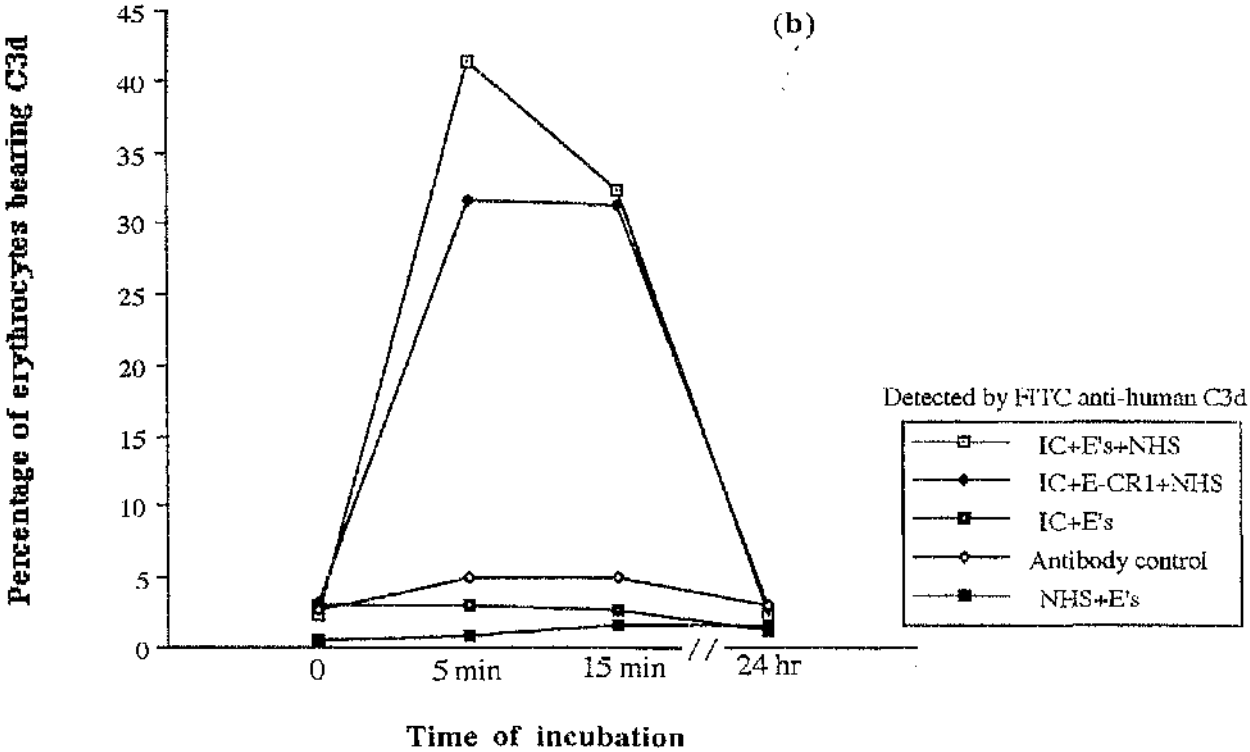
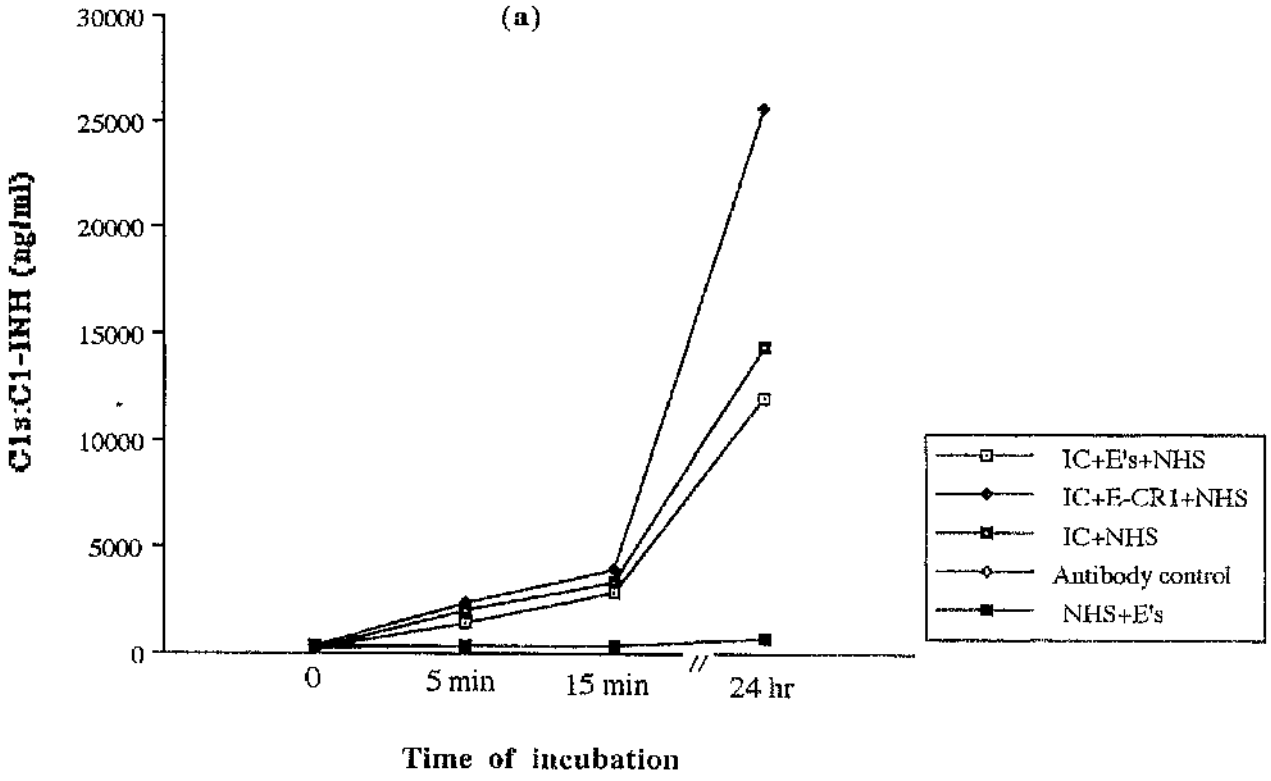


Figure 6-14: Kinetics of erythrocyte (E's) bound IgM incorporated in the streptokinase immune complexes (a) and E's bound C3d generated by human streptokinase ICs (b), detected by FACscan.



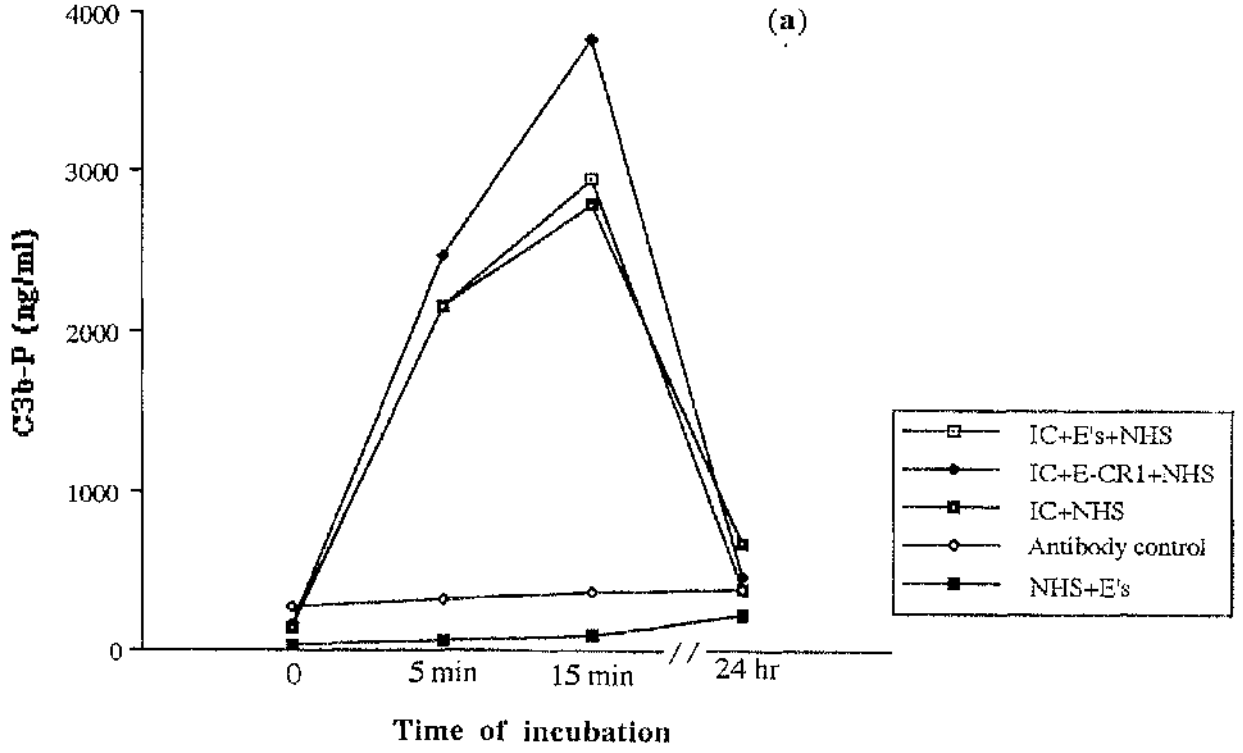


Figure 6-15: Kinetics of C1s:C1-INH (a) and C3b-P (b) generated by human streptokinase immune complexes in presence and absence of naive E's or E's treated with anti-CR1(E-CR1) .

The levels of bound human streptokinase immune complexes detected by FITC conjugated anti-human IgA increased and reached a peak at 5 minutes, after which the levels decreased and at 24 hours were not detectable on the erythrocytes (Figures 6-13b). In contrast to IgG in the presence anti-CR1 the levels of detectable surface IgA as detected by FITC conjugated anti-human IgA showed 4%, 18% and 3% reduction at 0, 5 and 15 minutes compared with the absence of anti-CR1 (Figure 6-13b). At 24 hours the binding of IgA was still detectable at 29 % (Figures 6-13b).

The levels of bound human streptokinase immune complexes detected by FITC conjugated anti-human IgM were lower compared to IgG and IgA (Figure 6-14a), the levels increased and reached a peak at 5 minutes, after which they decreased to undetectable at 24 hours (Figures 6-14a). In the presence anti-CR1 the levels of detectable surface IgM as detected by FITC conjugated anti-human IgM showed a 4% reduction at 5 minutes compared to in the absence of anti-CR1 (Figures 6-14a). The levels of immune complexes (IgM) were not detectable at 24 hours (Figures 6-14a).

The levels of E bound C3d, generated during complement activation by human streptokinase immune complexes detected by FITC conjugated anti-human C3d reached a peak at 5 minutes, after which the levels decreased slightly and at 24 hours were not detectable on the erythrocytes (Figures 6-14b). The presence of anti-CR1 did not influence the detectable levels of E's bound C3d (Figures 6-14b).

The complement activation products C1s:C1-INH, C3-b-P and free C3d were measured in accompanying supernatants. The levels of C1s:C1-INH generated by human streptokinase ICs rose up to 15 minutes, 10 minutes later than peak observed for erythrocyte binding of C3d, IgG and IgA on E's (Figures 6-15a). The levels of C1s:C1-INH at 24 hours were striking in their degree of elevation (12 μg/ml) (Figures 6-15a). The presence of E's did not influence the levels of C1s:C1-INH significantly (Figures 6-15a). The presence of anti-CR1(E-CR1) increased the levels of C1s:C1-INH compared to controls (Figures 6-15a). The levels of C1s:C1-INH generated by the

immune complexes in presence of E-CR1 and absence of erythrocytes at 24 hours were also striking in their degree of elevation (25.6 μg/ml and 14.4 μg/ml respectively) (Figures 6-15a). The presence of anti-CR1 generated almost two fold higher levels of C1s:C1-INH (25.6 μg/ml) as compared to the ICs in the presence of E's (12 μg/ml) or the absence of E's (14.4 μg/ml) (Figures 6-15a). The levels of C1s:C1-INH in the positive serum alone (contained anti-streptokinase antibodies) and NHS+E's (controls) were increased by 50% at 24 hours (0.720 μg/ml and 0.728 μg/ml respectively) which were still significantly lower than then levels of the ICs in all time points (Figures 6-15a).

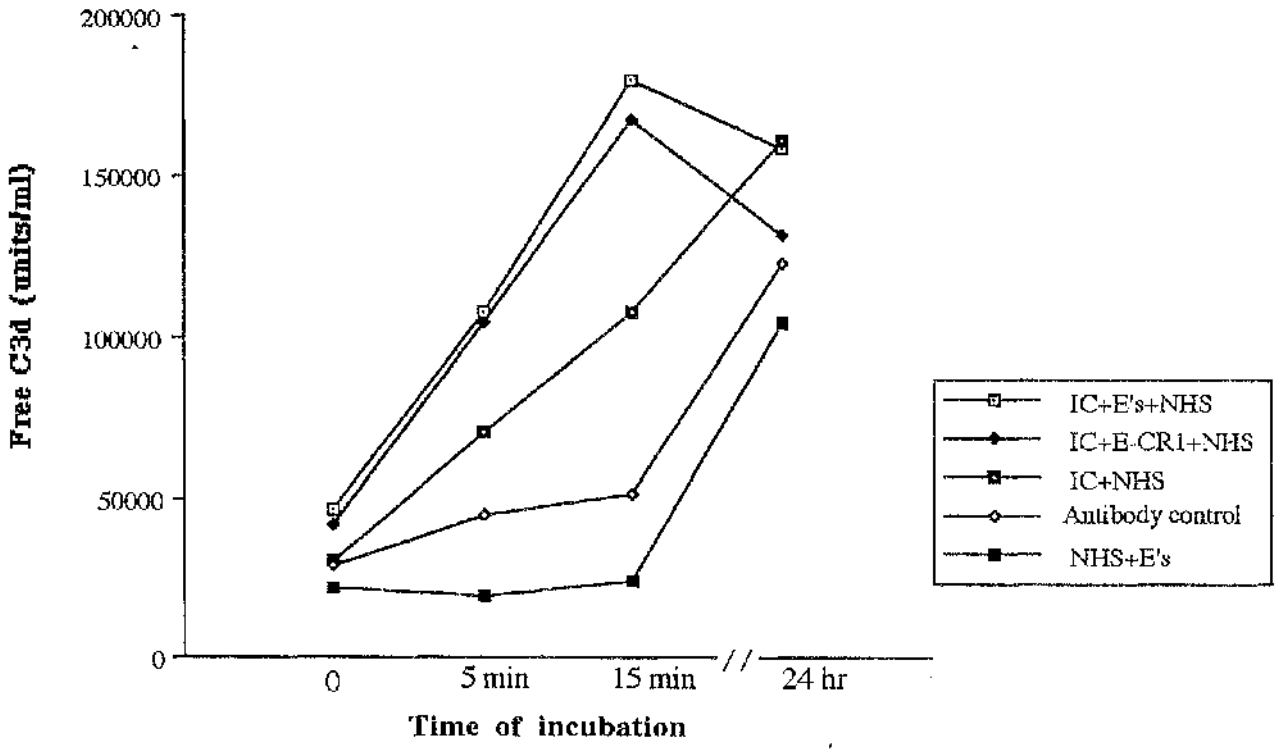


Figure 6-16: Kinetics of free C3d generated by human streptokinase immune complexes in presence and absence of naive E's or E's treated with anti-CR1 (E-CR1).

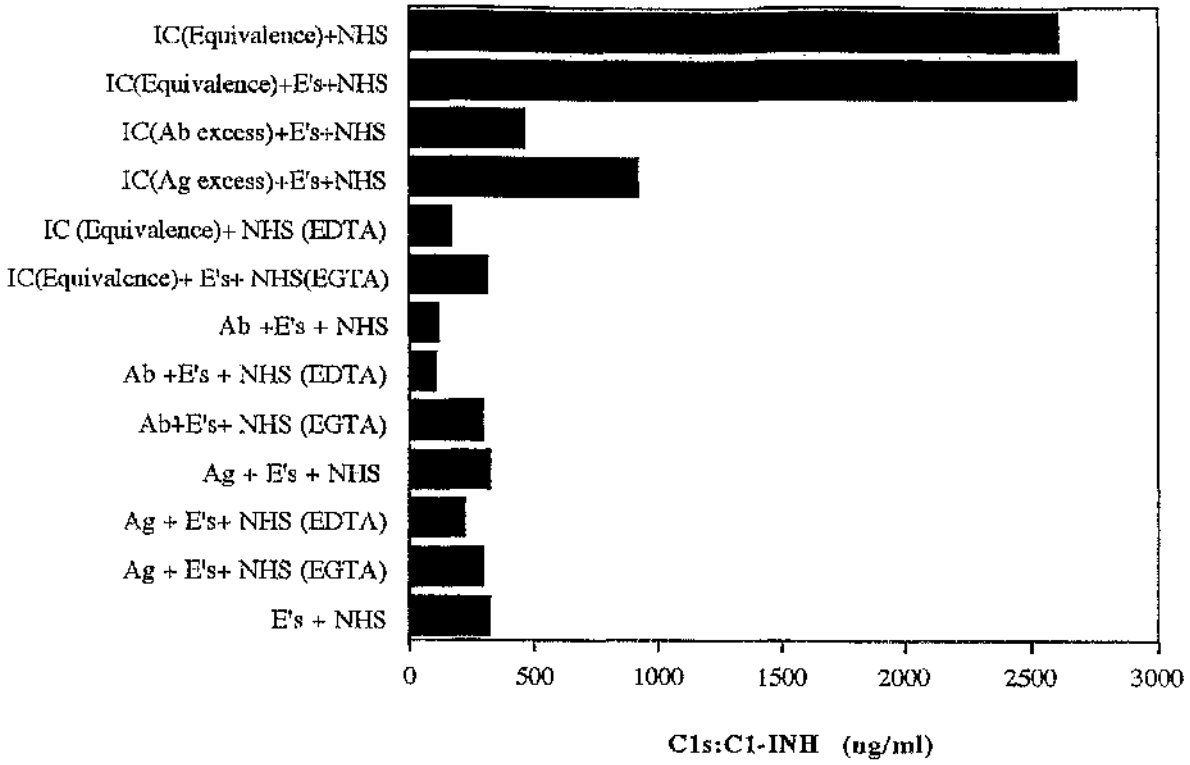
	Bound C3d FACscan	Bound C3d ELISA	C1s:C1-INH	C3b-P	Free C3d
Bound IC	r=0.94 (p<0.001)	0.62 (p<0.03)	r=0.66 (p<0.01)	r=0.80 (p<0.002)	r=0.67 (p<0.01)
Bound C3d FACscan		0.67 (p<0.01)	r=0.68 (p<0.01)	r=0.86 (p<0.001)	r=0.60 (p<0.03)
Bound C3d ELISA			r=0.62 (p<0.02)	r=0.67 (p<0.01)	r=0.63 (p<0.02)
C1s:C1-INH				r=0.83 (p<0.001)	r=0.83 (p<0.001)
C3b-P					r=0.83 (p<0.001)

Table 6-1: Correlation between the levels of erythrocyte (E's) bound immune complexes, E's bound C3d and free complement activation products, C1s:C1-INH,C3b-P, free C3d generated by preformed human streptokinase immune complexes at equivalence.

The levels of C3-b-P generated by human streptokinase immune complexes reached a peak at 15 minutes, 10 minutes later than peak binding C3d and ICs on E's (Figures 6-15b). In contrast to C1s:C1-INH, the levels of C3-b-P at 24 hours were not detectable (Figures 6-15b). The presence of erythrocytes did not significantly influence the levels of C3b-P generated by human streptokinase immune complexes (Figures 6-15b), this was in contrast with the previous experiment (6-8) were a 54 % reduction in the detectable levels of C3b-P generated by rabbit streptokinase ICs (Contained only IgG) was observed in the presence of erythrocytes (Figure 6-11). The presence of anti-CR1 increased the levels of C3-b-P by 23% at 15 minutes (Figures 6-15b). The levels of C3b-P generated by the immune complexes in presence of E-CR1 by 24 hours were also not detectable (Figures 6-15b).

The levels of free C3d generated by human anti-streptokinase immune complexes reached a peak at 15 minutes, 10 minutes later than peak observed for E's binding of C3d and ICs (Figures 6-16). The levels of free C3d by 24 hours were detectable and only slightly decreased (10%) compared to levels at 15 minutes (Figures 6-16). The presence of erythrocytes (Untreated E's and E-CR1) increased the levels of free C3d generated by the ICs at 15 minutes (40%) compared with levels in the absence of E's (Figure 6-16). The levels of free C3d in positive serum (antibody control) and NHS + erythrocytes (controls) were increased by 24 hours and reached to levels of ICs at 24 hours (Figure 6-16).

(a)



(b)

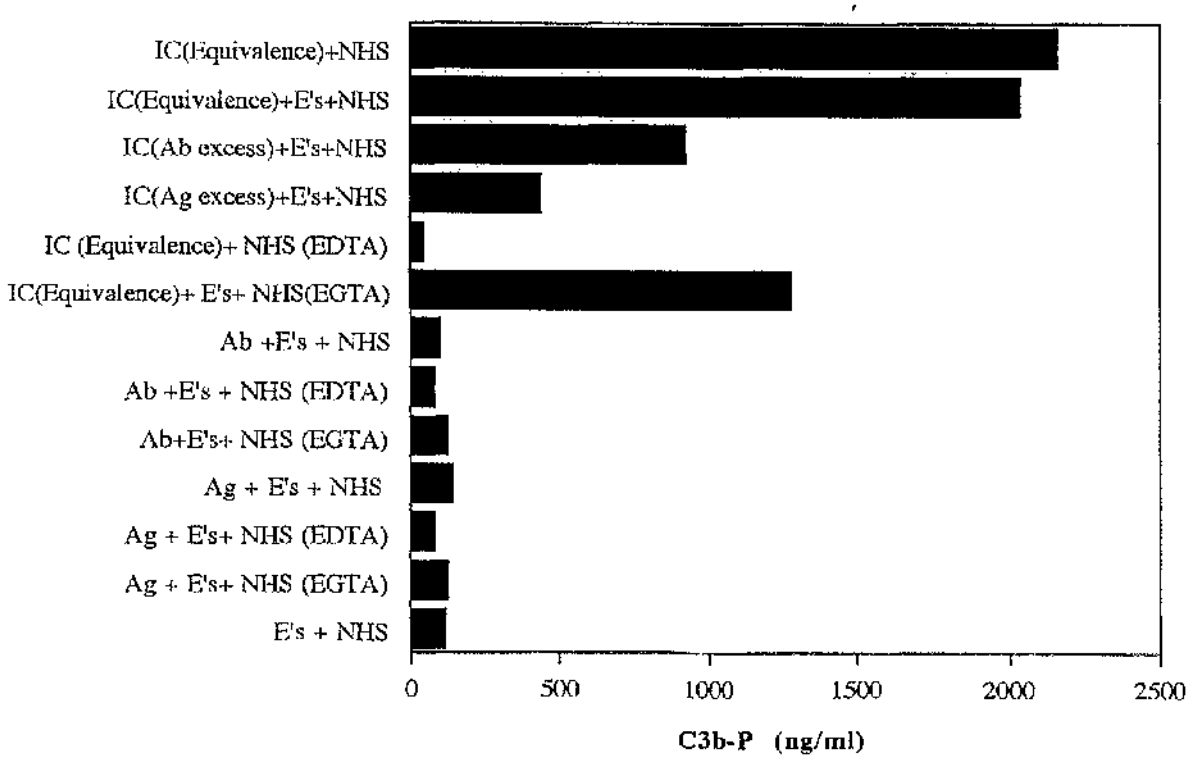


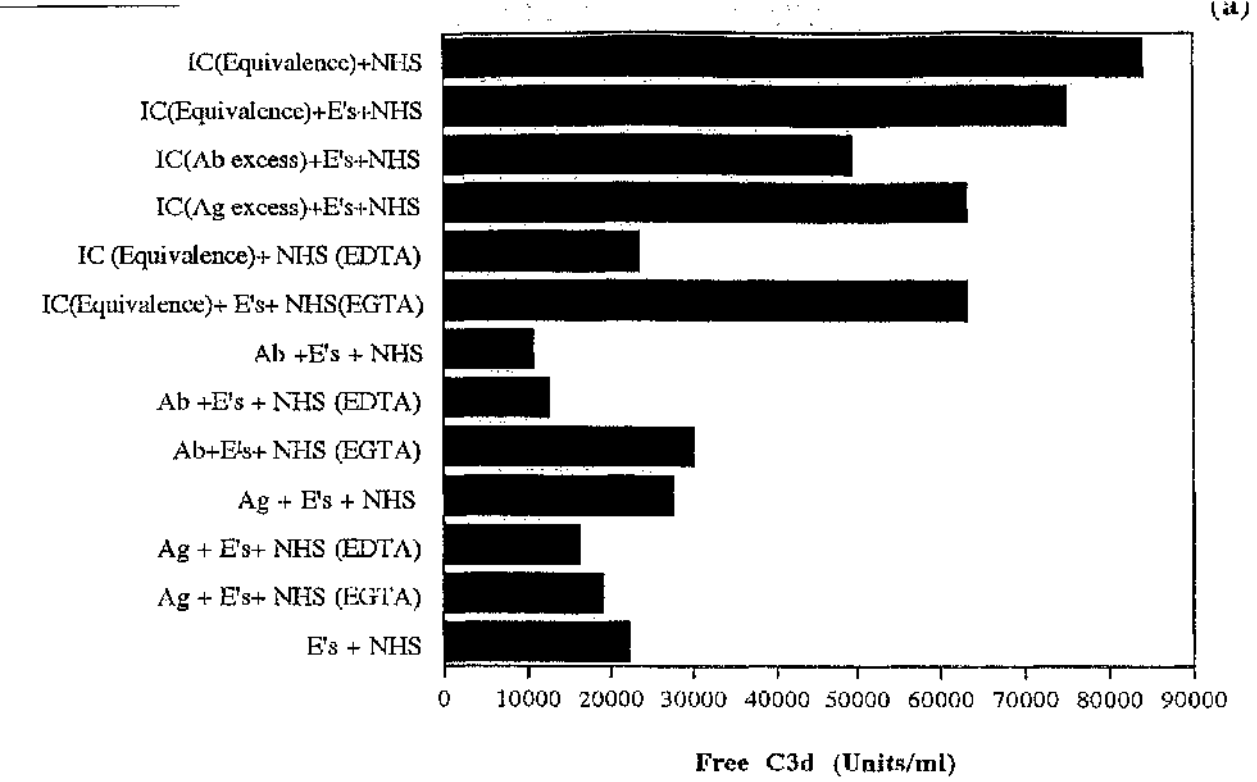
Figure 6-17: The levels of C1s:C1-INH (a) and C3b-P (b) generated by preformed human streptokinase immune complexes at equivalence, 4 times antigen (Ag) excess or 4 times antibody (Ab) excess at 5 minutes incubation at 37oC.

The levels of erythrocyte bound IgG, IgA, IgM and C3d as measured by FACscan did not correlate with the levels of free complement activation products C1s:C1-INH, C3b-P and C3d. The levels of E's bound IgG, IgA, IgM and C3d significantly correlated with one another ($r= 0.99$, $p<0.001$). The levels of free complement activation products C1s:C1-INH, C3b-P and C3d were also significantly correlated with one another ($r= 0.99$, $p<0.001$).

Effects of antigen and antibody excess on human streptokinase immune complexes binding to erythrocytes. Human anti-streptokinase IgG was purified from the sera of patients with elevated levels of anti-streptokinase antibodies by ion exchange chromatography (5-1) and preformed immune complexes were prepared (5-2). Binding of human streptokinase immune complexes to erythrocytes after 5 minutes incubation at 37°C (Time point chosen based on previous experiment), at equivalence, antigen excess and antibody excess to erythrocytes was studied by flow cytometry using FITC conjugated sheep anti-human IgG to find whether the binding is influenced by the antigen/antibody ratio (6-11). The levels of E's bound C3d was studied by flow cytometry using FITC conjugated rabbit anti-human C3d and modified ELISA (5-5). The relationship between bound immune complexes, bound C3d and free complement activation products was studied by measuring C1s:C1-INH, C3b-P and free C3d in the supernatants (5-3). The levels of complement activation products and binding of the ICs at equivalence to erythrocytes in presence of NHS treated with EDTA or EGTA was used as control (6-11).

Human streptokinase immune complexes at equivalence generated higher levels of C1s:C1-INH (2680 ng/ml) compared to antibody excess (464 ng/ml) and antigen excess (920 ng/ml) (Figure 6-17a). EDTA and EGTA completely inhibited the

generation of C1s:C1-INH and the presence of erythrocytes did not cause any significant effect on the levels of C1s:C1-INH (Figure 6-17a).



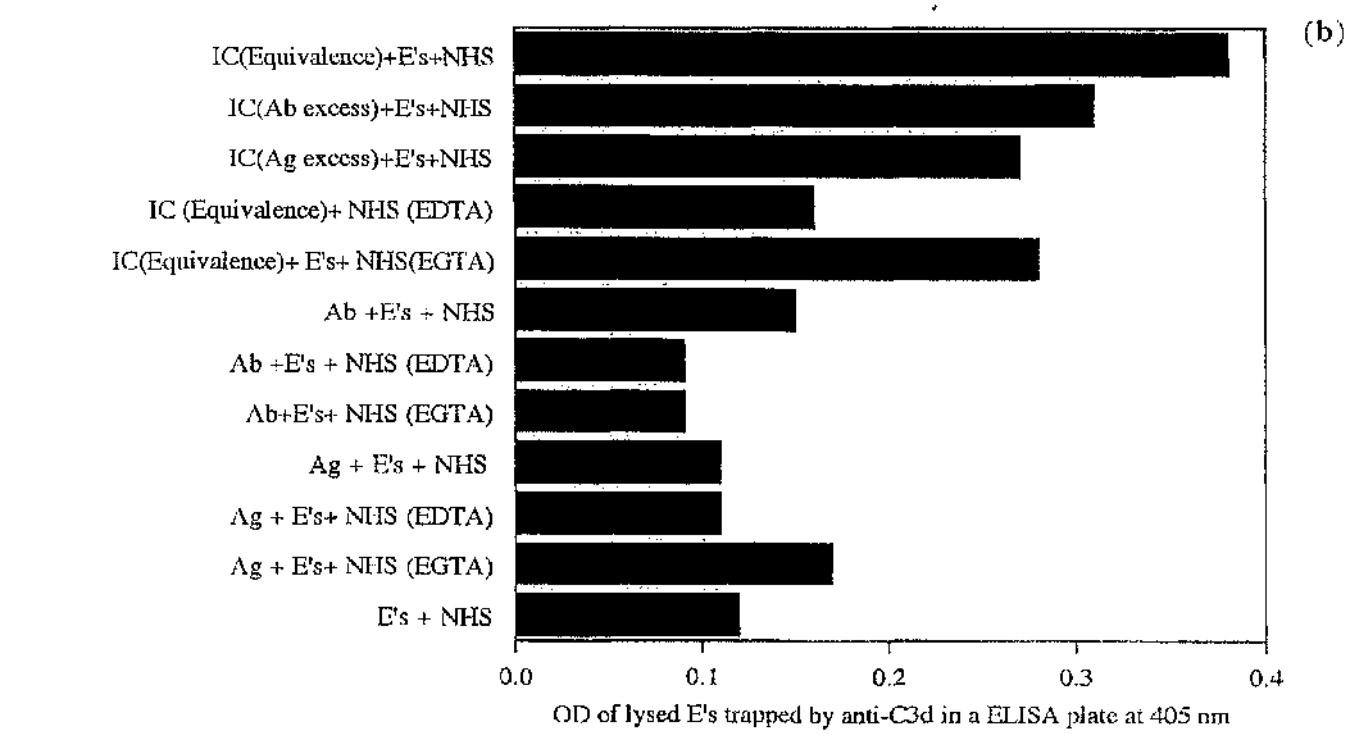


Figure 6-18: The levels of (a) free C3d and (b) erythrocyte (E's) bound C3d (b) generated by preformed human streptokinase ICs at equivalence, 4 times antibody (Ab)excess and 4 times antigen (Ag) excess by 5 minutes incubation at 37oC.

The ICs at equivalence generated higher levels of C3b-P (2040 ng/ml)compared to antibody excess (920 ng/ml) and antigen excess (432 ng/ml) (Figure 6-17b). EDTA completely inhibited the generation of C3b-P, however, EGTA only partially decreased the levels of generated C3b-P (Figure 6-17b). The presence of erythrocytes did not cause any significant effect on the levels of C3b-P (Figure 6-17b).

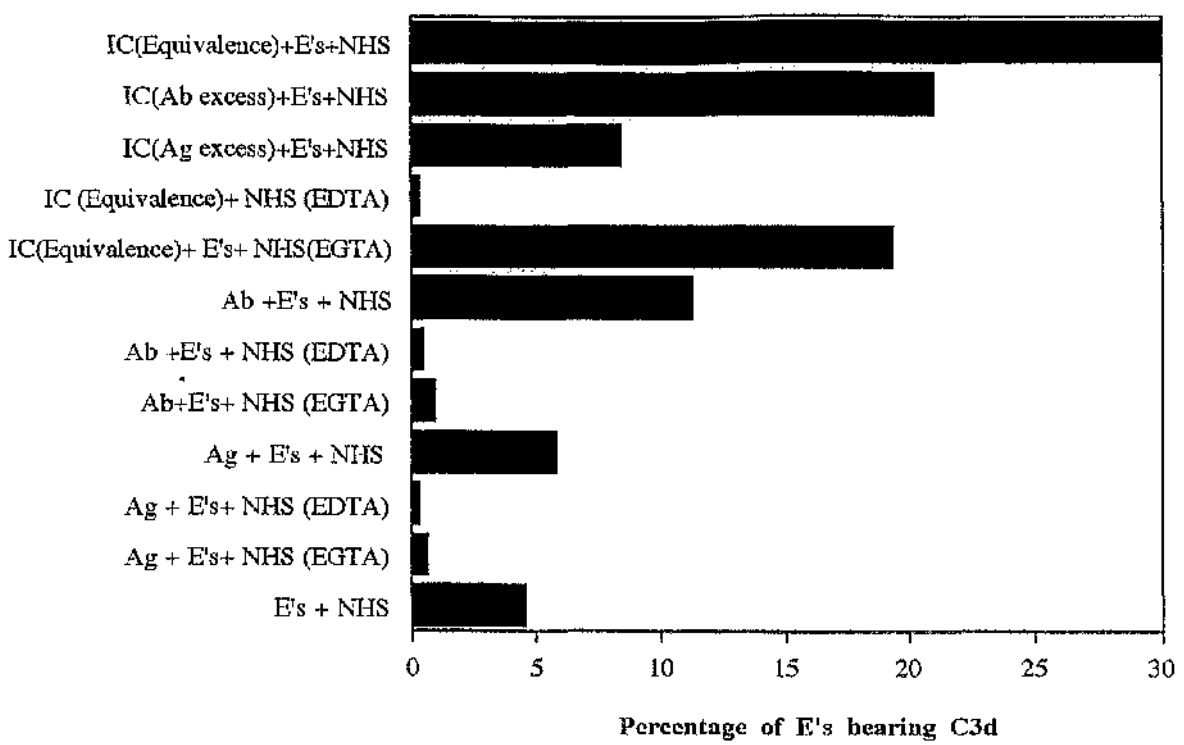
Immune complexes at equivalence generated higher levels of free C3d compared (75000 units/ml) to antibody excess (31200 units/ml) and antigen excess (63000 units/ml) (Figure 6-18a). Like C1s:C1-INH, immune complexes at antigen excess generated higher levels of C3d than antibody excess (Figure 6-18a). EDTA decreased the levels of C3d to the levels of controls, however, EGTA only partially decreased the levels of generated C3d (Figure 6-18a). The presence of erythrocytes did not cause any significant effect on the levels of C3d (Figure 6-18a).

The levels of bound C3d as measured by modified ELISA supported findings with free C3d and were higher at equivalence (OD_{405nm} = 0.38) compared to antibody excess (OD_{405nm} = 0.31) and antigen excess (OD_{405nm} =0.27) (Figure 6-18b). EDTA decreased the levels of C3d, however, EGTA partially decreased the levels of E's bound C3d (Figure 6-18b).

The levels of E's bound C3d as determined by FACscan analysis using FITC conjugated rabbit anti-human C3d showed higher levels at equivalence (29.92 % positive E's) than antibody excess (20.92 % positive E's) and antigen excess (8.44 % positive E's) (Figure 6-19a). At antibody excess the levels of E's bound C3d were higher than antigen excess (Figure 6-19a). EDTA inhibited the detectable levels of E's bound C3d by FACscan, however, EGTA only partially decreased the levels of bound C3d (Figure 6-19a).

Erythrocyte bound immune complexes as detected by FACscan analysis using FITC conjugated anti-human IgG at equivalence also showed a higher binding to erythrocytes (22.7% positive E's) than antibody excess (14.1% positive E's) or antigen excess

(2.8% positive E's) (Figure 6-19b). At antibody excess the levels of bound immune complexes were higher than the levels of bound immune complexes at antigen excess (49 % higher, Figure 6-20b). EDTA inhibited the binding of immune complexes to erythrocytes, however, EGTA only partially inhibited binding (Figure 6-19b).



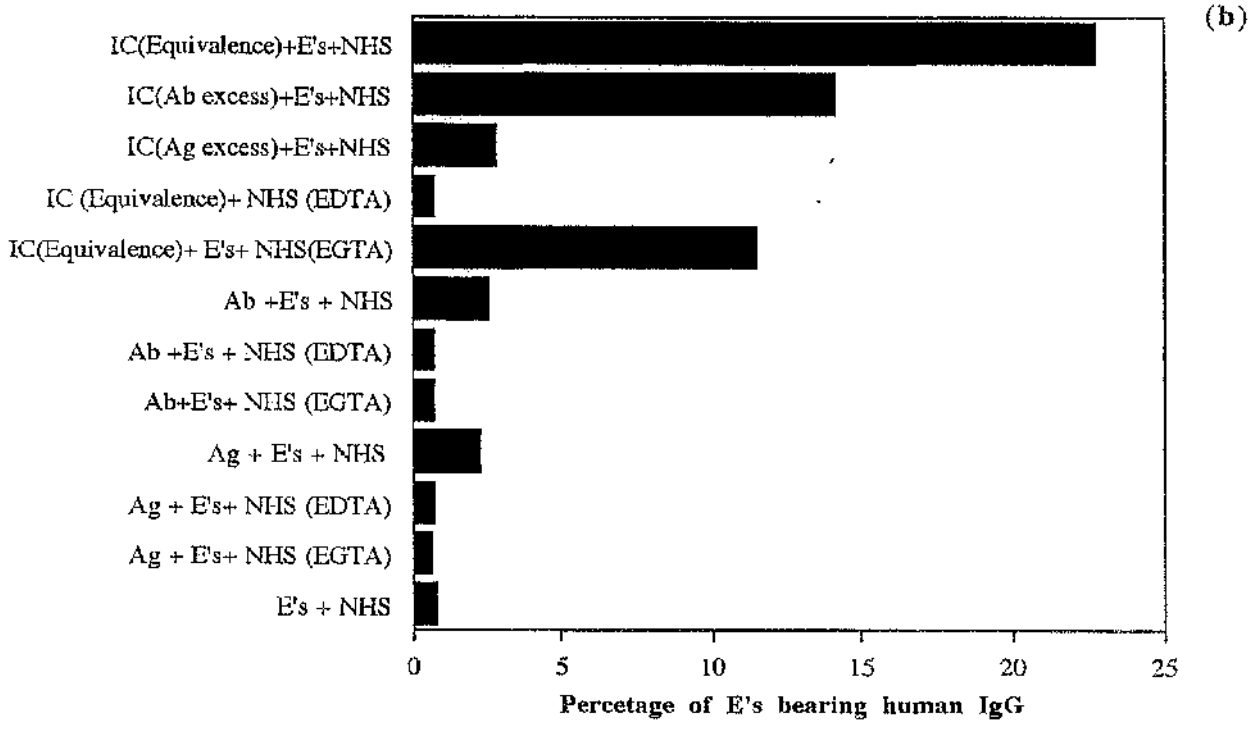


Figure 6-19: The levels of (a) erythrocyte (E's) bound C3d and (b) E's bound preformed human streptokinase ICs at equivalence, 4 times antibody (Ab) excess and 4 times antigen (Ag) excess after 5 minutes incubation at 37oC.

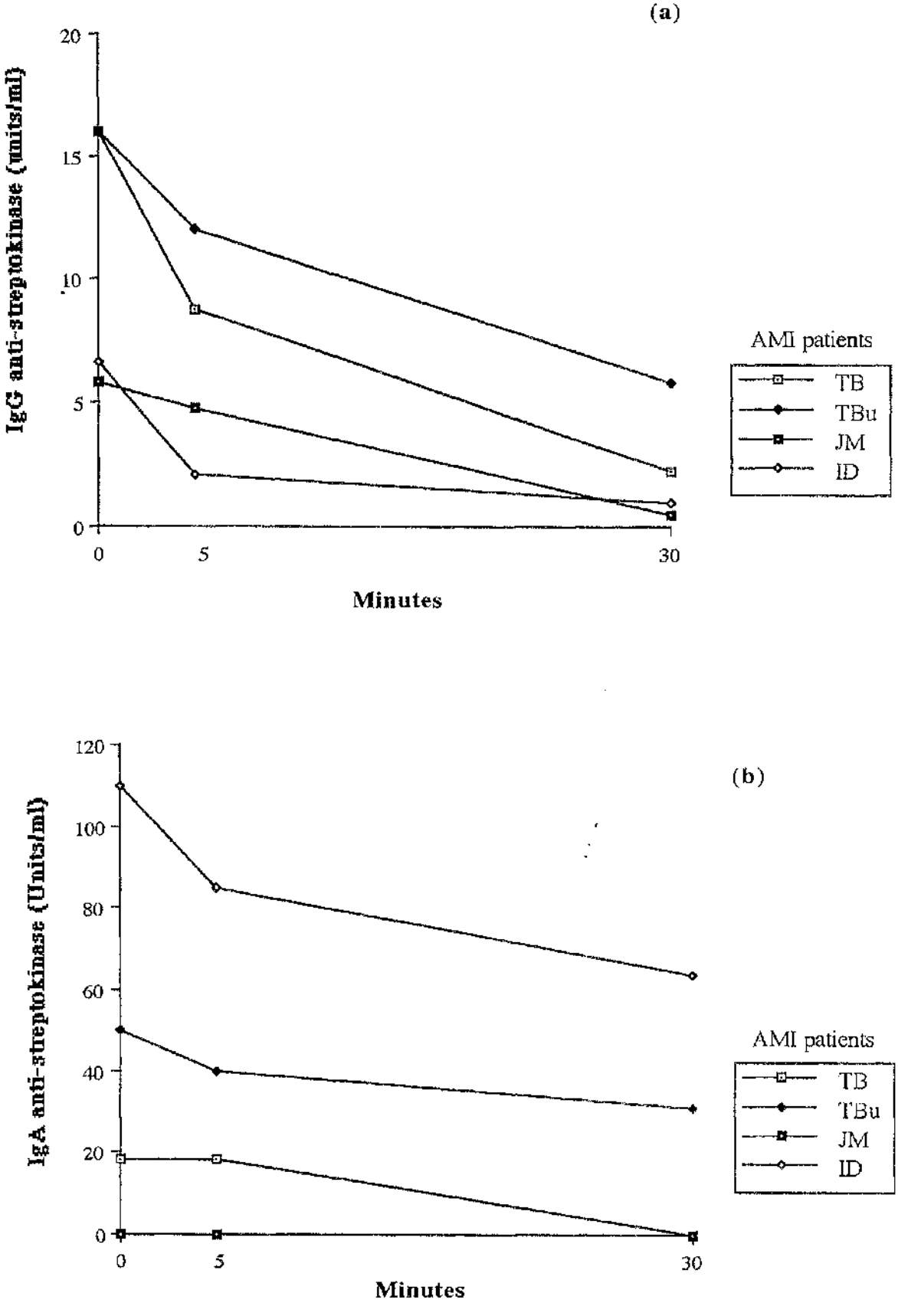
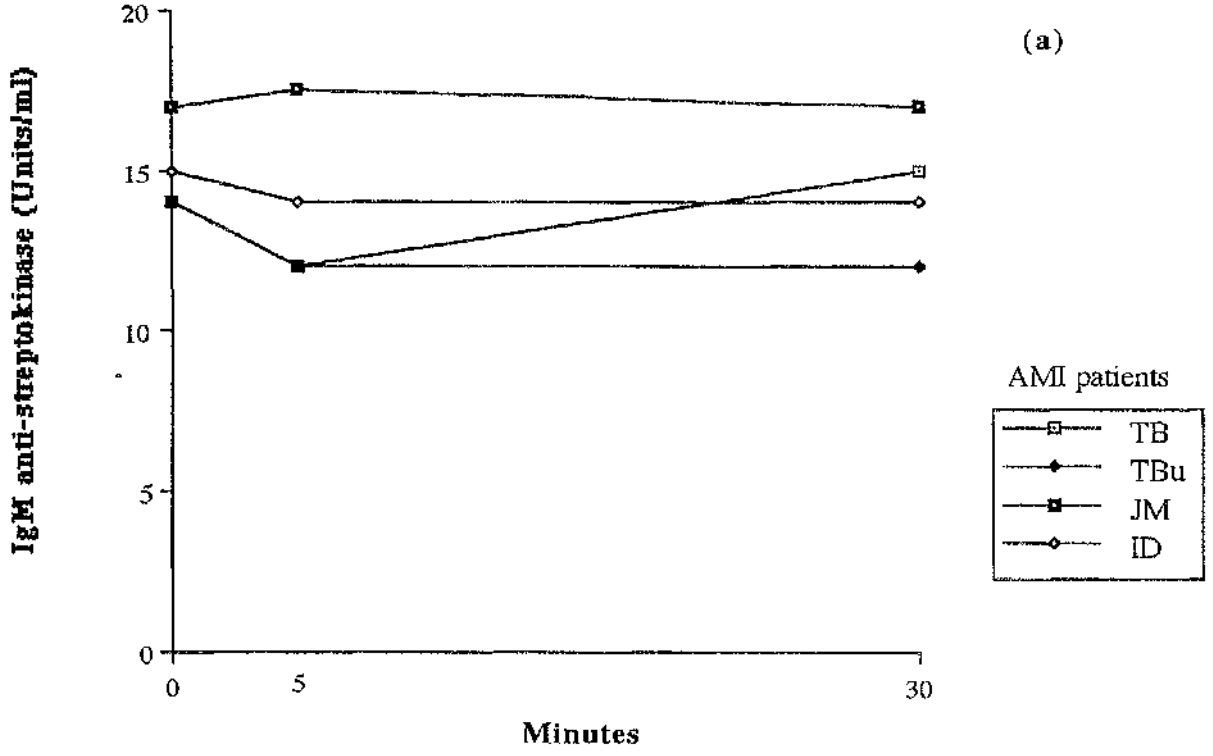


Figure 6-20: The levels of IgG (a) and IgA (b) anti-streptokinase antibodies in 4 patients with AMI treated with streptokinase.



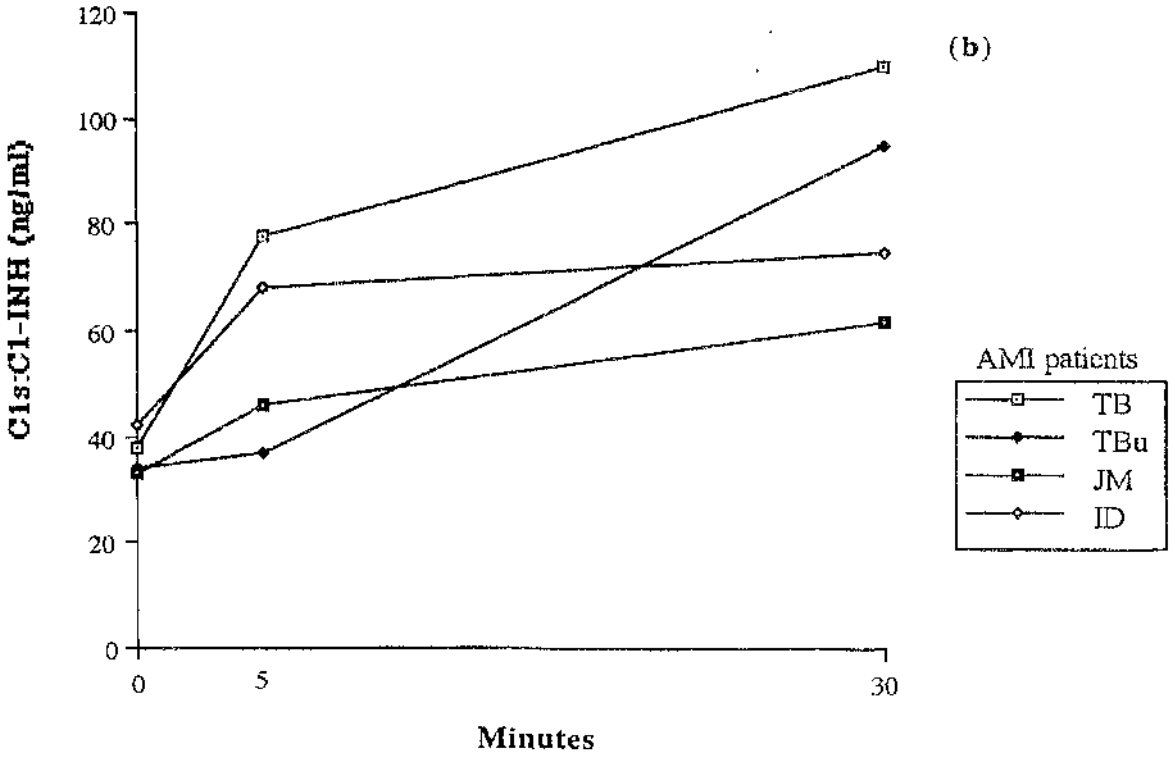


Figure 6-21: The levels of IgM anti-streptokinase antibodies (a) and C1s:C1-INH (b) in 4 patients with AMI treated with streptokinase.

Correlation between the E's bound ICs, C3d and free complement activation products, C1s:C1-INH, C3b-P and free C3d were analysed. The levels of E's bound immune complexes and E's bound C3d (Detected by FACscan and modified ELISA) correlated significantly with the levels of free complement activation products (Table 6-1). The levels of E's bound C3d detected by FACscan correlated with levels obtained by modified ELISA (Table 6-1). Free complement activation products C1s:C1-INH, C3b-P and free C3d also correlated significantly with one another (Table 6-1).

Study the streptokinase immune complexes binding to erythrocytes in patients with AMI. Using flow cytometry binding of human streptokinase immune complexes to erythrocytes formed during streptokinase treatment in 4 patients with AMI was studied, the levels of pre-existing anti-streptokinase antibodies and the extent of complement activation and its relationship with the levels of E's bound immune complexes were also studied (6-12). Blood samples were taken prior to administration of streptokinase and at 5 and 30 minutes after treatment.

The pre-treatment levels of IgG and IgA anti-streptokinase antibodies in the 4 patients were within the normal ranges (Mean value for IgG = 11.1 units/ml, normal range= 0.24-45.8 units/ml and mean value for IgA = 44.5 units/ml, normal range= 0.37-324 units/ml). All the patients had elevated levels of IgM anti-streptokinase antibodies (Mean value 15 units, normal range= 0.06-10.8 units/ml).

Administration of streptokinase resulted in an 38% reduction in the levels of IgG anti-streptokinase antibodies at 5 minutes (Mean value = 6.9 units/ml) compared with pre-treatment levels (Mean value 11.1 units/ml) at 30 minutes the levels were still depressed (Mean value = 2.3 units/ml, 84% reduction) (Figure 6-20).

Administration of streptokinase also caused a 20% and 47% reduction in the levels of IgA anti-streptokinase antibodies at 5 minutes (Mean value =35.7 units/ml) and 30

minutes (Mean value 23.7 units/ml) respectively, compared to pre-treatment levels (Mean value = 44.5 units/ml) (Figure 6-20).

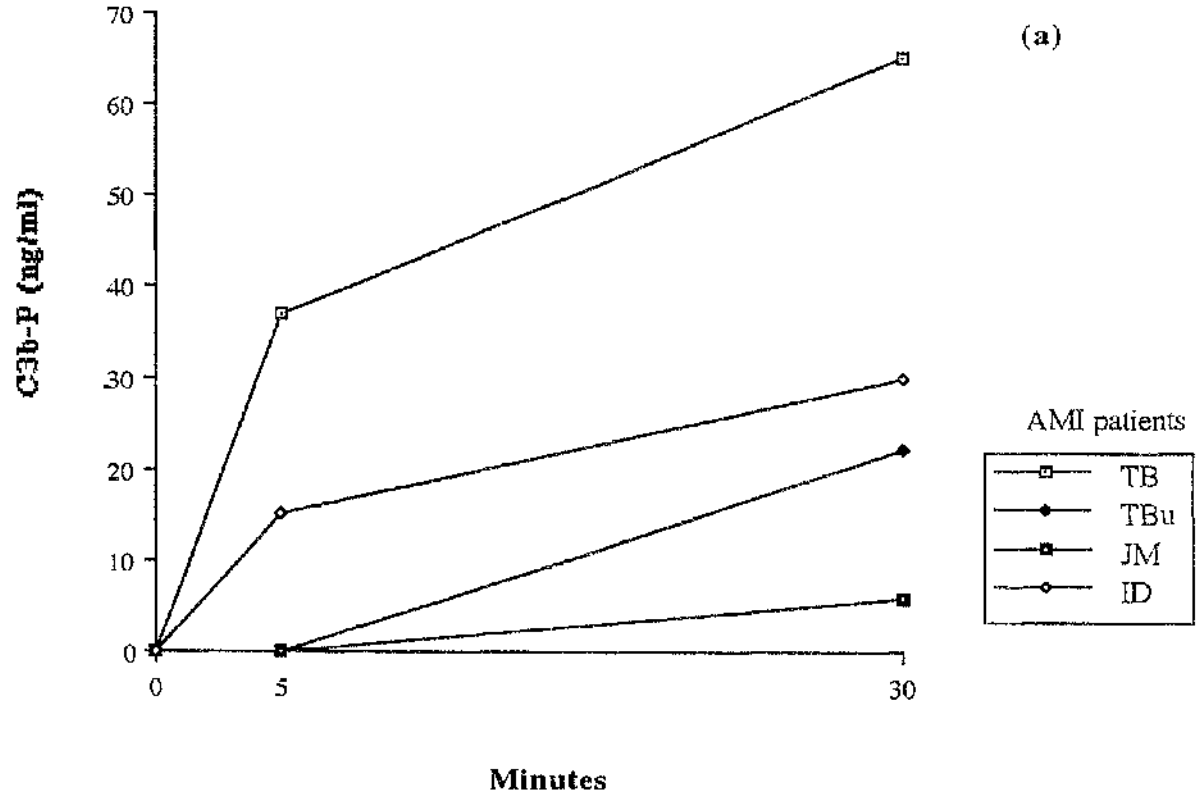
The levels of IgM did not show any significant changes at 5 (Mean value = 13.9 units/ml) or 30 minutes (Mean value = 14.5 units/ml) compared to the pre-treatment levels (Mean value = 15units/ml) (Figure 6-21).

Administration of streptokinase generated complement activation products C1s:C1-INH, C3b-P and free C3d as compared to pre-treatment levels (Figures 6-21 and 6-22). This was significant for C3b-P levels at 5 minutes when the levels compared to pre-treatment levels (Statistic=16, $p<0.05$). This was however, due to the rapid increase in 2 patients, in the remaining 2 patients no C3b-P was detectable at 5 minutes.

The levels of generated C1s:C1-INH correlated with the levels of generated C3b-P ($r=0.95$ $p<0.005$, at 5 minutes) and the levels of free C3d ($r=.99$ $p<0.001$, at 30 minutes). The levels of C3b-P correlated with the levels of C3d at 30 minutes ($r=0.99$, $p<0.001$).

The pre-treatment levels of IgG and IgM anti-streptokinase antibodies in the 4 patients showed a significant negative correlation with the levels of generated C1s:C1-INH at 30 minutes ($r= - 0.94$ $p< 0.05$) (Table 6-2).

IgA, IgM, C3c and C3d were not detectable on erythrocytes in the 4 patients, however, IgG was detectable on the erythrocytes of one patient (Patient T.B.) (Figure 6-23). In this patient the levels of anti-streptokinase IgG antibodies decreased after streptokinase administration and the levels of E's bound ICs as detected by FITC conjugated sheep anti-human IgG increased ($r=-0.99$, $p<0.001$) (Figure 6-23). This patient (Patient T.B.) also generated higher levels of C1s:C1-INH and C3b-P compared with the other 3 patients (Figures 6-21 and 6-22) and these levels significantly correlated with the levels of E's bound ICs ($r=0.99$, $p<0.001$) (Figures 6-21, 6-22 and 6-23).



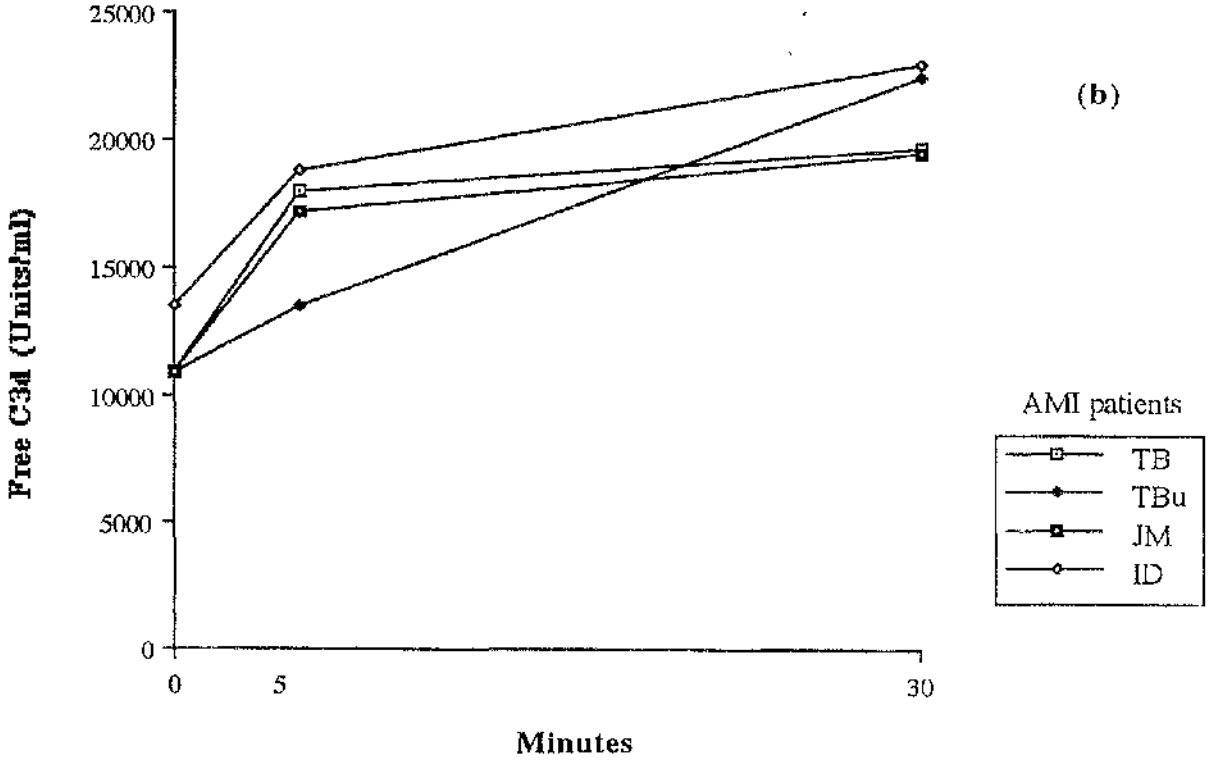


Figure 6-22: The levels of C3b-P (a) and free C3d (b) in 4 patients with AMI treated with streptokinase.

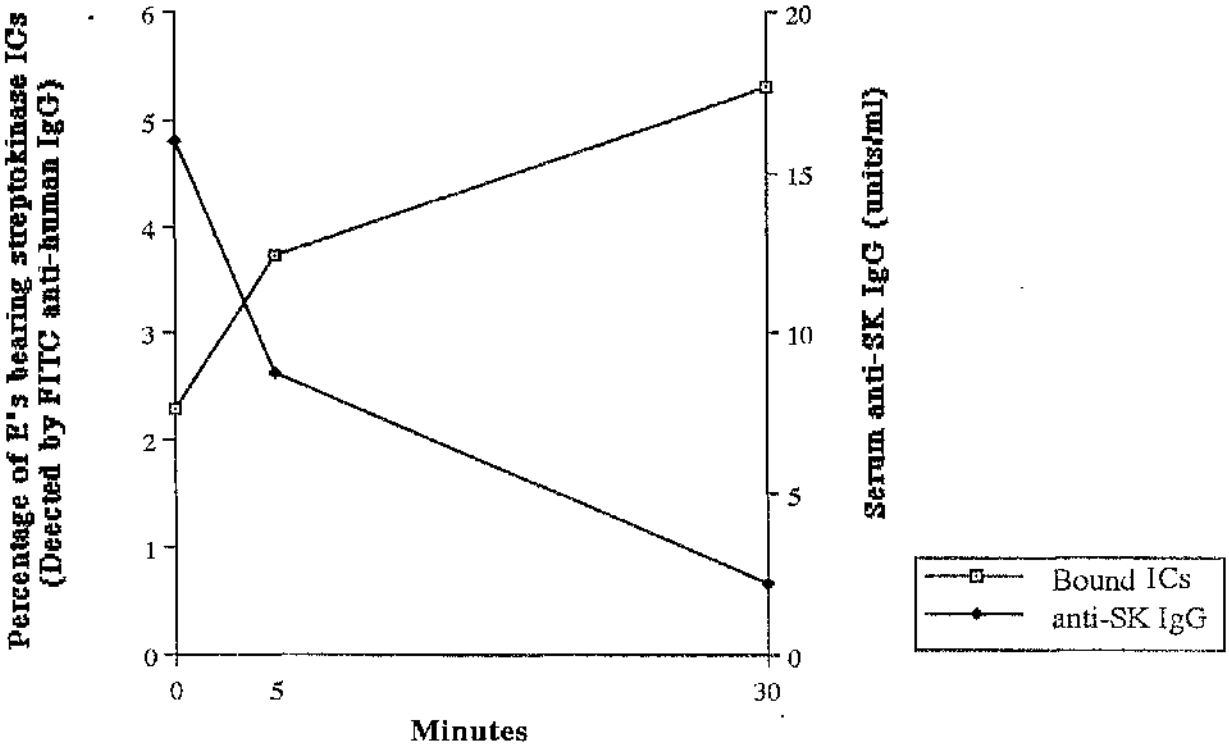


Figure 6-23: Fluctuations of IgG anti-streptokinase antibodies and erythrocytes (E's) bound streptokinase immune complexes (ICs) during streptokinase administration, in patient T.B. with AMI.

5 minutes

Isotype	C1s:C1-INH	C3b-P	Free C3d
IgG	-0.7 (NS)	0.38 (NS)	-0.2 (NS)
IgA	0.2 (NS)	0.2 (NS)	0.4 (NS)
IgM	0.73 (NS)	-0.39 (NS)	0.2 (NS)

30 minutes

Isotype	C1s:C1-INH	C3b-P	Free C3d
IgG	-0.94 ($p<0.05$)	0.63 (NS)	0.2 (NS)
IgA	0.2 (NS)	0.4 (NS)	0.89 (NS)
IgM	-0.94 ($p<0.05$)	-0.63 (NS)	-0.2 (NS)

Table 6-2: Correlation between the pre-treatment levels of anti-streptokinase antibodies (IgG, IgA and IgM) and the levels of complement activation products (C1s:C1-INH, C3b-P, free C3d) generated at 5 and 30 minutes after streptokinase infusion in 4 patients with AMI.

Discussion

Erythrocytes have an important role in clearance of immune complexes from circulation and prevention of tissue damage. This is mainly through the action of the complement receptor CR1 which binds to immune complexes coated with C3b and clears the immune complexes from circulation. These important interactions are governed by the complement system and the nature of immune complexes. Immune complexes which are not transported to liver or spleen will eventually be deposited outside the reticuloendothelial system and provoke inflammatory response (Schifferli et al 1988).

The kinetics and dose response of streptokinase immune complexes binding to erythrocytes was studied by flow cytometry and its relationship with complement activation was determined by measuring the C1s:C1-INH, C3b-P and C3d in the accompanying supernatants. This was compared with the thyroglobulin immune complexes system as antigens exhibit different molecular weights. Although many studies have looked at ICs and E's few models have had the opportunity which is given by streptokinase (In vitro or in vivo, rabbit or human systems).

The interactions between rabbit thyroglobulin immune complexes and erythrocytes showed that the binding of the ICs not only significantly correlates with the classical and alternative pathways activation (r values 0.98 and 0.91 respectively, $p<0.001$) (Figure 6-2) but also is upon the quantity of IC ($p<0.001$). The binding of rabbit streptokinase immune complexes to human erythrocytes also correlated significantly with the levels of generated C1s-C1-INH (Classical pathway activation) and C3b-P (Alternative pathway activation) ($r= 0.77$, $p<0.07$) (Figure 6-3).This interaction was also a dose dependent reaction ($r= 0.77$, $p<0.7$) as observed for the rabbit thyroglobulin ICs

The relationship of complement activation, the nature of the immune complexes and their binding to erythrocytes has been reported by other investigators. Lucisano &

Lanchmann (1991), using immune complexes consisting of ^{125}I NIP coupled with BSA and different antibody isotypes and subclasses to this antigen (IgG1, IgG2, IgG3, IgG4, IgM and IgA), showed that binding of the immune complexes to erythrocytes correlated with the degree of complement consumption (Using haemolytic assays) which was influenced by the immune complexes nature i.e. antibody isotype or subclass and the antibody/antigen ratio.

Rabbit IgG anti-streptokinase generated complement activation products to a lower extent compared to the that corresponding immune complexes but bound to human erythrocytes to higher extent (Figures 6-3 and 6-4). The generation of complement activation products by rabbit anti-streptokinase IgG can be explained by presence of aggregated antibodies formed during purification by caprylic precipitation method. The higher extent of rabbit IgG binding to the erythrocytes compared to the ICs binding was surprising since the levels of complement activation products generated by the rabbit IgG were substantially lower than the levels generated by the ICs. This may indicate that there is a second mechanism involved in the interaction between immune complexes or aggregated IgG and erythrocytes. This secondary mechanism will be discussed in detail in chapter 7.

The kinetics of ICs binding to E's was studied using 5 doses of rabbit streptokinase immune complexes (6-6, Figure 6-5a). Optimal binding was achieved with higher IC doses, binding peaked at 5 minutes and then dropped rapidly, this pattern corresponds to binding of ICs to E's followed by release. This pattern is similar to that originally described by Medof & Prince (1983) using BSA ICs system, and is explained by processing of ICs. The in vitro release of immune complexes from erythrocytes after 5 minutes can be explained by ongoing complement activation and degradation of C3b incorporated in ICs to iC3b, C3c and C3d. These have reduced or no affinity for CR1 receptors on the erythrocytes and therefore show no binding. During the kinetic study of ICs binding to erythrocytes the sampling of E's at 5 minutes (peak of binding),

followed by complement inactivation with EDTA showed that after several washing the ICs were still detectable on the erythrocytes surface. This supports the hypothesis that degradation of C3b by Factor I influences the release of bound ICs from E's and as Yokoyama & Waxman (1994) have shown that Factor I can accelerate the release of DNP-BSA ICs from erythrocytes by degradation of C3b to C3dg. Schifferli et al (1988), by infusion of preformed radio-labelled tetanus toxoid IC in human showed that the ICs bound to E's and transferred from circulation to the reticuloendothelial system. The release of ICs from E's due to degradation of C3b is believed to have an important effect on the transferring of E bound ICs to spleen and liver.

The levels of erythrocytes bound C3c and C3d generated during complement activation by streptokinase and thyroglobulin ICs was studied to find out whether the levels bound to E's reflect the extend of complement activation as measured free in serum. The levels of E's bound C3c as detected by flow cytometry using FITC rabbit anti-human C3c increased during complement activation and reached a peak at 5 minutes. After 5 minutes the levels of bound C3c decreased reflecting the degradation of the bound C3 (Figure 6-7). The levels of E's bound C3c correlated with the levels of generated C3b-P at 5 minutes ($r=0.97$, $p<0.001$).

The levels of E bound C3d (as detected by flow cytometry using FITC rabbit anti-human C3d or modified ELISA) during complement activation reached to a peak 15 minutes (10 minutes later than C3c) and in contrast to E bound C3c the levels did change significantly over the time course (Figures 6-8 and 6-9). This indicates that E bound C3d is more resistance to the effects degradation than E's bound C3c, and as C3d is covalently bound to E's can not be degraded further. The deposition of C3d on erythrocytes is therefore indicates that the complement system is activated (Davies et al 1990, Freysdottir et al 1993) and may be used in clinical situations.

The study of the kinetics and dose response of rabbit thyroglobulin and rabbit streptokinase immune complexes binding to erythrocytes provided a helpful background for the study of human streptokinase immune complexes (6-10).

Preformed human streptokinase immune complexes at equivalence prepared from the serum of a patient with elevated levels of anti-streptokinase antibodies and the levels of E bound IgG, IgA and IgM incorporated into streptokinase ICs was studied. Similar to the results observed with rabbit streptokinase ICs, the levels of bound human streptokinase ICs reached a peak at 5 minutes after which levels decreased. This was accompanied by generation of bound and free complement activation products, peaking 10 minutes later than E bound ICs (Figures 6-13, 6-14, 6-15 and 6-16). The presence of erythrocytes treated with anti-CR1 (E-CR1) did not inhibit the binding of immune complexes to erythrocytes as detected by FITC anti-human IgG, however, it decreased the levels of E's bound ICs detected by FITC anti-human IgA and IgM (Figures 6-14 and 6-15). As described the activation of complement is essential for the binding of immune complexes to erythrocytes. Although, this experiment showed that even by blocking CR1 receptor, ICs (Detected by FITC anti-IgG) bound to erythrocytes and also are resistance to the complement mediated degradation and release from the E's, as in the presence of anti-CR1 E's bound ICs (Detected by FITC anti-IgG) were significantly detectable at 24 hours despite of ongoing complement activation. Furthermore, immune complexes in absence of NHS bound to erythrocytes (6-5). This observation may be as a result of binding of immune complexes containing IgG to erythrocytes through a second mechanism, possibly Fcγ receptor and will be discussed in chapter 7 (Hajos et al 1978).

The antigen/antibody ratio influenced the binding of immune complexes to erythrocytes. Human streptokinase immune complexes at equivalence bound to erythrocytes to higher extent compared to antibody excess and antigen excess (Figure 6-20b) (6-11). At antibody excess the levels of bound immune complexes were 49 %

higher than the antigen excess (Figure 6-20b). The immune complex size and the nature of ICs and ratio of antibody/antigen affect the binding of immune complexes to erythrocytes and ultimately determine the fate of the immune complexes. Lucisano et al 1991 using NIP-BSA ICs showed that nascent ICs bind to E's to a lower extent than preformed ICs, the observed difference is thought to depend on the size of the ICs, since solubilization of preformed ICs takes time and nascent ICs are smaller than preformed ICs.

EDTA inhibited the binding of immune complexes to erythrocytes, however, EGTA only partially inhibited the binding (Figure 6-20b). The alternative pathway activation and immune complexes binding erythrocytes through this activation was rather unusual. This can be explained by insufficient concentration of EGTA for inhibiting the activation.

Studying the levels of anti-streptokinase antibodies the 4 patients with AMI treated with streptokinase and the extent of complement activation (6-12) showed similar results with the first 10 patients (Chapter 3), this is indicative that administered streptokinase caused immune complexes formation and complement activation. Freysdottir et al 1993 showed that the levels of bound C3d on erythrocytes were detectable at 1, 2 hours and day 7 post-administration of streptokinase, however, E bound streptokinase ICs were not detectable at that time points. This is not surprising as shown previously E bound ICs are cleared rapidly from circulation (Schifferli et al 1988). In this study although the levels of complement activation products were detectable at 5 minutes and 30 minutes (Figures 6-21a and 6-22) C3d was not detectable on the E's at this time points. On the other hand, streptokinase immune complexes were detectable on erythrocytes in one patient (Patient T.B.) (Figure 6-23). This patient generated higher levels of C1s:C1-INH and C3b-P compared to other 3 patients. The levels of E's bound ICs and serum anti-streptokinase IgG inversely correlated with one another

(r=.99, p<0.001) (Figure 6-23). This clearly showed that streptokinase ICs formed during the first 30 minutes of streptokinase infusion bound to erythrocytes.

Chapter 7

Non-complement dependent binding of immune complexes to erythrocytes

Introduction

Study of the kinetics and dose response of streptokinase immune complexes (ICs) binding to erythrocytes by flow cytometry and its relationship with complement activation as was determined by measuring the C1s:C1-INH, C3b-P and C3d in the accompanying supernatants showed that the binding of the ICs significantly correlates with complement activation and depends on the nature of the immune complex (Chapter 6).

The experimental protocols utilised however lead to the conclusion that an Fcγ receptor may also be involved as an alternative mechanism of binding of immune complexes to erythrocytes. The observation that immune complexes in the absence of NHS (Complement) bind to erythrocytes supported this hypothesis. Other supportive evidence was enhanced binding of aggregated rabbit IgG anti-streptokinase in the absence of NHS compared to in the presence of NHS. This observation indicates that the binding is not only a complement mediated interaction. Based on these pieces of evidence we decided to further investigate Fcγ mediated binding, to allow better understating of IC clearance mechanisms.

To study this phenomenon an indirect haemagglutinin test for rabbit IgG anti-streptokinase was developed to find whether the capacity of erythrocyt binding (in absence of complement) varied among the population. As described FcγR is expressed in three forms, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (1-7). Using

monoclonal anti-FcγR antibodies and flow cytometry technique the presence of these receptors on the erythrocytes were investigated. The binding of F(ab')2 fragment to erythrocytes was studied and compared with the intact IgG. As described the specificity of Fcγ receptors for binding to the IgG subclasses varied among the three forms of receptors (Table 1-2), therefore the interactions of mouse and human IgG subclasses with human erythrocytes were studied.

Materials and methods

7-1 Indirect (Passive) haemagglutinin test for rabbit anti-streptokinase IgG. As the rabbit IgG binds to human erythrocytes, to find whether the binding varied among the population, an indirect (Passive) haemagglutinin test was developed. This technique involves passive binding of IgG to erythrocytes (E's) then incubating with donkey anti-rabbit IgG to agglutinate E's.

Materials and Buffer: Sources of materials used here were described in 2-1.

Rabbit anti-streptokinase IgG

Donkey anti-rabbit IgG

Human erythrocytes

Phosphate buffered saline (PBS)

Round bottomed microplate

Method. Blood was collected by venesection from healthy volunteers and patients in EDTA (2-16). The normal group consisted of 8 individuals and groups of patients were as following; 18 patients with rheumatoid arthritis, 9 patients with SLE and 32 patients with miscellaneous diseases. The blood was spun at 1200 rpm, at 4°C for 10 minutes,

the buffy coat layer was completely removed and the cells were washed three times in PBS and resuspended in the same buffer. The cells were then standardised to 1X 10^8 cells/ml (2-17).

130 μg rabbit IgG anti-streptokinase (2-13 and 2-19) was incubated with the 5 ml erythrocytes (1X 10^8 cells/ml) at 37°C for half an hour with occasionally shaking. After incubation the cells were washed once with PBS and standardised to 1X 10^8 cells/ml (2-17).

Donkey anti-rabbit IgG (heat inactivated) diluted 1/5 in PBS was applied to a round-bottomed microplate and double diluted across 2 rows of the plate 23 times. The last well (number 24) contained only PBS and was used as control. 50 μL of the erythrocytes suspension were then applied per well, the plates were gently shaken and incubated at room temperature for 2 hours.

The plates were read using an inverted magnifying mirror. The last dilution which prevented the sedimentation of erythrocytes was considered as the end point of the agglutinin titre and expressed in reciprocal titre (Figure 7-1).

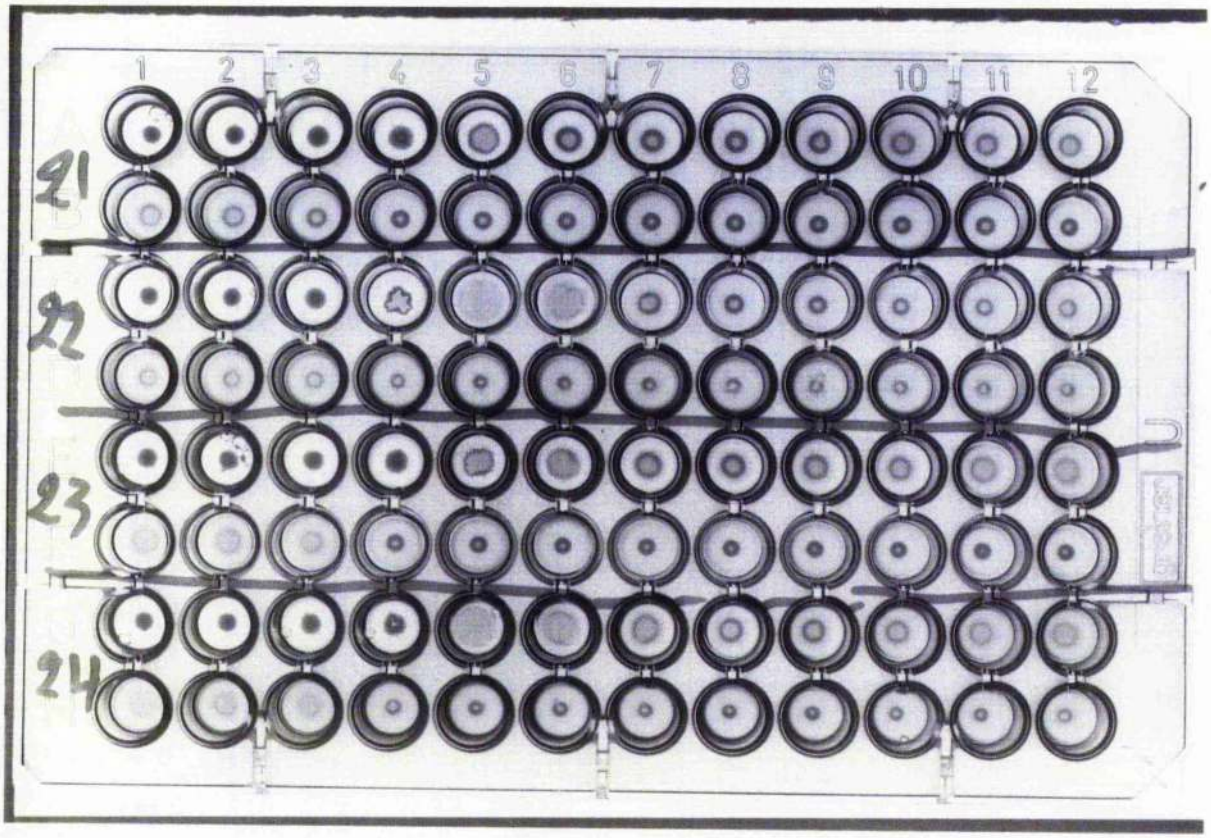


Figure 7-1: Indirect haemagglutinin test for rabbit IgG anti-streptokinase. Donkey anti-rabbit IgG applied to the two rows of microplate for each sample and double diluted from well 1 to well 23. The last well (24th well) is control.

7-2 Preparation of F(ab')2 fragment of IgG. $F(ab')_2$ was prepared by pepsin digestion, this digestion results in Fc and $F(ab')_2$ fragments of which the latter contains the intact antigen binding site (MW=100kD). Protein G-Sepharose was used to bind the Fc portion of IgG and unbound $F(ab')_2$ and bound Fc fractions can be then eluted (2-23).

Materials: Sources of materials used here were described in 2-1.

Rabbit IgG anti-streptokinase (2-13 and 2-19)

Pepsin

Protein G Sepharose 4B

Buffers:

Sodium phosphate 0.1M (pH 7.0) (2-23)

0.1 M sodium acetate (2-23)

0.1M glycine (2-23)

Method. 5 ml of rabbit IgG anti-streptokinase (13 mg/ml) was dialysed extensively against 0.1 M sodium acetate for 3 hours at 4°C. The pH of the IgG was adjusted with 0.1M acetic acid to 4.5 (2-19). 1 mg pepsin (1-2 mg per 100 mg IgG) was added to the IgG and incubated at 37°C overnight. The digested sample was then spun and any precipitate discarded, the pH was adjusted to 7.4 by addition of solid Tris. The sample was then dialysed extensively against PBS for 3 hours at 4°C to inactivate the enzyme. Protein G-Sepharose was pre-washed with 0.02M sodium phosphate buffer and resuspended in buffer to give a ratio of 25% 0.02M sodium phosphate buffer and 75% Sepharose. The digested IgG was then added to the protein G-Sepharose and mixed at room temperature for 90 minutes. The Sepharose was then spun and the supernatant containing $F(ab')_2$ removed and retained. The Sepharose was recycled by washing 3 times in 0.1M glycine and resuspended in 20% ethanol.

7-3 Binding of rabbit IgG anti-streptokinase and F(ab')2 fragment to human erythrocytes. Binding of rabbit IgG anti-streptokinase and F(ab')2 fragment to human erythrocytes(E's) in presence and absence of NHS was studied by flow cytometry. In presence of NHS complement activation products C1s:C1-INH and C3b-P were measured in the supernatants (5-3).

Materials: Sources of materials used here are described in 2-1.

Rabbit IgG anti-streptokinase (2-13 and 2-19)

Rabbit F(ab')2 anti-streptokinase (7-2)

FITC conjugated donkey anti-rabbit IgG

NHS

Human erythrocytes

Falcon round-bottomed tubes

Buffers

Phosphate buffered saline (PBS)

1% BSA in PBS

20 mM isotonic EDTA (2-2)

ELISA buffers (5-3)

Method. Erythrocytes (E's) were obtained by venesection from healthy volunteers, blood was clotted in EDTA (2-16) and spun at 1200 rpm, at 4°C for 10 minutes. The buffy coat layer was completely removed and remaining E's were washed three times in PBS. 580 µg of rabbit IgG anti-streptokinase or 580 µg rabbit F(ab')2 anti-streptokinase were added with 50 µl of packed erythrocytes and 500 µl NHS or 50 µl of packed erythrocytes and 500 µl PBS. The volume of mixtures were equalised to 700 µl with PBS and incubated at 37°C for 30 minutes. The assay was sampled at 0, 5, 15 and 30 minutes by removing 50 µl and adding to equal volume of ice cold 20 mM

isotonic EDTA. The samples were then spun at 4°C for 5 minutes, the supernatants were removed and analysed for the presence of the complement activation products (5-3). Erythrocytes were further washed in ice cold 1% BSA in PBS and resuspended in the same buffer to $1X10^8$ cells/ml (6-1). Erythrocytes were then incubated with an equal volume of FITC donkey conjugated anti-rabbit IgG (diluted 1/20 in PBS) for 30 minutes in an ice bath. The cells were washed twice with 1% BSA in PBS and analysed by a FACscan flowcytometer (6-4). The negative control contained 500 µl NHS and 50 µl of packed erythrocytes (Total volume = 700 µl with PBS) at 37°C and incubated with FITC conjugated donkey anti-rabbit IgG at above condition.

7-4 Inhibition of binding of rabbit IgG anti-streptokinase to human erythrocytes. To investigate whether human Fc fragment can inhibit the binding of rabbit IgG to human erythrocytes an inhibition assay was performed as following.

Materials: Sources of materials used here are described in 2-1.

FITC conjugated donkey anti-rabbit IgG

Human IgG Fc fragment

Rabbit IgG anti-streptokinase (2-13 and 2-19)

FITC conjugated donkey anti-rabbit IgG

Goat anti-human IgG (Fc fragment specific)

Human erythrocytes

Falcon round-bottomed tubes

Buffers

Phosphate buffered saline

1% BSA in PBS

Method. Erythrocytes (E's) were obtained by venesection from healthy volunteers, blood was clotted in EDTA (2-16) and spun at 1200 rpm, at 4°C for 10 minutes. The buffy coat layer was completely removed and remaining E's were washed three times in

PBS and standardised to $1X10^8$ cells/ml (6-1). The purity of human Fc fragment was checked by IEP using goat anti-human Fc fragment and anti-human IgG.

100 µl (500 µg) of human Fc and 500 µl erythrocytes ($0.5X10^8$ cells) or 100 µl 1% BSA in PBS per 500 µl erythrocytes ($0.5X10^8$ cells) were incubated at 37°C for 30 minutes. The mixtures were spun at 1200 rpm, at 4°C for 5 minutes, the supernatants were removed and the pellets resuspended with 500 µl 1% BSA in PBS. The suspensions of E's were then incubated with 260 µg of rabbit IgG anti-streptokinase at 37°C for 30 minutes. Erythrocytes were then washed once in ice cold 1% BSA in PBS and resuspended with 500 µl 1% BSA in PBS. The E's were then incubated with an equal volume of FITC conjugated donkey anti-rabbit IgG (diluted 1/20 in PBS) for 30 minutes in an ice bath. The cells were further washed in 1% BSA in PBS twice and analysed by a flowcytometer (6-4). Negative control consisted of a 500 µl E's suspension ($0.5X10^8$ cells) incubated with FITC conjugated donkey anti-rabbit IgG at above condition .

7-5 The binding of monoclonal anti-FcγR antibodies to human erythrocytes. To study whether monoclonal anti-human FcγR antibodies can recognise any epitopes on the human erythrocytes, the binding of a set of these antibodies directed against FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD 16) was examined by flow cytometry.

Materials and buffer: Sources of materials used here are described in 2-1

Human erythrocytes

Mouse monoclonal anti-human CD64 (FcγRI)

Mouse monoclonal anti-human CD32 (FcγRII)

Mouse monoclonal anti-human CD16 (FcγRIII)

Mouse IgG1 monoclonal anti-human AFP

FITC conjugated rabbit F(ab')2 anti-mouse immunoglobulin

Falcon round-bottomed tubes

Filtered phosphate buffered saline (PBS)

Method. Erythrocytes were prepared and standardised to 1X 10^8 cells/ml as described (7-3). 5μg of mouse monoclonal anti-human CD64 (FcγRI), mouse monoclonal anti-human CD32 (FcγRII), mouse monoclonal anti-human CD16 (FcγRIII) and mouse monoclonal anti-human AFP were incubated with 100 μl erythrocytes (contained $5X10^6$) at room temperature for half an hour. Erythrocytes were washed once in PBS and resuspended in 100μl PBS. The erythrocytes were then incubated with 20μl of FITC conjugated rabbit F(ab')2 anti-mouse immunoglobulin (diluted 1/20 in PBS) for half an hour at room temperature. The cells were washed in PBS and analysed by a flowcytometer as described (6-4). The negative control consisted of 100 μl erythrocytes (contained $5X10^6$) and 20μl of FITC conjugated rabbit F(ab')2 anti-mouse immunoglobulin (diluted 1/20 with PBS).

7-6 Purification of human IgG by Ion exchange chromatography.

Diethylaminoethyl cellulose (DE52, Whatman) binds to negative charged ions. The serum to be used should be dialysed into the column buffer (pH 7.6), under this condition IgG with a positive charge does not bind to DE52 and can be eluted, the remaining proteins being retained.

Materials: Sources of materials used here are described in 2-1.

Diethylaminoethyl cellulose (DE52, Whatman)

NHS, 30 ml pooled sera from normal individuals (protein concentration 78.6 mg/ml)

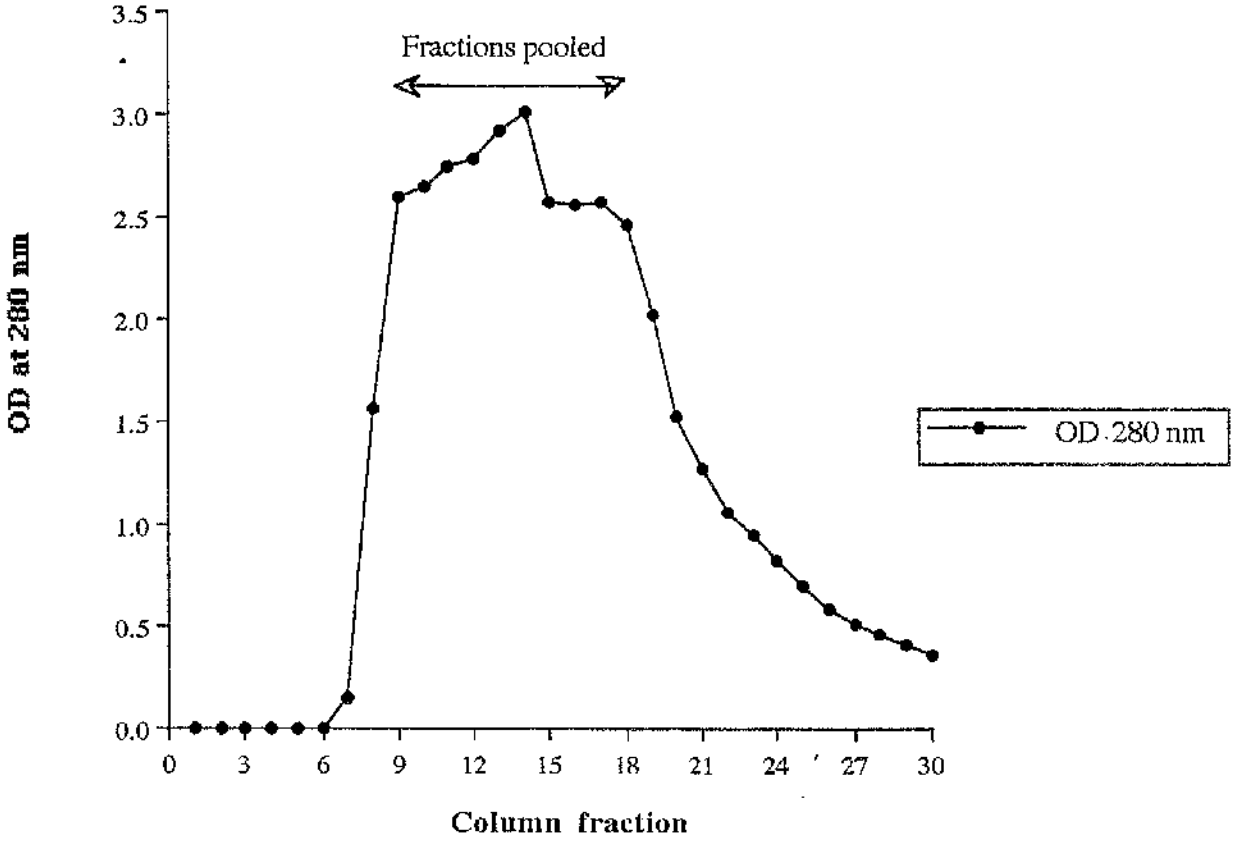
60 ml syringe barrel

Buffer: 0.01M sodium phosphate buffer pH 7.6 (5-1)
(Conductivity at 4°C, 0.5- 0.8 mS)

Column: 1.5 X 9 cm column (60 ml syringe barrel)
containing DE52 extensively equilibrated with buffer.

Method. After dialysis the pooled sera was applied and washed through the column with buffer and 3 ml fractions were collected, the protein concentration of the collected fractions were measured at 280 nm. The factions containing the protein peak were pooled (Figure 7-2).

The prepared IgG from pooled NHS was tested for purity by IEP, a single arc against anti-human IgG was observed and no precipitation with anti-human IgA and IgM was observed (2-9). The prepared sample was run on a 7.5% SDS-PAGE gel one band with molecular weight of 150 kDa was observed. Concentration of purified IgG was 5.1 mg/ml.



gure 7-2: Elution profile of human IgG purified by ion exchange chromatography on DE52 from pooled NHS.

7-7 Binding of human IgG to erythrocytes. To study the binding of human IgG to erythrocytes 3 sources of IgG were used, 1) Pooled NHS (5.1 mg/ml IgG, 7-6), 2) Pooled sera from patients with elevated levels of anti-streptokinase antibodies, purified by caprylic acid (14 mg/ml IgG, 2-19) 3) The serum from a patient with an IgG1 myeloma (21.3 mg/ml IgG, 7-6). Human IgG prepared from the sera of patients with elevated levels of anti-streptokinase antibodies on a 7.5% SDS-PAGE gel presented four bands with molecular weights of 150kD, 97kD, 78kD and 67 kD.
The proportion of IgG subclasses in the 3 IgG sources was determined by the Clinical Immunology Laboratory, Department of Immunology, Western Infirmary, Glasgow.

Materials: Sources of materials used here are described in 2-1

Human erythrocytes

Human IgG

FITC conjugated sheep anti-human IgG

Falcon round-bottomed tubes

Filtered phosphate buffered saline (PBS)

Method. Erythrocytes were prepared and standardised to $1X\ 10^8$ cells/ml (7-3). 5µg of IgG from the 3 IgG sources mentioned above were incubated with 100 µl erythrocytes (Contained $0.5X10^6$ cells) at room temperature for 30 minutes. Erythrocytes were washed in PBS and resuspended in 100µl PBS. The erythrocytes were then incubated with 20µl of FITC conjugated sheep anti-human IgG (diluted 1/20 in PBS) for half an hour at room temperature. The cells were washed twice in PBS and analysed by a flowcytometer (6-4). Negative control consisted of 100 µl erythrocytes (Contained $0.5X10^6$ cells) incubated with 20µl FITC conjugated sheep anti-human IgG (diluted 1/20 with PBS) at same conditions described.

7-8 Specificity of human IgG subclasses for human erythrocytes. The specificity of IgG subclasses for the 3 forms of Fcγ receptors is different (Table 1-2), the binding of human IgG subclasses to human erythrocytes was studied.

Materials: Sources of materials used here are described in 2-1

Human erythrocytes

Monomeric human IgG subclasses IgG1, IgG2, IgG3 and IgG4

Aggregated human IgG subclasses IgG1 and IgG3

FITC conjugated sheep anti-human IgG

Falcon round-bottomed tubes

Filtered phosphate buffered saline (PBS)

Method. Erythrocytes were prepared and standardised to $1X\ 10^8$ cells/ml (7-3). 100μg of human subclasses IgG1, IgG2, IgG3 and IgG4 or 50μg of aggregated human IgG1 and IgG3 were incubated with 100 μl erythrocytes (Contained $0.5X10^6$ cells) at room temperature for half an hour. Erythrocytes were washed once in PBS and resuspended in 100μl PBS. The erythrocytes were then incubated with 20μl of FITC conjugated sheep anti-human IgG (diluted 1/20 with PBS) for half an hour at room temperature. The cells were washed twice in PBS and analysed by a flowcytometer (6-4). A mixture of $0.5X10^6$ erythrocytes and 20μl FITC conjugated sheep anti-human IgG (diluted 1/20 with PBS) was used as negative control. Negative control consisted of 100 μl erythrocytes (Contained $0.5X10^6$ cells) incubated with 20μl FITC conjugated sheep anti-human IgG (diluted 1/20 with PBS) under the same conditions.

7-9 Specificity of mouse IgG subclasses for human erythrocytes. The specificity of IgG subclasses for the 3 forms of Fcγ receptors is different (Table 1-2), the binding of mouse IgG subclasses to human erythrocytes was studied.

Materials: Sources of materials used here are described in 2-1

Human erythrocytes

Mouse IgG subclasses IgG1, IgG2a, IgG2b and IgG3

Aggregated mouse IgG subclasses IgG1, IgG2a, IgG2b and IgG3

FITC conjugated rabbit F(ab')2 anti-mouse immunoglobulin

Falcon round-bottomed tubes

Filtered phosphate buffered saline (PBS)

Method. Erythrocytes were prepared and standardised to $1X\ 10^8$ cells/ml (7-3). 64μg of the mouse subclasses IgG1, IgG2a, IgG2b and IgG3 or 16μg of the corresponding aggregated antibodies were incubated with 100 μl erythrocytes (Contained $0.5X10^6$ cells) at room temperature for half an hour. The erythrocytes were then washed once in PBS and resuspended in 100μl PBS. The erythrocytes were incubated with 20μl of FITC conjugated rabbit F(ab')2 anti-mouse immunoglobulin (diluted 1/20 with PBS) for half an hour at room temperature. The cells were washed twice in PBS and analysed by a flowcytometer as described (6-4). Negative control consisted of 100 μl erythrocytes (Contained $0.5X10^6$ cells) incubated with 20μl FITC conjugated sheep anti-human IgG (diluted 1/20 with PBS) at same conditions described.

7-10 Erythrocytes surface biotinylation and purification of putative erythrocyte IgG binding protein. The aim of this procedure was to biotin label the surface proteins of erythrocytes, lyse the cells and elute specific IgG binding proteins from a IgG-Sepharose column.

Materials and buffers

Human erythrocytes

Biotin N-hydroxy-succinimide ester (2-1)

Dimethyl Sulphoxide (DMSO)

Phenyl methyl solphonyl fluoride (PMSF) (Sigma)

Nonidet P40 (NP40)

Affinity chromatography column (Sepharose 4B coupled to human IgG) (4-3)

SDS-PAGE materials and buffer (2-11)

Western blotting materials and buffers (2-12)

Method. Erythrocytes were prepared and standardised to $1X\ 10^8$ cells/ml in PBS (7-3). 0.4 mg biotin N-hydroxy-succinimide ester (From stock solution 5mg/ml biotin N-hydroxy-succinimide ester in DMSO) was added to 4 ml erythrocytes (Contained $5X10^6$/ml erythrocytes) and the mixture was shaken gently at room temperature for 15 minutes. The erythrocytes were then washed 3 times in PBS and lysed at a concentration of 10^7 cells per ml in PBS containing 2mM PMSF and 1% NP40. The erythrocytes were then spun at 18000 rpm for 20 minutes.

Affinity chromatography column contained Sepharose 4B coupled to human IgG was prepared as described before (4-3). The supernatant of lysed erythrocytes were applied and washed through the column with PBS, 1 ml fractions were collected, the protein concentration of fractions were measured at 280 nm.

The specifically bound material was eluted by washing the column with 0.1 M Glycine-HCl (pH 2.5) (4-3) and 1 ml fractions were collected, the protein concentration of fractions were measured at 280 nm.

The fractions which contained the protein peak were pooled and run on a 7.5% SDS-PAGE gel . The gel was then transferred to a nitro-cellulose membrane for Western blotting. The blot was blocked with 1% Marvel in TBS (2-12) and after washing incubated with alkaline phosphatase Avidin-D. The colour was developed using phosphatase substrate (BCIP/NBT) (2-1).

Results

Indirect (Passive) haemagglutinin test for rabbit anti-streptokinase IgG. To find whether the binding of rabbit IgG to human erythrocytes varied among the population, an indirect (Passive) haemagglutinin test was developed and the binding of IgG to erythrocytes from 8 normal individuals, 18 patients with rheumatoid arthritis, 9 patients with SLE and 32 patients with miscellaneous diseases was studied (7-1). The results were expressed as reciprocal of end titre, in the normal group the end point titres were all clustered around a value of 32768. Little spread was seen in the levels of binding as reflected by the reciprocal titre (Figure 7-3). In rheumatoid arthritis patients the mean of reciprocal titre (985560) was greater than other groups (3.5 times greater than normals, 5.6 times greater than SLE patients and 2.9 times greater than miscellaneous group) (Figure 7-3). In SLE patients the mean of reciprocal titre (17312) was lower than other groups (1.5 times lower than normals, 5.6 times lower than RA patients and 1.9 times lower than miscellaneous group) (Figure 7-3). The trend was a greater spread of values in patients groups (Figure 7-3).

Binding of rabbit IgG anti-streptokinase and F(ab')2 fragment to human erythrocytes. The binding of rabbit IgG anti-streptokinase and the F(ab')2 fragment to human erythrocytes was studied in presence and absence of NHS together with generated levels of complement activation products (7-3). The purity of prepared rabbit F(ab')2 (contained 3.9 mg /ml protein, 7-2) was checked by IEP (2-9) which showed a different mobility when compared with intact rabbit IgG (Figure 7-4).

IgG and F(ab')2 fragment when incubated with NHS did not result in generation C1s:C1-INH levels compared to NHS alone (Figure 7-5a). In contrast, IgG and F(ab')2 fragment when added to NHS resulted in predominant of C3b-P peaking at 15 minutes (Figure 7-5b).

In the presence of NHS the IgG fraction of rabbit anti-streptokinase bound to human erythrocytes peaking at 11% at 5 minutes. The levels of bound IgG then began to decrease to 5% at 30 minutes (Figure 7-6a). F(ab')2 fragment of rabbit anti-streptokinase IgG in presence of NHS showed a similar pattern of binding to erythrocytes, however the peak binding at 5 minutes was 5% (Figure 7-6a). The levels of bound IgG did not significantly correlate with the levels of complement activation products.

In the absence of NHS the levels of erythrocyte bound IgG were significantly higher than in the presence of NHS (Figure 7-6b). In the presence of NHS peak binding occurred at 5 minutes and then declined, however, in the absence of NHS binding of IgG steadily increased over the 30 minutes incubation (Figure 7-6). At 30 minutes 80% of the E's showed positive for IgG. in sharp contrast F(ab')2 fragment did not show high levels of E's binding. However, at 15 minutes the levels of binding were similar to those for IgG in the presence of NHS (Figure 7-6a, b). The binding of F(ab')2 fragment unlike IgG was not stable (Figure 7-6b).

Inhibition the binding of rabbit IgG anti-streptokinase to human erythrocytes. To investigate whether human Fc fragment can inhibit the binding of rabbit IgG to human erythrocytes (E's), erythrocytes (0.5 X 10^6 E's) were pre-treated with Fc or PBS and the incubated with rabbit IgG (7-4). The human Fc fragment used here was pure as checked by IEP using goat anti-human Fc fragment and anti-human IgG (plate not presented) and was used at a concentration of almost twice (500 μg) that of rabbit IgG (260 μg) to give approximately an equal number of the molecules (500 μg Fc = $6X10^{15}$ molecules and 260 μg rabbit IgG = 1.04 X 10^{14} molecules).

The binding of rabbit IgG was determined using FITC anti-rabbit IgG as the probe. The fluorescent probe alone gave 2.6% binding, this considered as background.

Incubation of E's with rabbit IgG resulted in 55% positive E's, blocking with Fc reduced this value to 29% (Figure 7-7).

Binding of monoclonal anti-FcγR antibodies to human erythrocytes. To study whether monoclonal anti-human FcγR antibodies can recognise any epitopes on the surface of human erythrocytes a set of these mouse monoclonal antibodies directed against FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) were incubated with erythrocytes and probed with FITC anti-mouse detected by flow cytometry (7-5). The negative control consisted of replacing the anti FcγR antibodies with mouse monoclonal anti-human AFP (IgG1).

Of the three monoclonal anti-FcγR used, only anti-FcγRI (CD64) gave a significant positive signal compared to irrelevant mouse monoclonal antibody control (Figure 7-8). Binding with anti-CD64 was 29% compared to 1.9% with anti-AFP (Figure 7-8).

Binding of human IgG to erythrocytes. The binding of three sources of human IgG to erythrocytes. IgG was studied with 3 sources of IgG, prepared by ion exchange chromatography (7-7), caprylic acid precipitation method (2-19) and the serum from a patient with an IgG1 myeloma (7-8). The distribution of the subclasses within these IgG preparations are shown in table 7-1.

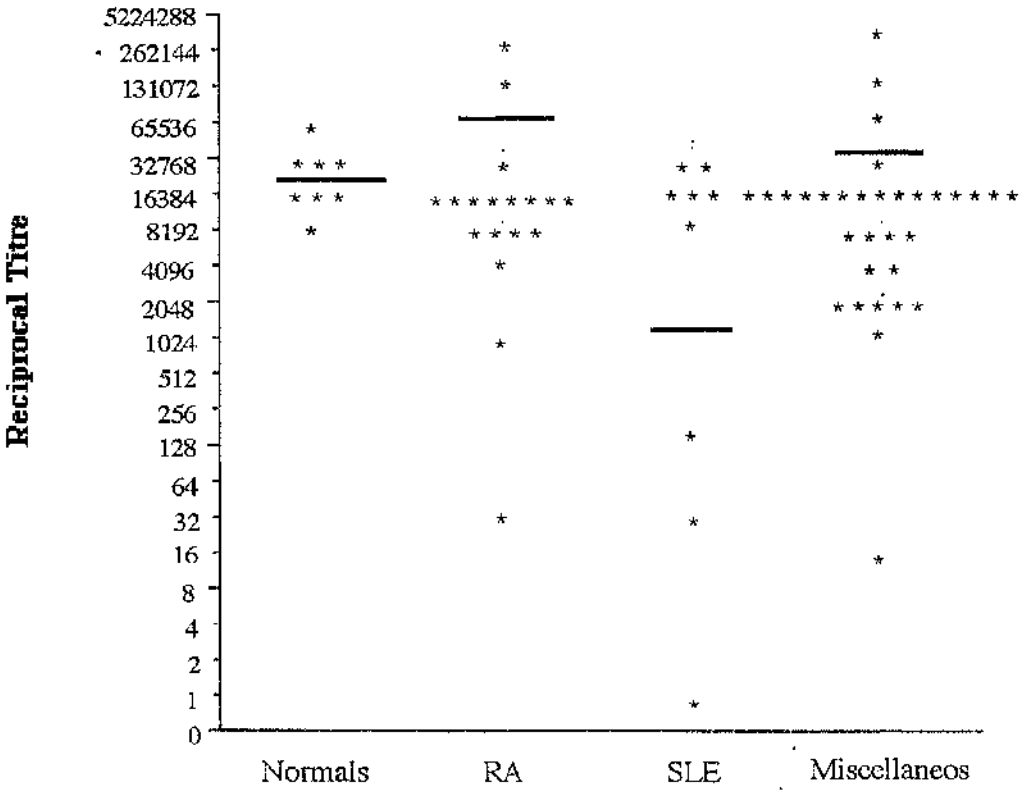


Figure 7-3: Reciprocal titres of indirect haemagglutinin test for binding of rabbit IgG anti-streptokinase to human erythrocytes in different groups.

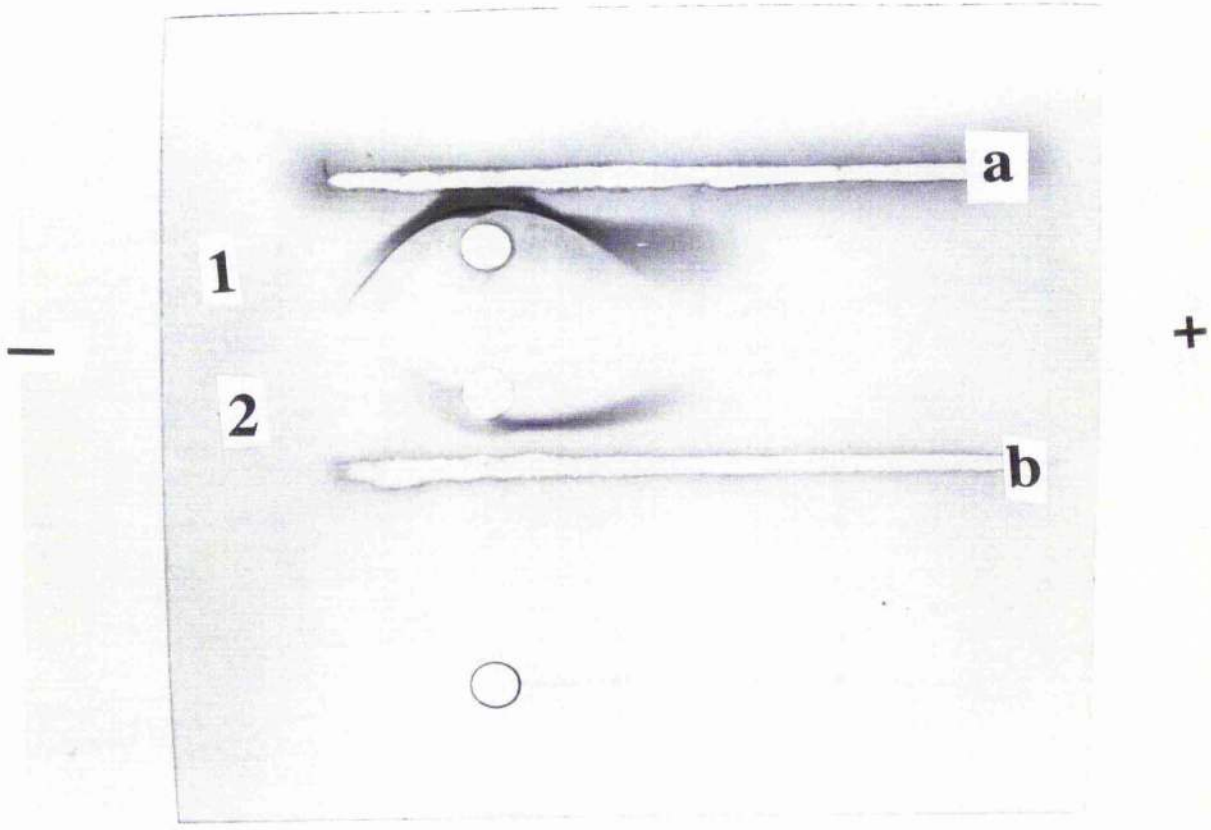
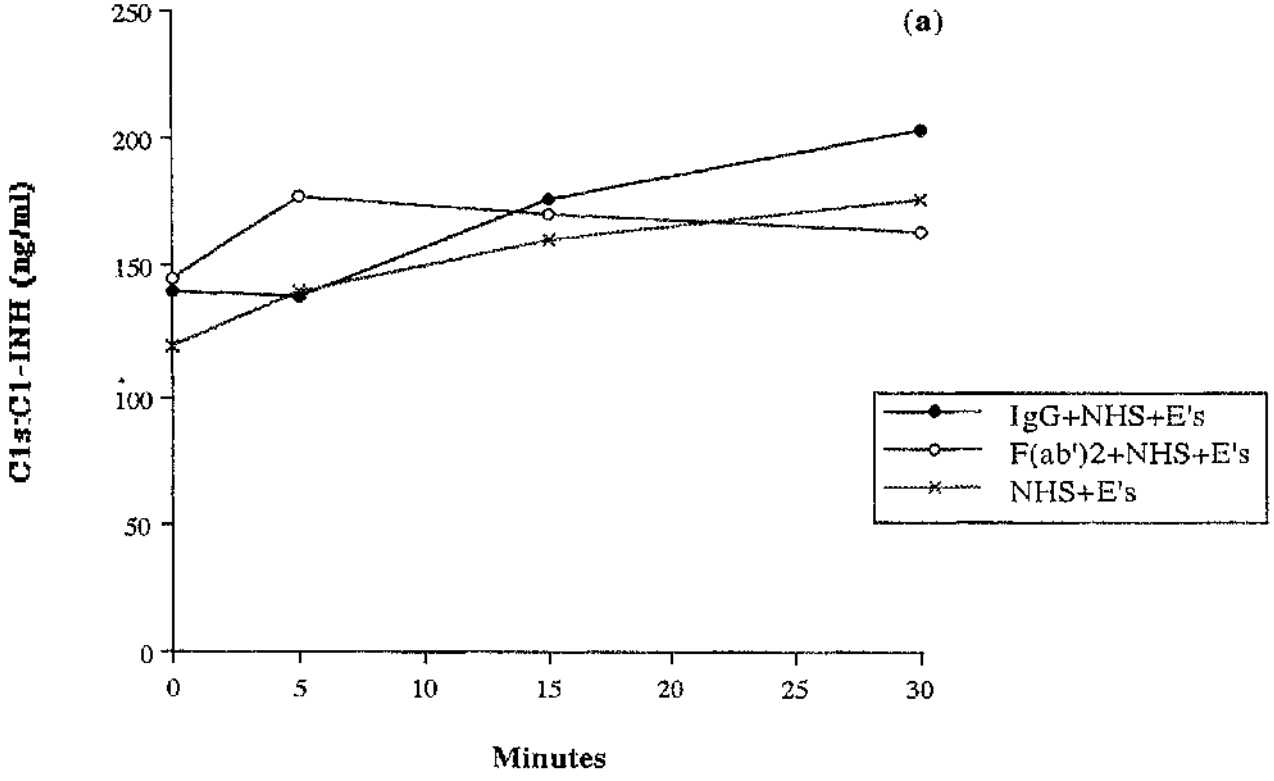


Figure 7-4: Immunoelectrophoresis of rabbit IgG anti-streptokinase and F(ab')2 fragment prepared by pepsin digestion and protein G coupled to Sepharose (Well 1 IgG fraction, well 2 F(ab')2 fragment). After electrophoresis, donkey anti-rabbit IgG was applied to the trough a and sheep anti-rabbit F(ab')2 specific was applied to trough b.



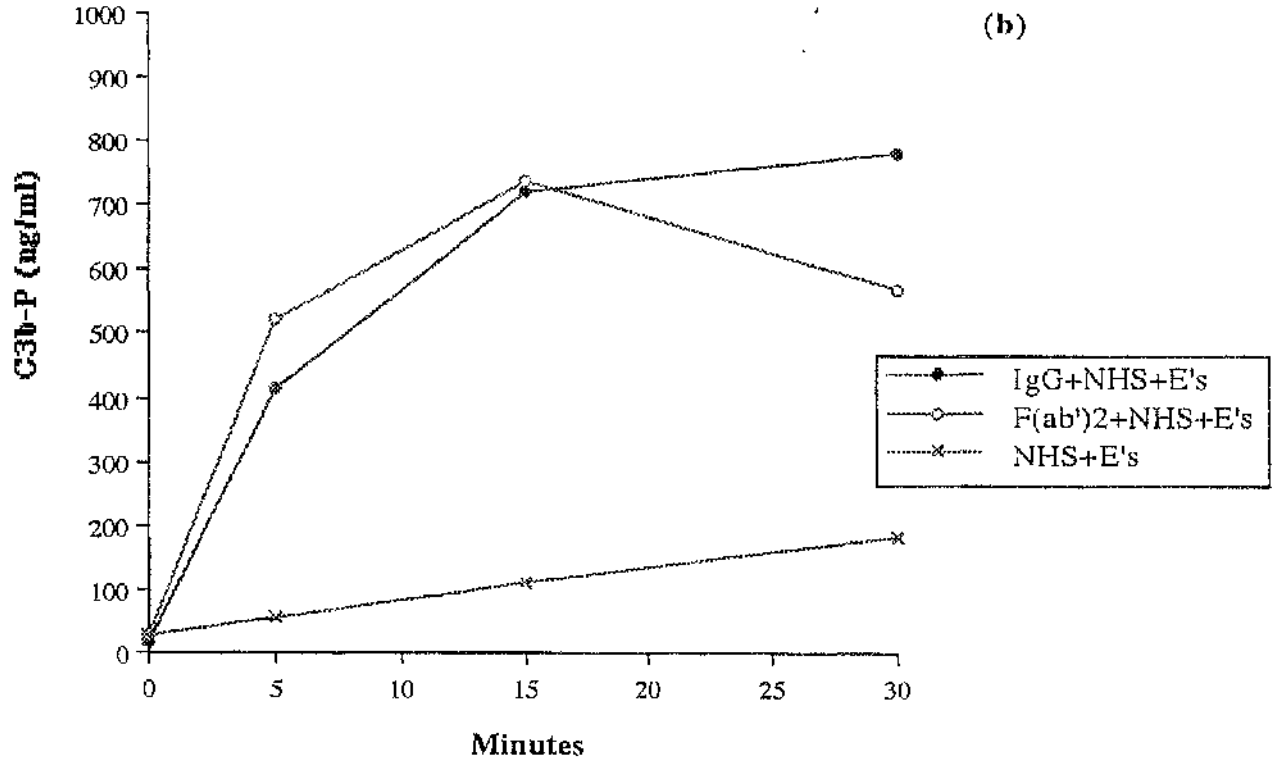


Figure 7-5: Kinetics of complement activation products by rabbit IgG and F(ab')2 fragment of anti-streptokinase antibody.

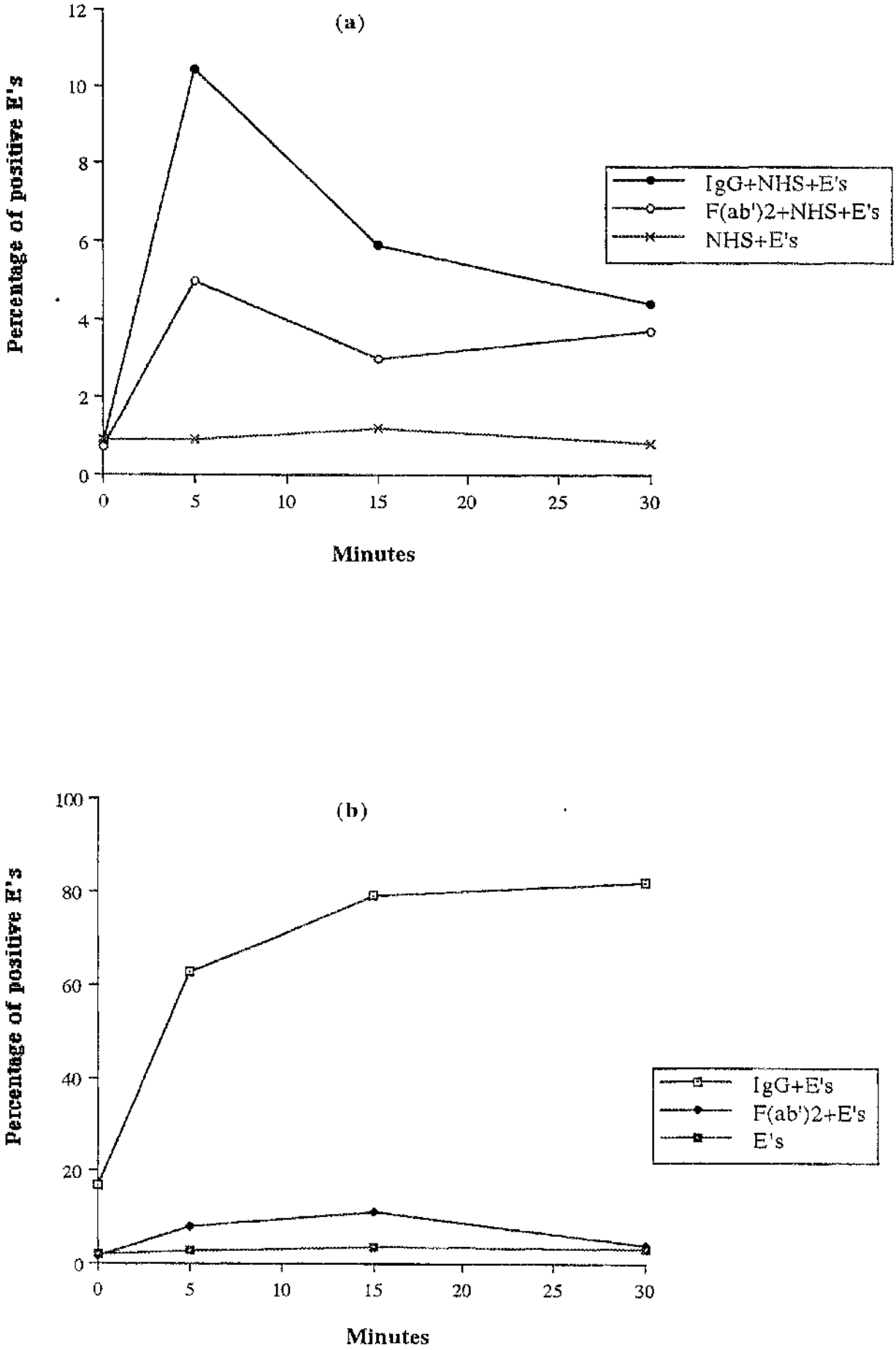


Figure 7-6: Kinetics of IgG and F(ab')2 fragment of rabbit anti-streptokinase binding to human erythrocytes (E's) in presence (a) and absence (b) of NHS detected by FITC conjugated donkey anti-rabbit IgG.

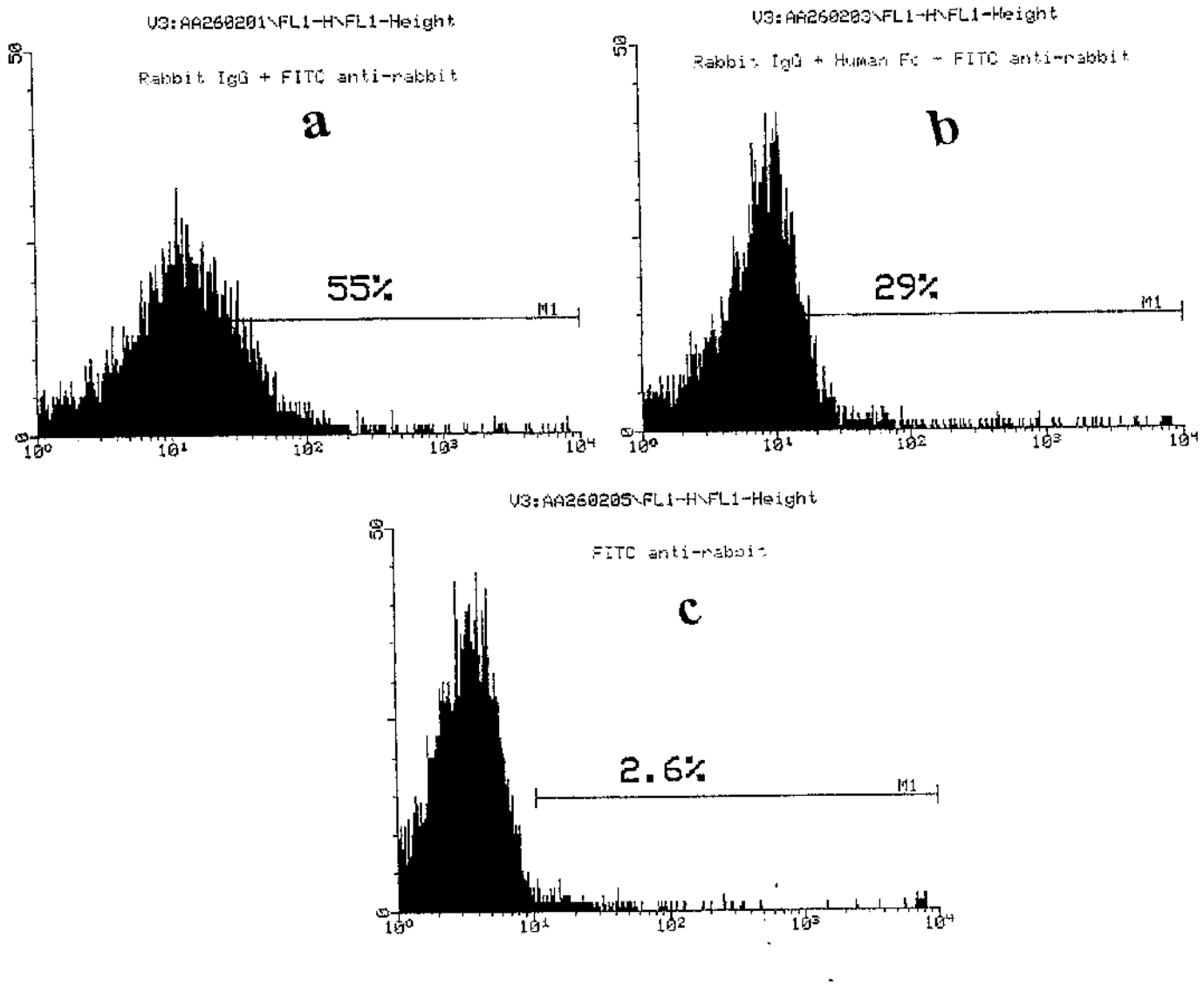


Figure 7-7: Histograms of fluorescence intensity (log scale x axis) of FITC anti-rabbit IgG binding to human erythrocytes (E'). a) E's Incubated with rabbit IgG b) E's pre-incubated with human Fc then incubated with rabbit IgG c) FITC conjugate alone.

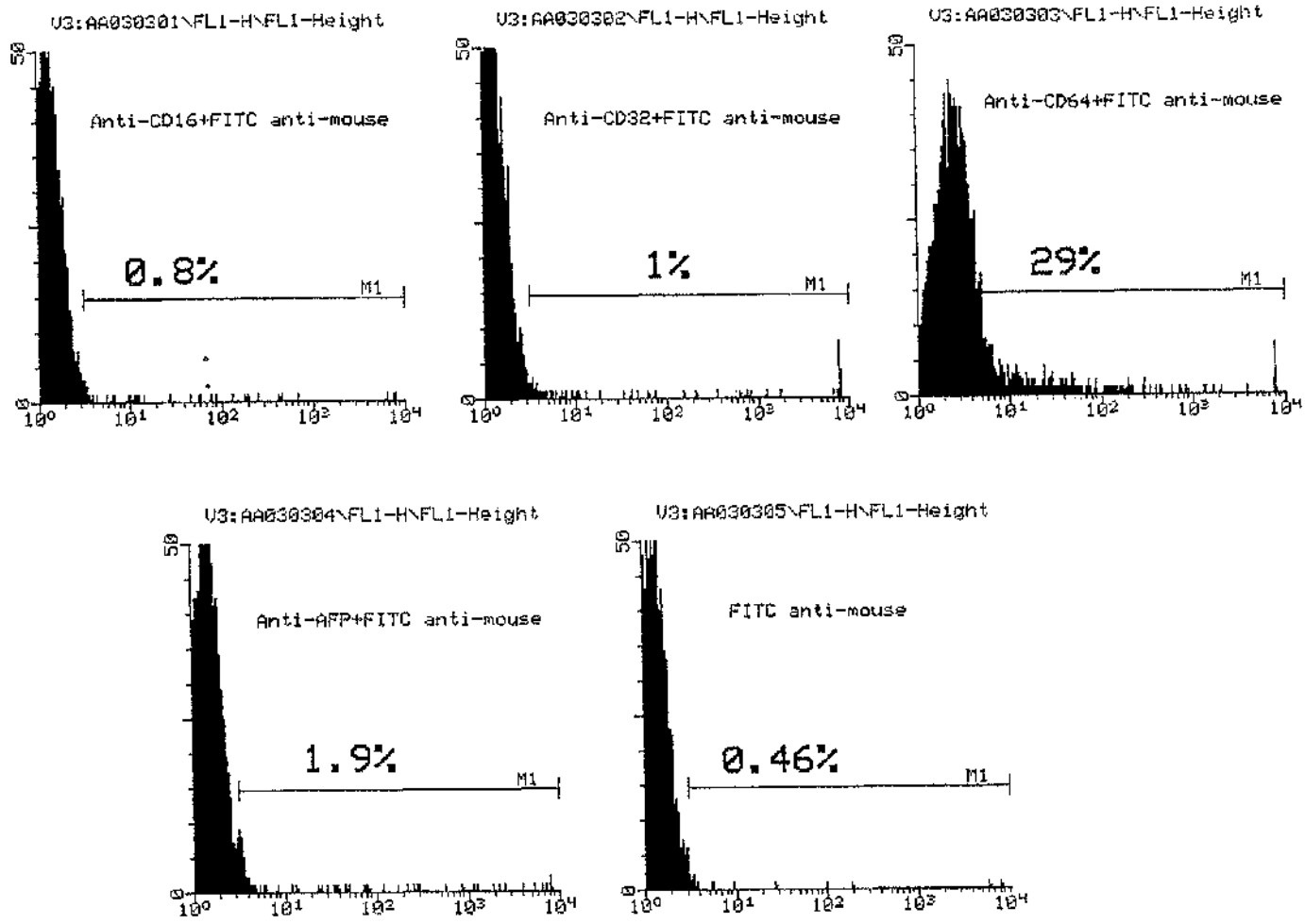


Figure 7-8: The binding of monoclonal anti-FcγR antibodies, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), to human erythrocytes expressed as percentage binding of FITC anti-mouse. Mouse IgG1 monoclonal anti-human AFP incubated with the erythrocytes or erythrocytes alone with FITC anti-mouse IgG were used as

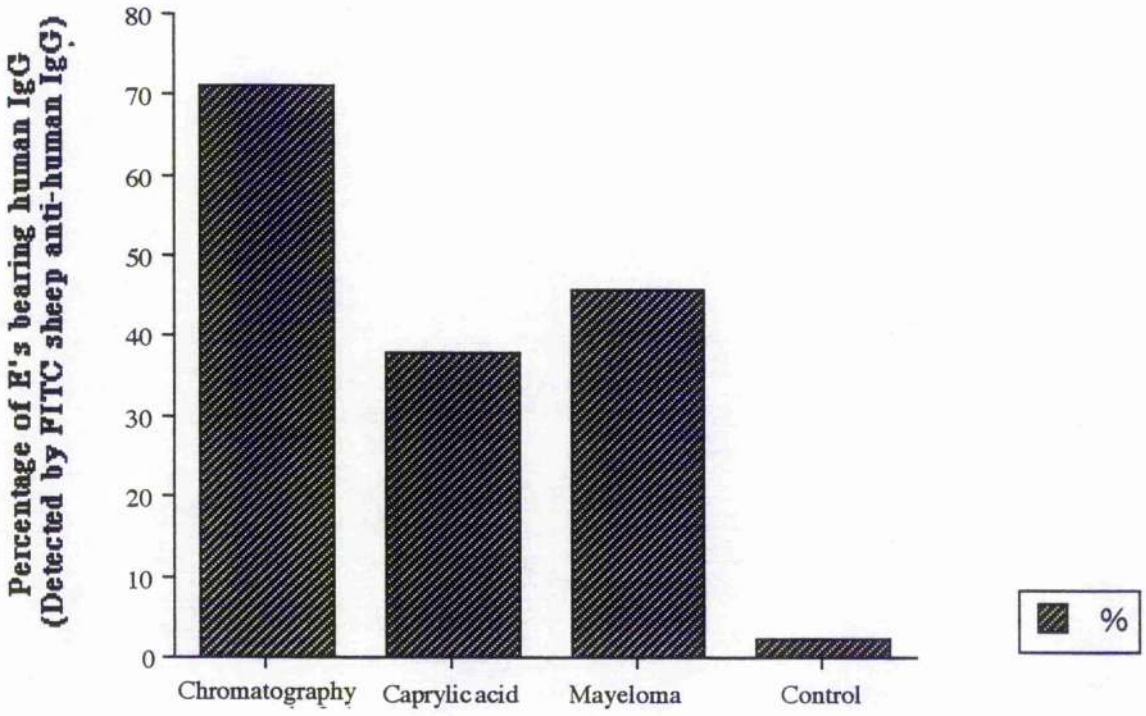


Figure 7-9: The binding of human IgG prepared by ion exchange chromatography, caprylic acid and from myolema patient to erythrocytes (E's), was studied by flow cytometry.

The IgG obtained by caprylic acid purification contained predominantly IgG1, similarly the sample from myeloma patient contained predominantly IgG1. IgG prepared by ion exchange chromatography contained 55% IgG1, 34% IgG2, 7.7% IgG3 and 1.3% IgG4 (Table 7-1). These values are closer to the values observed in normals (58% IgG1, 29.7% IgG2, 6.9 % IgG3 and 4.9 % IgG4).

IgG prepared by ion exchange chromatography bound to a higher extent to E's compared to the other two IgG preparation. By flow cytometry IgG purified by DE52 showed 70% of E's positive, IgG purified by caprylic acid 38%, and myeloma IgG showed 45% binding (Figure 7-9). This reduced erythrocyte binding of IgG was observed when IgG1 concentrations were elevated.

Binding of human IgG subclasses to human erythrocytes. The specificity of IgG subclasses for the 3 forms of Fcγ receptors is well known to differ (Table 1-2), and as IgG from different sources bound differential to E's (see above) the binding of pure human IgG subclasses to erythrocytes was studied (7-9).

Commercial supplies of human IgG subclasses were used to study their binding to human E's. Using the same concentration of each isotype IgG2 showed higher percentage binding to E's (81%) as measured by FACscan (Figure 7-10). This was followed by IgG1 (51%), IgG3 (26%) and IgG4 (5.4%). Little binding of aggregated IgG subclasses was observed (Figure 7-10, Table 7-2).

Binding of mouse IgG subclasses to human erythrocytes. The pattern of mouse subclasses binding to human FcgR is well investigated (Table 1-2). Commercial supplies of the four mouse IgG subclasses were obtained and the binding of a constant amount of the four subclasses to human erythrocytes was studied (7-10). Binding was detected using FITC conjugated rabbit F(ab')2 anti-mouse immunoglobulin, and FACscan analysis (7-10).

All four isotypes in their monomeric form showed some binding to human E's compared to the control (Figure 7-11). Highest binding was seen with monomeric IgG2b (12.44%). Optimal binding however was seen with heat aggregated IgG3 (Figure 7-11, Table 7-2).

Erythrocytes surface biotinylation and purification of putative erythrocyte IgG binding protein. The aim of this procedure was to label the surface proteins of erythrocytes with biotin then lyse the cells in NP40 and affinity purify the IgG binding protein on IgG-Sepharose (7-11). 4 ml of erythrocytes ($5X10^6$/ml erythrocytes) were labelled and the lysate applied a column containing Sepharose 4B coupled to human IgG, 1 ml fractions were collected and the protein concentration was measured at 280 nm (Figure 7-12a).

The bound material was then eluted from the column with Glycine-HCl (pH 2.5) and 1 ml fractions were collected, the protein concentration measured at 280 nm (Figure 7-12b). Application of Glycine-HCl resulted in the elution of a broad protein peak tube 1-12, and single tube peak at 24. The peak protein fractions (Fractions 6 and 24, Figure 7-12b) were pooled and applied to a 7.5 % SDS-PAGE gel and after electrophoresis was transferred to a nitro-cellulose membrane for Western blotting. After blocking and incubating with alkaline phosphatase Avidin-D the no colour was developed.

Discussion

Study of interactions between anti-streptokinase immune complexes and erythrocytes revealed that the binding of the immune complexes is not only a complement mediated interaction but they also can bind to erythrocytes in the absence of complement. Other investigators have shown that immune complexes bind to human erythrocytes in the

absence of complement (Hajos et al 1978, Virella et al 1983), although the involved mechanisms are not clear, and the idea not widely supported.

To study the binding of IgG to erythrocytes two methods were utilised, an indirect haemaglutination assay and FACscan analysis. The indirect haemagglutinin test was used to assess the levels of IgG binding protein on surface of E's among normal individuals and patients groups. This revealed that the binding capacity of human erythrocytes for rabbit IgG differs (Figure 7-4). The patients showed a wider spread in their ability to bind IgG and erythrocytes of SLE patients trended to have lower binding capacity for IgG, the erythrocytes of rheumatoid arthritis patients had a higher binding capacity (Figure 7-4). These findings suggest that the capacity of erythrocytes to bind IgG varies, and greater variation in binding is observed in patients population (Figure 7-4). These findings indicated that the binding was not occurring non-specifically.

To further elucidate binding a comparison between rabbit IgG anti-streptokinase and F(ab')2 fragment binding to human erythrocytes (7-3) revealed that rabbit anti-streptokinase IgG bind significantly to human erythrocytes, however, F(ab')2 fragments do not (Figure 7-6b). These observations implied that specific binding of IgG occurs through Fc portion and not F(ab')2 fragment.

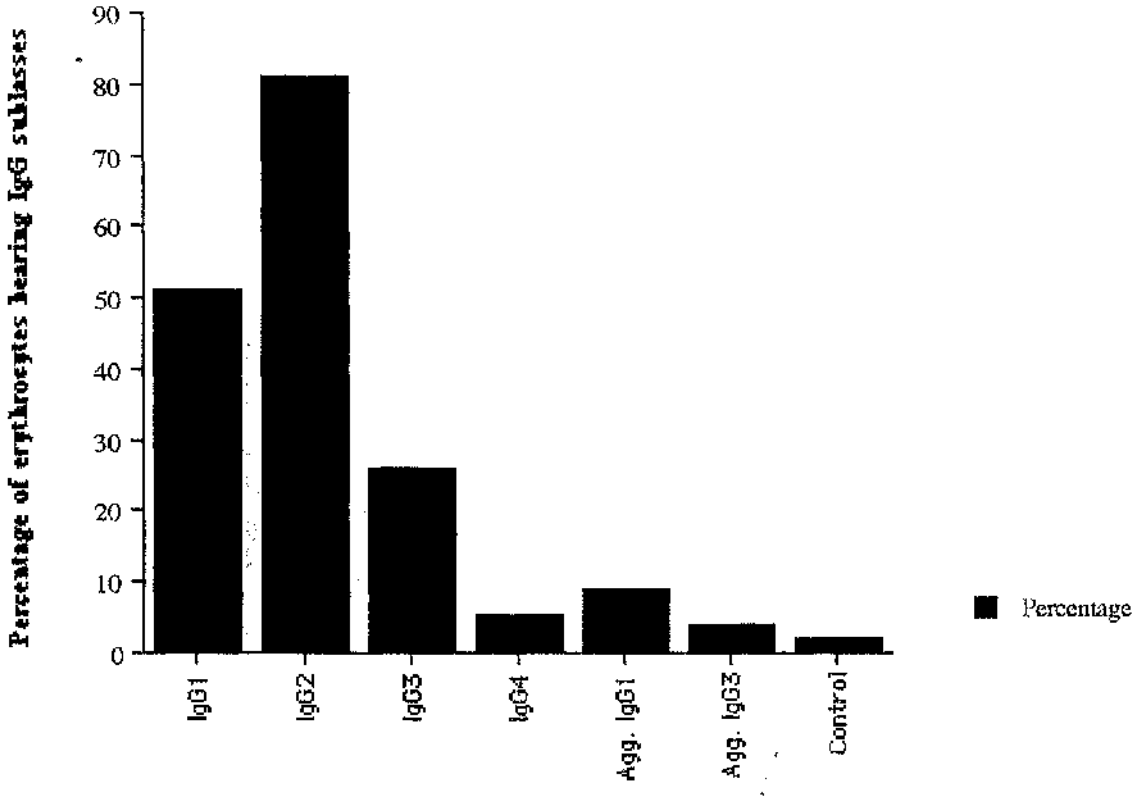


Figure 7-10: The percentage binding of human IgG subclasses to erythrocytes, as measured by flow cytometry (Agg.=aggregated, E's=erythrocytes)

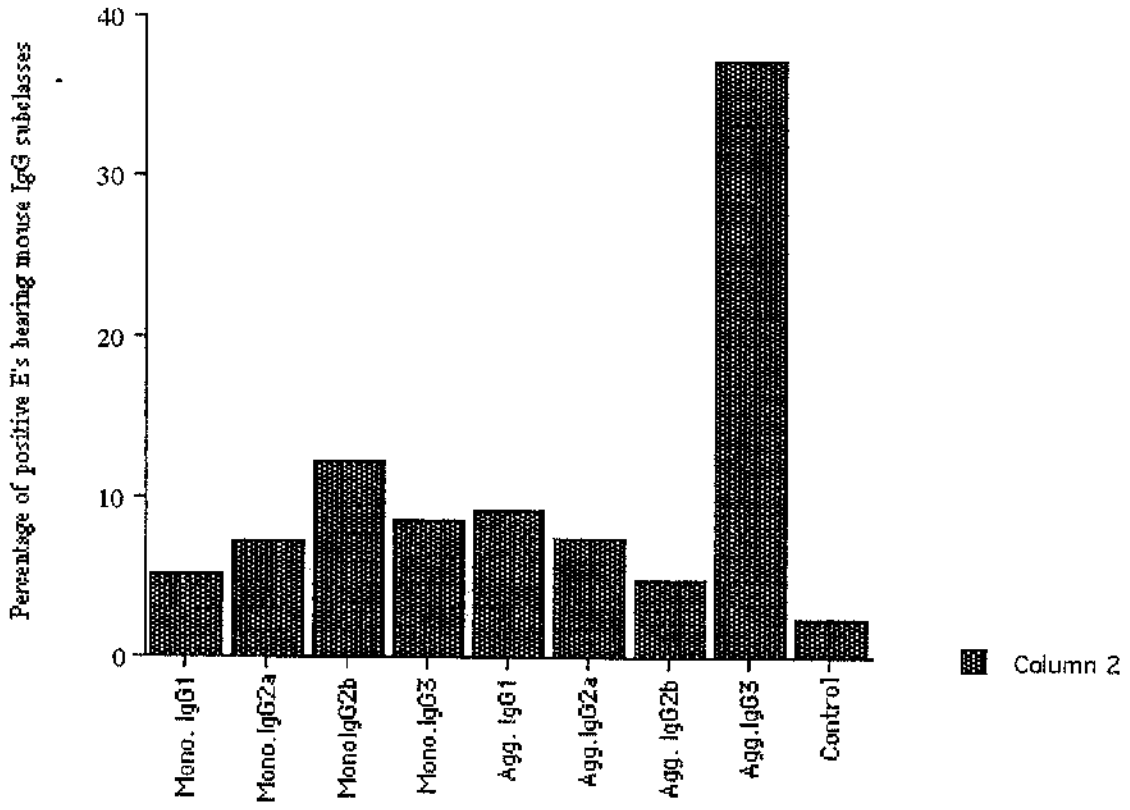


Figure 7-11: Percentage binding of mouse IgG subclasses to human erythrocytes (E's). (Mono=monomeric, Agg.=aggregated)

	Percentage of subclasses			
IgG Source	IgG1	IgG2	IgG3	IgG4
Chromatography	55%	34%	7.7%	1.3%
Caprylic acid	90%	5.7%	2.4%	1.8%
Myeloma	95%	2.7%	0.8%	1.3%

Table 7-1: The proportion of IgG subclasses in 3 sources of IgG, prepared by ion exchange chromatography, by caprylic acid precipitation and the serum of a patient with myeloma.

IgG subclass	Monomer	Aggregated
Mouse	IgG2b>IgG3, IgG2a>IgG1	IgG3>>>IgG1>IgG2a>IgG2b
Human	IgG2>IgG1>IgG3>>>IgG4	IgG1 > IgG3

Table 7-2: Affinity of human and mouse IgG subclasses for binding to human erythrocytes using FITC rabbit F(ab')2 anti-mouse immunoglobulin and FACscan analysis.

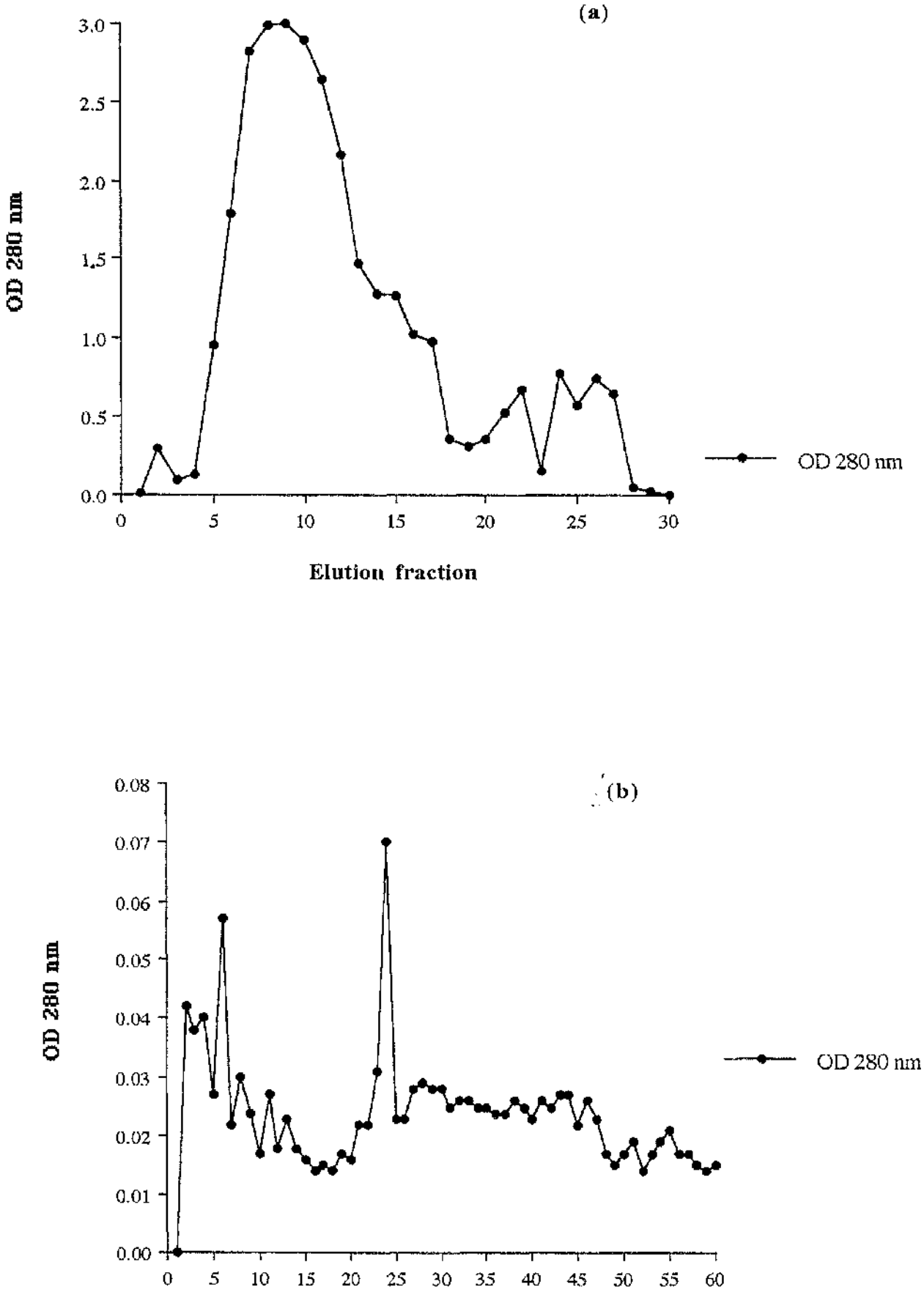


Figure 7-12: Elution profile of lysed erythrocytes washed through a Sepharose column coupled to IgG (a) and the bound material to the column was then eluted by Glycine-HCl pH 2.5 (b).

Using flow cytometry human IgG (Fc fragment) was shown to inhibit the binding of rabbit IgG to human erythrocytes by 26% (Figure 7-7) indicating that both rabbit IgG and Fc fragment of human IgG bind to the same sites on the erythrocytes. This was in agreement with Hajos et al 1978 who using a Coombs test showed that Fcγ fragment and not the Fab fragment inhibits the binding of human IgG and immune complexes to erythrocytes.

The presence of Fc R on the red cells in rabbit, guinea pig, sheep, birds, reptiles, amphibian has been reported previously (Hajos et al 1978), however, in human the presence of FcR on the erythrocytes is still very controversial. Using a set of monoclonal anti-human FcγR antibodies [(FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16)], revealed that anti- FcγRI (CD64) is detectable on human erythrocytes (Figure 7-8). Fcγ RI (CD 64) (MW = 72 kD) is a high affinity receptor for monomeric IgG although the binding of aggregated IgG to the receptor is reported to have the same affinity as monomeric IgG (Hulett & Hogarth 1994). This receptor that it is expressed on monocytes and naive macrophages has 3 isoformes (isoforms A, B and C) which only the specificity of IgG subclasses for isoform A has been documented (Table 1-2). Currently one method of FcγR typing on leukocytes and monocytes is to study the binding of human and mouse IgG subclasses, for which their are recognised binding patterns (Table 1-2). Studies by other groups confirm that the specificity and affinity of IgG subclasses for Fcγ receptors (CD16, CD32 and CD64) is different (Hulett & Hogarth 1994) (Table 1-2), however, the ligand specificity for many of the Fcγ receptor isoforms is not fully elucidated.

The binding of human subclasses to erythrocytes were studied by FACscan, this revealed optimal binding with IgG2, followed by IgG1 and IgG3 with little binding of IgG4 (Figure 7-10) (Table 7-2). This pattern was not consistent with the patterns of IgG subclasses specificity and affinity for binding to any of the recorded Fcγ receptor isoforms.

The affinity of mouse IgG subclasses for binding to human erythrocytes showed optimal binding with aggregated IgG3 (Figure 7-11). All the monomeric and aggregated mouse IgG subclasses showed some binding compared to the control. The preferential binding of IgG3 (Aggregated), compared to IgG2b and IgG2a (Figure 7-11, Table 7-2) is consistent with the binding pattern of CD16 (isoforms A and B) and CD64 (isoform A).

These results together would support the presence of CD64 on the surface of erythrocytes (Figures 7-8 and 7-11, Table 7-2) or an IgG binding protein with a similar specificity as CD64. However, human IgG subclasses binding to erythrocytes did not follow a pattern consistent to CD64, in fact the binding pattern was not consistent with binding sequences to any known Fcγ receptor.

Initial attempts to purify the IgG binding protein on erythrocytes were not successful, however clarifying the existence of such a protein will have an important physiological role in handling immune complexes and IgG.

Chapter 8
Final Discussion

Streptokinase is now a commonly administered thrombolytic agent in patients with AMI. The administration of streptokinase in patients suffering from AMI results in heart muscle reperfusion and reduces the mortality rate in these patients. Streptokinase has the same efficacy as alternative thrombolytic agent i.e. r-tPA (GISSI-2, 1990, White et al 1990) but is often chosen preferentially as it is about ten times cheaper (Sharma & Sashara1982). One of the main disadvantages of the streptokinase administration is that as foreign antigen it may provoke hypersensitivity reactions due to previous streptococcal infections. In addition pre-existing antibodies are thought to reduce the lytic effect of streptokinase (Garner & Tillet 1935, Lew et al 1984, Massel et al 1991, Bom et al 1993).

The work carried out in this thesis aimed to address humoral immunity to streptokinase. Several areas of research were encompassed. These will be discussed and drawn together in this final chapter.

Firstly, ELISA's were developed to measure the levels of IgG, IgA, IgM, IgE and neutralising anti-streptokinase antibodies in a normal population (246 normal individuals), to find out to what extend these antibodies exist in the normal population. Serum samples were also analysed for neutralising anti-streptokinase antibodies using a commercial ASK-kit (Functional assay). This assay is still commonly used and more importantly it has been developed into screening microcards by TAS (Thrombolytic assessment system), to enable bed side rapid testing of new AMI patients for antibody levels to streptokinase.

The ELISA's were developed using as antigen the streptokinase used for therapeutic use. Molecular weight and purity of the commercial streptokinase used in different assays by SDS-PAGE revealed two close bands with molecular weights

of 47 kD and 50 kD (Figure 3-1). This is in agreement with previous reports on 2 close band corresponding to streptokinase of molecular weight at approximately of 47 kD (Lynch et al 1993). The presence of two bands has been observed by other investigators and reported to be due to the effect of de-amidation of the streptokinase molecule by alkaline buffer (Dillon & Wannamaker 1964, Hugh 1965, Einarsson et al 1979, Lynch et al 1993).

IgG, IgM, IgA, IgE and neutralising anti-streptokinase antibodies levels were measured in the normal group and normal ranges for each antibody isotype were established (Table 3-2, Figure 3-8a, b, c and d). Only a small proportion of the normal individuals had elevated levels of anti-streptokinase antibodies (IgG 1.68%, IgA 2.7%, IgM 9.9% and neutralising anti-streptokinase antibodies 0.4%) (Table 3-2).

The patients with AMI showed a higher incidence of elevated anti-streptokinase antibodies in particular for IgM (80% streptokinase patients group and 50% r-tPA group). This observation was confirmed in a further 4 AMI patients (Chapter 6) and would suggest that this is not to limited studied sample size of AMI. IgM response to streptokinase can be accounted for a recent streptococcal infection in these individuals and 2) As fibronectin and streptokinase share a similar epitope (Gonzalez-Gronow et al 1993) cross-reactivity of antibodies against fibronectin (or fibronectin combined with other body constituents) with streptokinase.

In present study the levels of neutralising anti-streptokinase antibodies which measuring the efficiency of anti-streptokinase to prevent clot lysis by streptokinase correlated with the levels of IgG anti-streptokinase antibodies ($r=0.55$ $p<0.001$), this is in agreement with Hoffmann et al 1988 who studied a group of 61 individuals with AMI, there was a significant correlation between the levels of IgG and neutralising anti-streptokinase antibodies ($r= 0.43$, $p<0.001$). The group of normals that were studied represented a group of Caucasians from limited demographic area, therefore the proportion of the population with elevated anti-

streptokinase antibodies in other normal population groups may vary according to their prevalence of streptococcal infections and normal ranges must be established based on population being screened.

In vitro and in vivo studies of quantitation of neutralising anti-streptokinase in presence of r-tPA, impairs the detection of these antibodies (Chapter 3). This effect has not to date been reported and indicates that the neutralising assay is not a suitable method for clinical diagnosis of anti-streptokinase antibodies levels in patients since any alteration to the coagulation system due to anticoagulant therapy (i.e. heparin, r-tPA etc.) may interfere with these assays.

Administration of streptokinase in 10 patients studied, resulted in an immediate fall in level of IgG (at 15 minutes, $p<0.01$) and neutralising anti-streptokinase antibodies (at 30 minutes, $p<0.01$) (Figure 3-10). The observed reduction in the levels of the antibodies could not be accounted for by changes in plasma volume during the clinical procedure (Figure 3-13) and is therefore considered to be due to sequestering of specific anti-streptokinase antibodies into an immune complex with the administered streptokinase. The sequestering of antibodies into an immune complex is further supported by C1s:C1-INH formation occurs in ongoing classical pathway activation predominately through ICs. The decrease in levels of specific antibodies after streptokinase administration has been observed by other workers Lynch et al 1991, Fears et al 1992).

There was no significant difference in rate of reperfusion between patients given streptokinase or r-tPA. All the patients when received streptokinase and reprofused within 2 hours of streptokinase administration (early reperfusion), had levels anti-streptokinase antibodies which were within the normal ranges, however, the mean values increased in late reperfusion and none reperfusion groups (Table 3-6). To ensure that circulating anti-streptokinase antibodies do not prevent the therapeutic efficiency of the administered streptokinase, the prescription is generally given as a large single dose intravenously (usually 1200000 units) over one hour (Crossland

1980). Two of patients treated with streptokinase had elevated levels of anti-streptokinase IgG prior to treatment (patients EM and CD), the levels of IgG anti-streptokinase antibodies in these individuals took longer to drop than those with lower levels (Figure 3-11a), patient CD reperfused late and patient EM did not reperfuse. The results from this small patient group study implies that elevated levels of antibodies prevent the thrombolytic effects of streptokinase. In individuals with large antibody levels the amount of streptokinase administered is probably not sufficient to overcome these antibodies therefore a second dose may be necessary. An immune response in all the patients administered streptokinase was observed since all of them had elevated levels of specific antibodies to streptokinase by day 5 (Figure 3-10a and b). This finding would suggest that if further thrombolytic therapy is required and streptokinase is the choice, this must be administered with hours of first bolus. White et al 1990 showed that patients with AMI re-treated with streptokinase 3-5 days after initial treatment do not exhibit a better response, he noted that develop immunological reactions were more common than after the initial dose. This is not surprising since at this period the levels of antibodies are elevated in response to streptokinase administration, this would be amplified of the patients already had pre-existing elevated levels.

Patient EM with elevated levels of IgG and neutralising anti-streptokinase antibodies, did not develop adverse reaction to streptokinase administration but was in the group of non reperfusion patients (Table 3-6). The poor response to streptokinase treatment due to elevated anti-streptokinase antibodies has been reported by other investigators (Lew et al 1984, White et al 1990, Massel et al 1991 and Bom et al 1993). In this study three of the patients with normal levels of anti-streptokinase antibodies did not respond to streptokinase treatment and belong to the 25% of patients who fail to reperfuse (Anderson et al 1991, Chouhan et al 1992) as the antibody levels are low. The cause of this failure must be due to other factors that prevent the thrombolytic effect of streptokinase. One candidate is elevated levels of lipoprotein-a, this area has not been fully investigated (1-3).

Of the 10 patients given streptokinase, one patient (Patient CD, with levels of IgG and IgA above the normal range) developed serum sickness. In this patient significant reduction (80%) in the levels of IgA at 30 minute after streptokinase administration was observed (Figure 3-11b). This is in contrast to the result of patient EM who had elevated IgA, but in this case the levels did not drop as rapidly. Stewart et al 1990 using DNP-BSA ICs showed that ICs complexes containing IgA isotype precipitate more easily than IgG and IgM isotypes. This may imply a very important role for IgA anti-streptokinase isotype in provoking serum sickness in the patients treated with streptokinase.

From the present study it is clear that elevated levels of the pre-existing anti-streptokinase antibodies are responsible for both failure of reperfusion and initiating adverse immunological reactions, and among many types of methodology described for measuring anti-streptokinase responses, the ELISA assays can predict both failure of treatment and adverse reactions. However, a larger sample size of patients should be studied to evaluate the role of specific isotype in provoking adverse immune reactions to administered streptokinase more clearly.

The general population is commonly exposed to streptokinase as this antigen is released during streptococcal infections (throat, skin, nasal, ears, etc.). After analysis of a normal population and a group of AMI we were keen to establish what levels were like in patients with auto-immune diseases (RA, SLE, thyroditis and HSP). The levels of IgG, IgM, IgA and neutralising anti-streptokinase antibodies in the patient group described were measured and the values compared to the established normal ranges (Table 4-1b Figures 4-1, 4-2 and 4-3). Although the levels of anti-streptokinase antibodies in these patients were not statistically elevated a significant proportion of the patients had elevated levels of the antibodies compared with the normal population.

Patients with RA had significantly elevated levels of specific IgM anti-streptokinase antibody levels (Figure 4-2, Table 4-2) even when rheumatoid factor was removed.

The elevated anti-streptokinase antibodies in R.A. patients could be attributable to: a) the association of streptococcal infections b) the effect of immuno-suppressive therapy on these patients c) as fibronectin and streptokinase share a similar epitope (Gonzalez-Gronow et al 1993). In this chronic inflammatory disease, the presence of auto-antibodies against fibronectin (or fibronectin combined with other body constituents) may show cross-reactivity with streptokinase and d) the presence of excess amounts of rheumatoid factor in the sera of theses patients (IgM isotype) enhances the results which are obtained by ELISA's.

Specific anti-streptokinase antibodies were not related to any concomitant rise in the super antigen mHSP65 in RA, SLE, thyroiditis and HSP patients. Elevated levels of anti-mHSP65 have been demonstrated in RA patients (Bahr et al 1988). We could not support this observation in the RA patients, or patients with SLE, thyroiditis or HSP as the levels of IgG anti-mHSP65 in this group were not statistically elevated compared to the normal individuals (Figure 4-5). On the other hand, in patients with Henoch Schonlein Purpura there was a correlation between IgG anti-mHSP65 levels and IgG, IgA and neutralising anti-streptokinase antibodies levels (Figures 4-6 and 4-7). This although was not highly significant based on a small number of patients, it however, dose raise the possibility that streptococcal infections and the release of heat shock proteins may play an important role in the aetiology of HSP and from this data it can be concluded that streptococcal infections have an association in the pathogenesis of Rheumatoid Arthritis.

From the study of the anti-streptokinase antibodies levels in AMI patient it was not clear why elevated levels of anti-streptokinase antibodies were not always accompanied by adverse immunological reactions. The complement system has a central role in processing immune complexes and preventing tissue damage, so the interactions of complement system and immune complexes was studied (1-8). This was performed by measurement of complement activation products C1s:C1-INH,

C3b-P and C5b-9 generated by thyroglobulin and streptokinase ICs in NHS. An indirect ELISA's for measuring serum C3d levels and modified ELISA for detecting erythrocytes bound C3d were developed to evaluate the relationship of bound C3d and the levels of free complement components (5-4).

As mentioned previously in vivo studies in patients administered streptokinase showed generation of C1s:C1-INH while if any detectable C3b-P, C5b-9. In patients treated with r-tPA no change in the levels of mentioned complement activation observed. The activation of complement system after r-tPA administration has been reported although the extent of this activation is substantially lower than streptokinase (Agostoni et al 1994).

The correlation of C1s:C1-INH with IgG, IgA levels and neutralising antibodies clearly confirmed that the activation of classical pathway was due to formation of anti-streptokinase immune complexes during treatment. This is in agreement with Freydottir et al (1993), who analysed the extent of complement activation in patients administered streptokinase, the levels of complement activation were assessed by the extent of C3d production and occurred in those individuals with highest levels of anti-streptokinase antibodies.

The antigen streptokinase is somewhat unusual in that it may trigger complement activation by two mechanisms, through plasminogen activation and generation of plasmin or through immune complexes formation (1-10). Generation of plasmin by streptokinase in turn influences the detectable levels of complement activation products by activation of the these pathways, by binding to C1 inhibitor or by proteolytic effects (Levi et al 1993, Cugno et al 1993). In vitro study confirmed this and showed that not only streptokinase at therapeutic dose causes detectable levels of C1s:C1-INH and C3b-P but streptokinase or plasmin can significantly change the levels of these complement activation products generated by thyroglobulin ICs (Chapter 5).

In vitro studies of the effect of ICs on complement activation showed that the nature of immune complexes and the way they are formed influence the extent and the rate of complement activation pathways, i.e. preformed streptokinase ICs (human and rabbit) at antigen excess activated the classical pathway to a greater extent than antibody excess. This was reversed for the alternative pathway, preformed thyroglobulin ICs generated higher levels of C3b-P than the nascent ICs (Chapter 5). This study clearly illustrated that different ICs activate complement system in different ways and results can not be interpreted from model ICs.

Many in vitro systems for studying complement activation do fail to consider the effect of erythrocytes. As this mirrors more closely the in vivo situation, the effect of E's were examined. The presence of erythrocytes did not modulate significantly free and bound C3d or C1s:C1-INH levels generated by streptokinase and thyroglobulin ICs, but exert an influence on C3b-P (Chapter 5).

Erythrocytes have an important role in clearance of immune complexes from the circulation and the prevention of tissue damages. This is predominately through the action of complement receptor CR1. The interactions between ICs and E's is governed by the complement system and the nature of immune complexes, ineffective processing may lead to immune complexes deposition in tissues outside the liver and spleen (Schifferli et al 1989).

The kinetics and dose response of streptokinase immune complexes binding to erythrocytes were studied by flow cytometry and its relationship with complement activation was determined by measuring the C1s:C1-INH, C3b-P and C3d in the accompanying supernatants. The study of thyroglobulin immune complexes and erythrocytes interaction showed that the binding of the ICs not only significantly correlates with the classical and alternative pathways activation (r values 0.98 and 0.91 respectively, $p<0.001$) (Figure 6-2) but is dose dependent ($p<0.001$). The kinetics and dose response of rabbit streptokinase ICs binding to erythrocyte gave similar result (Figure 6-3). This was in agreement with the results of Lucisano et al

1991, who showed that binding of immune complexes to erythrocytes correlated with the degree of complement consumption and related to ICs nature. Using a model of NIP-BSA ICs (IgG, IgM and IgA isotypes) he noticed that optimal binding occurred by isotypes that cause significant classical pathway activation (IgG1, IgG2 and IgM). In the present study the binding of human streptokinase ICs to E's by the classical pathway activation did not significantly differ from the alternative pathway activation (Figure 6-19), however, it is important to study the effect of alternative pathway to a greater extent.

The kinetics of E-bound streptokinase ICs (Human and rabbit) during 30 minutes incubation at 37°C showed a peak by 5 minutes and after which the levels declined corresponding to their release from the erythrocytes surface (Figures 6-5a, Figures 6-14a, 6-14b and 6-15a). The release of ICs has been previously reported by Medof & Prince 1983 and can be explained by ongoing complement activation and degradation of C3b, incorporated in ICs, to the fragments C3c and C3d which have no affinity for CR1 receptors on the erythrocytes. This pattern of binding was seen with C3c and the results could indicate its corresponding pattern of the ICs, as both E bound ICs and C3c followed a similar pattern (Figure 6-7). However, C3d remained on erythrocytes surface as detected by FACscan analysis or modified ELISA (Figure 6-8). C3d binding is a covalent and these results suggest that the binding predominately is not dependent on CR1 and is generalised on E's surface. The deposition of C3d on erythrocytes therefore indicates that the complement system is activated and may be used in clinical situations. Davies et al 1990 showed that C3d was detectable on the surface of E's during serum sickness due to streptokinase treatment. Freysdottir et al 1993 by studying 13 AMI patients treated with streptokinase reported that C3d is detectable on E's from 1hour to day 7 after treatment.

The presence of erythrocytes treated with anti-CR1 (E-CR1) did not inhibit the binding of human ICs to erythrocytes as detected by FITC anti-human IgG,

however, decreased the levels of E's bound ICs detected by FITC anti-human IgA and IgM (Figures 6-14 and 6-15). Immune complexes in absence of NHS bound to erythrocytes (6-5). This observation may account for binding of ICs containing IgG to erythrocytes through a second mechanism, possibly Fcγ receptor (Hajos et al 1978).

By studying the levels of anti-streptokinase antibodies the 4 patients with AMI treated with streptokinase and the extent of complement activation similar results were obtained to those obtained for the first 10 patients studied in chapter 3 which were indicative of immune complex formation and complement activation during streptokinase treatment (6-12). The levels of bound IgA, IgM, C3c and C3d were not detectable on erythrocytes in the 4 patients. Freysdottir et al 1993 showed that the levels of bound C3d on erythrocytes are detectable from the first hour of administration of streptokinase, however I could not detect E-bound C3d from 5 minutes to 30 minutes. In contrast to Freysdottir et al 1993 streptokinase immune complexes as detected by FITC anti-human IgG were detectable on erythrocytes in one of the patients (Patient T.B.) (Figure 6-23). In this patient the levels of serum anti-streptokinase IgG were not significantly higher than other patients however this patient generated higher levels of C1s:C1-INH and C3b-P and the levels of both E-bound ICs and serum anti-streptokinase IgG inversely correlated with one another ($r=.99$, $p<0.001$) (Figure 6-23). This observation confirmed the in vitro studies that the clearance of streptokinase ICs was dependent on the extend of complement activation. Furthermore, as IgA is a poor activator of the classical pathway (Law et al 1988, Holme et al 1989, Lucisano et al 1991). Patients with elevated levels of IgA anti-streptokinase are at potential risk of developing serum sickness and this may account for adverse reactions seen in patient CD who had elevated levels of anti-streptokinase IgA with high affinity (Chapter 3).

The study of streptokinase immune complexes interaction and erythrocytes has revealed that the binding of the immune complexes is not only a complement

mediated interaction but they can also bind to erythrocytes in absence of complement, this is in agreement with other investigators (Hajos et al 1978, Virella et al 1983), although the involved mechanisms are not clear, and not generally considered

The presence of erythrocyte surface IgG binding was studied (Chapter 7). An indirect haemagglutinin test among normal individuals and patients groups revealed that the capacity of human erythrocytes to bind rabbit IgG is different (Figure 7-4) indicating that the binding is not non-specific adsorption phenomena. This binding is Fc specific as, F(ab')2 fragment do not bind to the erythrocytes (Figure 7-6b). Furthermore, using flow cytometry human IgG (Fc fragment) inhibited the binding of rabbit IgG to human erythrocytes (Figure 7-7) indicating that both rabbit IgG and Fc fragment of human IgG bind to the same sites on the erythrocytes. This is in agreement with Hajos et al 1978 who using a Coombs test showed that Fcγ fragment and not Fab inhibits the binding of human IgG and ICs to erythrocytes.

Using a set of monoclonal anti-human FcγR antibodies revealed that anti- FcγRI (CD64) is detectable on human erythrocytes (Figure 7-8). It is known that the specificity and affinity of IgG subclasses for binding to the Fcγ receptors is different (Table 1-2). The binding of human IgG subclasses to erythrocytes (Figure 7-10) (Table 7-2) was not consistent with the patterns of IgG subclasses specificity and affinity for binding to any of the recorded Fcγ receptors isoforms. However, the affinity of mouse IgG subclasses for binding to human erythrocytes (Figure 7-11) (Table 7-2) followed the pattern of CD16 (isoforms A and B) and CD64 (isoform A).

These results together would support the presence of CD64 on the surface of erythrocytes or an IgG binding protein with a similar specificity as CD64. However, human IgG subclasses binding to erythrocytes did not follow a pattern consistent to CD64, in fact the binding pattern was not consistent with binding sequences to any known Fcγ receptor.

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