Clin Exp Immunol 1993; **94**:286–290

Evaluation of *in vivo* immune complex formation and complement activation in patients receiving intravenous Streptokinase

J. FREYSDOTTIR, S. ORMARSDOTTIR* & A. SIGFUSSON *Departments of Immunology and *Medicine, The National University Hospital, Reykjavik, Iceland*

(Accepted for publication 6 August 1993)

SUMMARY

The usefulness of several different methods for detecting immune complex formation and complement activation in the circulation were applied to samples from patients receiving intravenous Streptokinase therapy for myocardial infarction. Streptokinase is a foreign antigen and can cause immune reactions. We collected samples from 13 patients, before Streptokinase administration (baseline), at the end of infusion (1 h), 12 h later and on day 7. We measured IgG containing immune complexes (IgG-IC), free C3d and antibodies to Streptokinase by ELISA, and CR1, C3d and C4d on erythrocytes by flow cytometric assay. Antibodies to Streptokinase are common, as all but two of the patients had measurable antibody levels. During Streptokinase treatment there was a drop in antibody levels, most prominent in those patients who had high baseline levels. At the same time increased levels of free C3d and erythrocyte-bound C3d were observed. After 12 h free C3d was usually back to baseline level, but C3d on erythrocytes was still raised. These data indicate the formation of Streptokinase immune complexes in patients with high Streptokinase antibody levels, and show that these complexes are cleared rapidly from the circulation, leaving more persistent signs of complement activation. We conclude that free C3d is a good indicator of ongoing complement activation, whereas C3d on erythrocytes indicates that complement activation has recently taken place.

Keywords complement complement activation immune complexes Streptokinase

INTRODUCTION

Immune complex formation and subsequent complement activation play a crucial role in the pathogenesis of several diseases such as systemic lupus erythematosus (SLE) and are also of importance in the disease mechanism of many other illnesses like rheumatoid arthritis (RA), chronic infections, AIDS and drug reactions. Several serological parameters are helpful in diagnosing and classifying immune complex-mediated diseases. Measurement of these parameters has been used extensively to evaluate disease activity and severity. Most commonly used are measurements of autoantibody titre and reactivity pattern (e.g. anti-nuclear antibodies and anti-DNA antibodies), quantitative or functional measurements of complements (e.g. serum C3, serum C4 and CH_{50}) and measurement of serum immune complexes (IC). Although these measurements often correlate with disease activity, there is wide individual variation and quite often these laboratory values do no more than support the clinical evaluation [1–3]. Over the last few years several reports have described the use of new methods for assessing disease activity in SLE and other diseases. Amongst these are measurements of complement activation products in serum [4–7], assessment of C3 metabolic turnover [8] and changes in the amount of complement receptor CR1 (CD35) on erythrocytes [9–11]. These measurements seem to correlate better with disease activity than the more classical tests.

We have established assays for measuring IC formation and complement activation products in the circulation. Amongst these assays are flow cytometric measurements of the complement fragments C3d and C4d and their receptor (CR1) on the surface of erythrocytes, and ELISA assays for measuring free C3d and C3d containing IC in plasma. We wanted to see how well these methods could detect IC formation and complement activation compared with a traditional assay, C3 rocket electrophoresis, widely used by clinicians to evaluate disease severity. Before applying these methods on selected patient groups we established a simple model in which we could be sure that complement activation would take place and at what time it happened. Therefore, we investigated patients receiving Streptokinase as treatment for myocardial infarction.

Streptokinase (SK) is a 47-kD protein produced by β-haemolytic streptococci group C. Since streptococcal infections are not rare, antibodies to SK are common [12]. For the last

Correspondence: Asbjörn Sigfusson, Department of Immunology, The National University Hospital, Landspitalinn, 101 Reykjavik, Iceland.

decade SK has been widely used in patients with myocardial infarction or peripheral artery occlusion because of its thrombolytic properties. It is most commonly administered intravenously in large quantities over a short period of time. When myocardial infarction patients who have antibodies to SK receive 600 mg of SK intravenously over 1 h, IC formation and subsequent complement activation are likely to take place, making these patients ideal for evaluating our assays.

PATIENTS AND METHODS

Patients

Thirteen patients who received intravenous SK for myocardial infarction were studied. Both EDTA blood and whole blood were collected before treatment (baseline value), at the end of the 1-h SK infusion, 12 h later and 7 days after the SK administration. Plasma and sera were aliquoted and stored at −70°C until measured, but erythrocytes were analysed within 48 h of sampling. Four patients with myocardial infarct who did not receive SK treatment were included for comparison.

Streptokinase antibodies

Antibodies to SK were measured by an ELISA assay. ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 2·5 μg/ml of Streptokinase (Strepase, Behring, Marburg, Germany), diluted in bicarbonate buffer (pH 9·6). Serum samples at 1:100 dilution and standard in two-fold serial dilutions starting at 1:100 were incubated for 5 h at room temperature. Then alkaline phosphatase (AP)-conjugated rabbit anti-human IgG (D336, Dako), diluted 1:2000 was incubated overnight at room temperature. The substrate, para-nitrophenylphosphate (NPP; Sigma, St Louis, MO), 5 mg/ml in 10% diethanolamine buffer (pH 9·8), was then added and the optical density of the developing colour measured at 405 nm in an ELISA reader (Titertech Multiscan, Flow Labs). The standard was made from serum obtained from a person receiving intradermal injections of SK. The 1:100 dilution of the standard was given 100 arbitrary units (AU) and a standard curve established, from which AU units for the samples were read.

Serum C3

Serum C3 was measured by a rocket immunoelectrophoresis. Samples, diluted 1:20, and Standard-Human-Serum (Behring) were run in 1·2% agarose containing 0·5 μl/cm² rabbit anti-human C3c (A062, Dako, Glostrup, Denmark) for 3·5 h at 10 V/cm. The amount (g/*l*) of C3 in the samples was read from a standard curve made from the Standard-Human-Serum.

Free C3d in plasma

The method used was a slight modification of a C3d ELISA previously reported [13]. ELISA plates (Maxisorp, Nunc) were coated with rabbit anti-human C3d (A063, Dako), diluted 1:1000 in bicarbonate buffer overnight at 4°C. The plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. To precipitate IC bound C3d, plasma samples and standard were mixed with equal volume of 22% polyethylene glycol (PEG 6000) in borate buffer (pH 8·3) and kept on ice for 60 min and then centrifuged at 1500 ***g*** at 4°C for 30 min. The supernatants, containing free C3d, were diluted 1:100 for plasma samples and the standard in two-fold serial dilutions starting at 1:200, and incubated for 5 h at room temperature. AP-conjugated rabbit anti-human C3d (A063, AP-conjugated at our laboratory) diluted 1:500 was incubated overnight at room temperature. NPP was used as the developing reagent as described before. Zymosan-activated serum (in which C3 had been cleaved to generate C3d) was used as standard and the 1:200 dilution was given 500 AU.

IgG-IC in plasma

IgG isotype-specific IC were measured by an ELISA assay. ELISA plates (Immulon 1, Dynatech) were coated overnight at 4°C with Fab_2 rabbit anti-human C3d (A063, Dako), diluted 1:500 (2·5 μg/ml) in bicarbonate buffer. Plasma samples, diluted 1:50, and standard in two-fold serial dilutions starting at 1:40, were incubated for 5 h at room temperature. Then AP-conjugated rabbit anti-human IgG (D336, Dako), diluted 1:500, was incubated overnight at room temperature. Developing was done as described before. The standard was made by incubating heat-aggregated normal human serum (NHS) with fresh NHS at 37°C for 2 h which ensured complement opsonization of the aggregate. A dilution of 1:40 was given 100 AU.

Erythrocyte-bound CR1, C3d and C4d

CR1, C3d and C4d on erythrocytes were measured by a flow cytometric assay as previously described [14]. Erythrocytes were washed three times in PBS with 1% fetal calf serum (PBS/FCS) and a 1% solution made by adding PBS/FCS to packed erythrocytes. Ten microlitres of 1% erythrocytes were mixed with 50 μl of either mouse anti-human CR1 (M710, Dako), 5·5 μg/ml, rat anti-human C3d (clone 3, kindly provided by Professor Peter Lachmann, Cambridge, UK), ascites diluted 1:10000 or mouse anti-human C4d (clone T2C5, kindly provided by Dr Gordon Ross, University of North Carolina, NC), 1·0 μg/ml, and incubated on ice for 45 min. Erythrocytes were then washed twice, mixed with 30 μl of FITC-conjugated rabbit anti-mouse 1:50, or rabbit anti-rat 1:20, and incubated on ice for 30 min. Erythrocytes were washed again and resuspended in 800 μl of 0·5% paraformaldehyde. Two thousand erythrocytes were analysed in a flow cytometer (FACScan, Becton Dickinson, Stockholm, Sweden) using FACScan program. Results were expressed as median fluorescence (MFCN).

RESULTS

Streptokinase antibodies

Figure 1 shows the distribution of SK antibody levels in samples from 13 patients receiving SK infusion with patients numbered in order of decreasing baseline SK antibody levels. Figure 1 also shows the changes in SK antibody levels during and after the SK infusion. The baseline levels did not differ from what we had previously found in healthy individuals (data not shown). These changes followed a similar pattern in all the patients who had measurable baseline levels of SK antibodies, where antibody levels dropped dramatically during SK infusion and stayed low during the next 12 h. By day 7 the antibody levels had risen, often enormously. It is interesting that in some patients with very low baseline levels of SK antibodies, the antibody levels increased many-fold, but in others the rise was not substantial. The four control patients not receiving SK infusion had intermediate baseline levels of SK antibodies (Table 1).

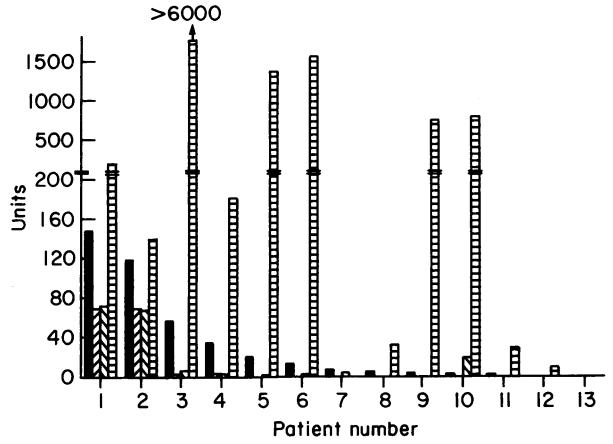

Fig. 1. Changes in Streptokinase (SK) antibody levels during and after SK infusion. The patients are numbered in order of decreased baseline SK antibody levels. ■, 0 h; ■, 1 h; ▧, 12 h; ■, 7 days.

Serum C3, free C3d and C3d on erythrocytes

Table 1 shows the relation between baseline levels of SK antibodies and changes in C3, free C3d and erythrocyte-bound C3d during the SK infusion both for patients who received SK treatment and for the control patients who did not receive SK treatment. It is clear that those patients with the highest levels of SK antibodies had the strongest signs of complement activation, i.e. substantial increases in both free and erythrocyte-bound C3d. On the other hand, C3 did not change significantly during the SK infusion. Figure 2 shows the changes in C3, free C3d and erythrocyte-bound C3d during and after the SK infusion in samples from selected patients: the three patients with the highest baseline levels of antibodies to SK, the two patients with the lowest baseline levels, and two control patients. Figure 2a shows that C3 did not change significantly during and after the SK infusion, with no value below the normal range (0·50–0·95 g/*l*). A rise was sometimes seen at day 7 at the same time as SK antibody levels increased dramatically (see Fig. 1). It is clear that patients with high levels of antibodies who received SK treatment had obvious signs of complement activation in their circulation reflected in rise of both free C3d and erythrocyte-bound C3d (Fig. 2b, c). The upper limit of normal for free C3d is 50 AU and 94 MFCN for erythrocyte-C3d. At the end of SK infusion the three patients with high SK antibodies had values well above these normal ranges (patient 1, 370 AU and 157 MFCN; patient 2, 470 AU and 112 MFCN; patient 3, 390 AU and 114 MFCN for free C3d and erythrocyte-C3d respectively). Free C3d had reached baseline levels on day 7 in all three patients, but erythrocyte-bound C3d only in one. The two patients with no measurable antibodies to SK showed little or no signs of complement activation in their circulation, as did the two control patients not receiving SK treatment, even though they had intermediate baseline levels of antibodies to SK (Table 1).

Table 1. Relation between levels of Streptokinase (SK) antibodies and changes of serum C3, free C3d and erythrocyte-bound C3d during SK infusion

Patient number	Baseline levels of SK antibodies (AU)	Changes in complement fragments during SK infusion*		
		Serum C3 (g/*l*)	Free C3d (AU)	Erythrocyte-bound C3d (MFCN)
SK1†	148	−0·07	325	65
SK2	118	−0·24	435	51
SK3	56	−0·13	354	49
SK4	34	−0·10	272	NT
SK5	21	−0·13	155	10
SK6	13	−0·04	0	10
SK7	7	0·0	63	NT
SK8	6	−0·05	21	5
SK9	4	0·05	53	−7
SK10	3	0·06	7	20
SK11	2	−0·19	11	7
SK12	0	−0·05	52	8
SK13	0	0·0	7	0
C1†	24	NT	−1	−3
C2	19	0·05	1	1
C3	17	−0·05	NT	NT
C4	10	0·05	0	NT

* Difference between the baseline sample and the sample taken just after the SK infusion. Changes from baseline levels never exceeded these values at any time points, except that a rise in C3 was commonly detected at day 7.

† SK, Patients receiving SK treatment; C, patients not receiving SK treatment. NT, Not tested.

Erythrocyte-bound CR1 and C4d and circulating IgG-IC

Erythrocyte-bound CR1 and IgG-IC levels did not change following the SK treatment (data not shown), and baseline levels were in accordance with those seen for normal individuals. In patients with high baseline levels of SK antibodies a minimal rise was detected for erythrocyte-bound C4d. This was much less obvious than the rise in erythrocyte-bound C3d and never exceeded 10%.

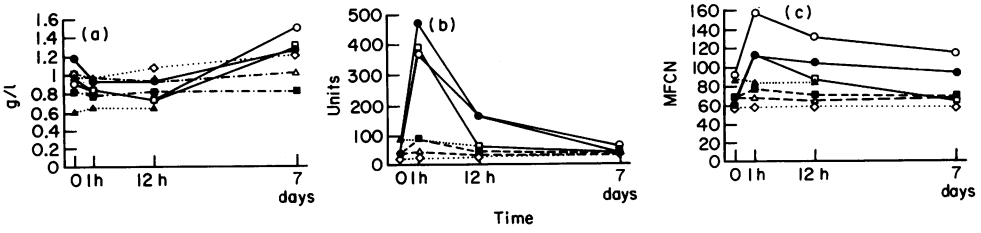

Fig. 2. Changes in serum C3 (a), free C3d (b) and erythrocyte-bound C3d (c) during and after Streptokinase (SK) infusion in seven patients; three patients with the highest baseline SK antibody levels are indicated by unbroken lines, two patients with the lowest baseline levels by broken lines and two control patients not receiving SK treatment by dotted lines. ○, SKpt 1; ●, SKpt 2; □, SKpt 3; ■, SKpt 12; △, SKpt 13; ▲, Cpt 1; ◇, Cpt 2. MFCN, Median fluorescence.

DISCUSSION

Several investigators have established elegant experimental models to investigate *in vivo* IC formation and clearance and complement activation in humans [15–17]. These models are rather laborious and invasive, however, and demand special reagents like radiolabelled antigens or infusion of specific foreign antibodies. The aim of this study was to establish a more simple model to explore some of the same problems, but mainly to evaluate the clinical value and sensitivity of new laboratory assays.

As recorded, many of our myocardial infarction patients had serum antibodies to SK before receiving SK treatment [12]. The antibody levels dropped markedly during SK infusion, and this drop is best explained by formation of IC between the infused SK and serum SK antibodies and subsequent clearance of the complexes by the reticulo-endothelial system. This decrease in SK antibody levels could also be explained by the presence of free SK in the post-infusion samples interfering with the SK antibodies in the ELISA assay by competition. This was controlled for by mixing pre-infusion serum from a patient with high level of SK antibodies and 1-h serum from a patient with no SK antibodies. No drop in SK antibody level was obseved, although SK antibody detection could easily be inhibited by adding a large amount of exogenous SK to a pre-infusion serum containing SK antibodies (data not shown).

It was rather surprising that we could not detect any increase in circulating IC just after the SK infusion. This cannot be explained by lack of sensitivity of our ELISA IC assay, as IC formed *in vitro* by mixing SK and serum containing SK antibodies could easily be detected. The most plausible explanation is that the amount of IC formed was well within the clearance capacity of the immune system, and was therefore removed very rapidly from the circulation. This is supported by the fact that none of the SK-treated patients suffered any serum sickness-like symptoms. Although the pharmacological half-life in individuals without SK antibodies is about 80 min, it has been reported that the presence of circulating antibodies shortens this time dramatically [18]. Others have shown that when preformed IC are infused intravenously, the majority is eliminated from the circulation within minutes [15–17].

Of the seven different parameters evaluated in this study, only measurement of free C3d and erythrocyte-bound C3d showed significant changes during and after the SK infusion. Demonstrable increases in free C3d occurred only in those patients with high or moderate baseline SK antibody levels, and increases in erythrocyte-bound C3d only occurred in the three patients with the highest baseline SK antibody levels. The increase in these two parameters correlated with the baseline levels of SK antibodies and therefore the probable amount of IC formed. This shows that both assays are sensitive and to some extent quantitative indicators of IC formation and subsequent complement activation. After IC formation stopped and IC had been cleared from the circulation, free C3d dropped rapidly and was already near to or at baseline levels after 12 h. In contrast, erythrocyte-bound C3d normalized more slowly, with only one of the three patients reaching baseline levels on day 7. Like changes in free C3d and erythrocyte-bound C3d, decreases in serum C3 correlated somewhat with the baseline levels of SK antibodies, but the drop was always very unimpressive, and serum C3 never fell below the normal range. It is therefore obvious that a decrease in serum C3 is a much less sensitive indicator of complement activation than the increase in both free C3d and erythrocyte-bound C3d. Moreover, the rise in serum C3 on day 7 suggests that in a clinical situation, where complement activation is only moderate, the expected drop in serum C3 due to its consumption could easily be masked by an acute-phase increase in C3 production.

It is well documented that in active immune complex diseases, erythrocyte CR1 decreases in number and erythrocyte-bound C3d and C4d increase [19]. We did not see any changes in erythrocyte CR1 or erythrocyte-bound C4d, probably due to the small and transient IC load in our model.

Our conclusion is that patients receiving SK infusion make a simple but useful model to evaluate methods to monitor *in vivo* IC formation and complement activation. Measurements of free C3d by ELISA and erythrocyte-bound C3d by flow cytometry are sensitive, reliable and quantitative methods to detect *in vivo* complement activation, and are much more sensitive than a drop in serum C3, which can also be influenced by increased C3 production as a result of an acute-phase response. Free C3d is more sensitive, but both methods can detect and quantify even subclinical short-lasting complement activation. The differences between free C3d and erythrocyte-bound C3d is not only sensitivity, as free C3d normalizes rapidly after complement activation stops, but erythrocyte-bound C3d stays raised at least for a few days. Thus the former parameter only reflects ongoing complement activation, but a rise in erythrocyte-bound C3d can also reflect a recent although terminated complement activation.

REFERENCES

1 Lloyd W, Schur PH. Immune complexes, complement, and anti-DNA in exacerbations of systemic lupus erythematosus (SLE). Medicine (Baltimore) 1981; **60**:208–17.

2 Valentijn RM, van Overhagen H, Hazevoet HM *et al.* The value of complement and immune complex determinations in monitoring disease activity in patients with systemic lupus erythematosus. Arthritis Rheum 1985; **28**:904–13.

3 Isenberg DA, Colaco CB, Dudeney C *et al.* The relationship of anti-DNA antibody idiotypes and anti-cardiolipin antibodies to disease activity in systemic lupus erythematosus. Medicine (Baltimore) 1986; **65**:46–55.

4 Hopkins P, Belmont HM, Buyon J *et al.* Increased levels of plasma anaphylatoxins in systemic lupus erythematosus predict flares of the disease and may elicit vascular injury in lupus cerebritis. Arthritis Rheum 1988; **31**:632–41.

5 Negoro N, Okamura M, Takeda T *et al.* The clinical significance of iC3b neoantigen expression in plasma from patients with systemic lupus erythematosus. Arthritis Rheum 1989; **32**:1233–42.

6 Petersen NE, Elmgreen J, Teisner B *et al.* Activation of classical pathway complement in chronic inflammation. Elevated levels of circulating C3d and C4d split products in rheumatoid arthritis and Crohn's disease. Acta Med Scand 1988; **233**:557–60.

7 Wild G, Watkins J, Milford A *et al.* C4a anaphylatoxin levels as an indicator of disease activity in systemic lupus erythematosus. Clin Exp Immunol 1990; **80**:167–70.

8 Swaak AJG, Han H, van Rooyen A *et al.* Complement (C3) metabolism in rheumatoid arthritis in relation to the disease course. Rheumatol Int 1988; **8**:61–65.

9 Ross GD, Yount WJ, Walport MJ *et al.* Disease-associated loss of erythrocyte complement receptors (CR1, C3b receptors) in patients with systemic lupus erythematosus and other diseases involving

autoantibodies and/or complement activation. J Immunol 1985; **135**:2005–14.

10 Thomsen BS, Nielsen H, Andersen V. Erythrocyte CR1 (C3b/C4b receptor) levels and disease activity in patients with SLE. Scand J Rheumatol 1987; **16**:339–46.

11 Thomsen BS, Heilmann C, Jacobsen SEH *et al.* Complement C3b receptors on erythrocytes in patients juvenile rheumatoid arthritis. Athritis Rheum 1987; **30**:967–71.

12 Lynch M, Littler WA, Pentecost BL *et al.* Immunoglobulin response to intranvenous streptokinase in acute myocardial infarction. Br Heart J 1991; **66**:139–42.

13 Mollnes TE. Quantification of the C3d split product of human complement by a sensitive enzymelinked immunosorbent assay. Scand J Immunol 1985; **21**:607–13.

14 Freysdottir J, Sigfusson A. A flow cytometric assay for measuring complement receptor 1 (CR1) and the complement fragments C3d and C4d on erythrocytes. J Immunol Methods 1991; **142**:45–52.

15 Cornacoff JB, Hebert LA, Smead WL *et al.* Primate erythrocyte-immune complex-clearing mechanism. J Clin Invest 1983; **71**:236–47.

16 Paccaud J-P, Steiger G, Sjöholm AG *et al.* Tetanus toxoid–anti-tetanus toxoid complexes: a potential model to study the complement transport system for immune complex in humans. Clin Exp Immunol 1987; **69**:468–76.

17 Davies KA, Hird V, Stewart S *et al.* A study on *in vivo* immune complex formation and clearance in man. J Immunol 1990; **144**:4613–20.

18 Grierson DS, Bjornsson TD. Pharmacokinetics of streptokinase in patients based on amidolytic activator complex activity. Clin Pharmacol Ther 1987; **41**:304–13.

19 Hammond A, Rudge AC, Loizou S *et al.* Reduced numbers of complement receptor type 1 on erythrocytes are associated with increased levels of anticardiolipin antibodies. Findings in patients with systemic lupus erythematosus and the antiphospholipid syndrome. Arthritis Rheum 1989; **32**:259–64.