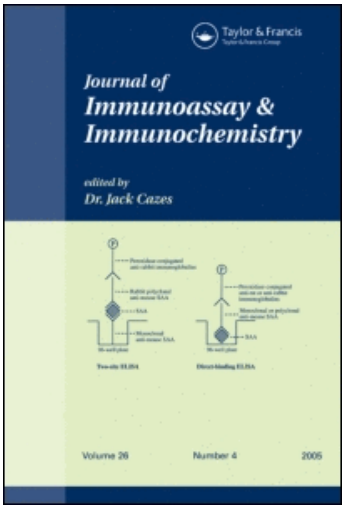

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A Simple Enzyme Immunoassay for Measurement of Immune Complex Solubilization Utilizing Preformed Peroxidase-Antiperoxidase Complexes

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A SIMPLE ENZYME IMMUNOASSAY FOR MEASUREMENT OF IMMUNE COMPLEX SOLUBILIZATION UTILIZING PREFORMED PEROXIDASE-ANTIPEROXIDASE COMPLEXES

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ABSTRACT

An enzyme immunoassay (EIA) was developed for measurement of complement-mediated solubilization of immune complexes. Preformed complexes consisting of horseradish peroxidase (Pe) and anti-Pe antibodies were incubated in diluted serum. After centrifugation the soluble complexes in the supernatant were quantitated by adding peroxidase substrate followed by measurement of absorbance. Kinetic analysis of revealed significant difference between normal and EDTA-treated serum similarly to a standard assay utilising immune complexes containing radiolabelled bovine serum albumin. The difference was most pronounced after incubation of immune complexes with serum for 40 minutes. Immune complex solubilization measured by EIA was reduced in sera from 10 patients with active systemic lupus erythematosus (SLE). In serially followed patients, flares of SLE were preceeded by reduction of solubilization capacity. The EIA is a simple method for determining serum capacity to solubilize preformed immune complexes and might be considered as a routine test for assessment of complement function in diseases such as SLE.

Key words: Immune complex, Solubilization, Enzyme immunoassay, SLE

INTRODUCTION

Impaired complement-dependent modification and transport of immune complexes is thought to be of pathogenetic importance in conditions such as systemic lupus erythematosus (SLE) and could contribute to the association between complement deficiency and disease (1). Inhibition of immune complex formation and solubilization of preformed immune aggregates varies with severity and activity during disease course in SLE (2,3,4). Methods for such measurements have generally been cumbersome and dependent on radiolabelling of the antigen used in the immune complexes. Enzymes are widely used as an alternative to radiolabelling to increase sensitivity of immunoassays. This approach was suggested for application to measurement of immune complex solubilization by Vikingsdottir et al., 1990 (5). We developed and validated a simple enzyme immunoassay (EIA) for immune complex solubilization using peroxidase - IgG-anti-peroxidase complexes. The assay proved to be reliable and could be used for routine purposes.

MATERIALS AND METHODS

Buffers

VBS: Veronal buffered saline: 0.7 mol/l NaCl, 9.1 mmol/l Na-5.5-dietylic barbiturate, 15.6 mmol/l 5,5 dietylic barbiturate.

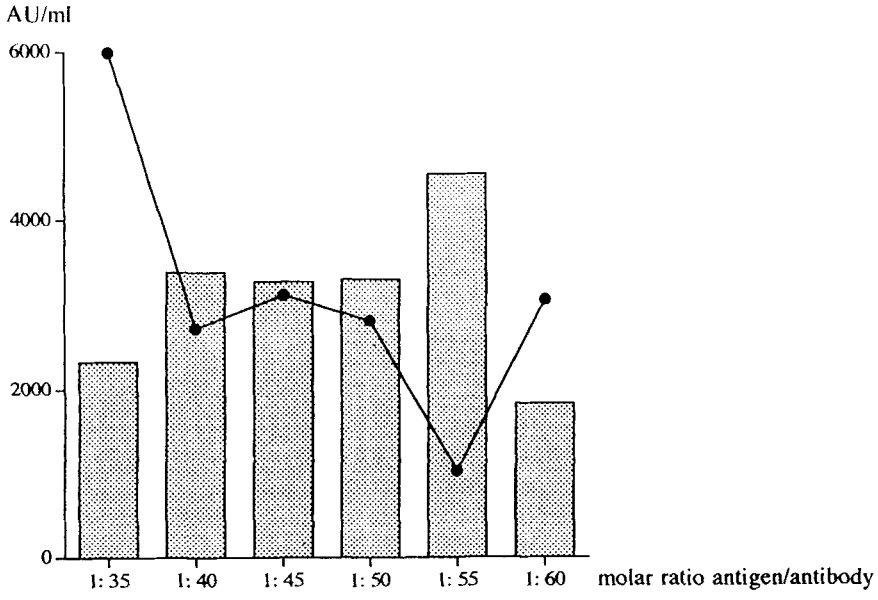

FIGURE 1. Precipitation of Peroxidase (Pe)-antiperoxidase (anti-Pe) complexes at various ratios of antigen and antibodies from rabbit immune serum. The line shows the absorbance of the supernatants obtained when Pe-anti-Pe complexes were precipitated in EDTA treated NHS. The columns represent the difference of absorbance in supernatants obtained bewteen Pe-anti-Pe complexes precipitated in NHS and EDTA-treated NHS respectively. AU : arbitrary units.

VBS-CaMg: VBS diluted 1/5 with Ca^{2+} (2mmol/l) and Mg^{2+} (1 mmol/l).

VBS-EDTA: VBS diluted 1/5 with 20 mmol/l EDTA (Titriplex III, Merck, Darmstadt, Germany)

PBS-Tween: 2.5 mmol/l NaH_2PO_4,H_2O, 7.5 mmol/l $Na_2HPO_4,12H_2O$, 0.5 mol/l NaCl, 0.1% (w/v) Tween 20, pH 7.2.

Normal Sera

Serum samples were obtained from 10 healthy volunteers among the Hospital

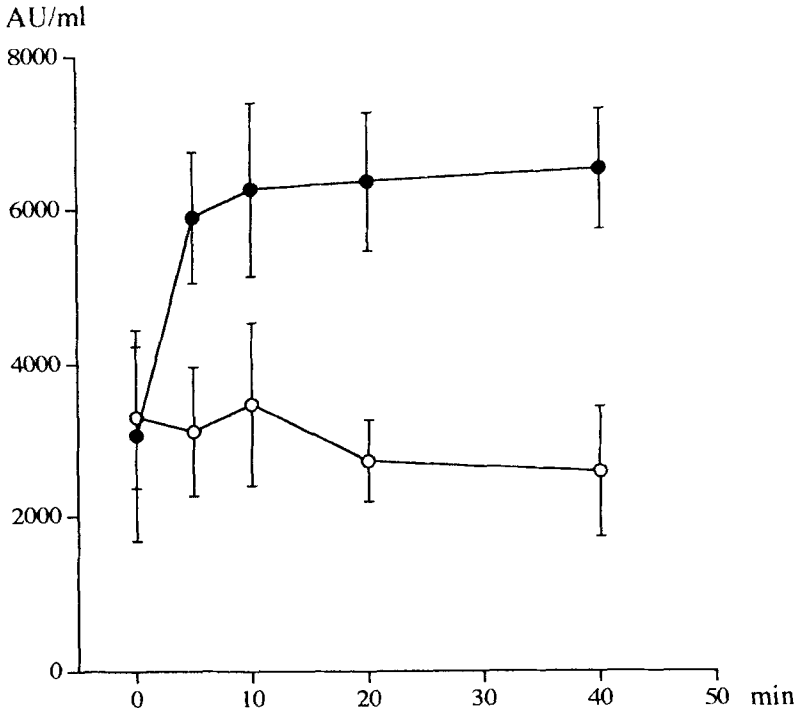

FIGURE 2a. Solubilization of Pe-anti-Pe complexes in serum from 10 healthy individuals, with (open symbols) and without (filled symbols) EDTA treatment. The mean and 2 standard deviations (2 SD) are given.

staff, nine of whom were women, aged 22 - 60 years. Serum from one of these blood donors was used as a control in all experiments. As a positive serum control, normal EDTA serum (0.084 ml 0.34 mol/l EDTA in 0.5 ml of serum) was used.

SLE Sera

Serum from 10 patients , 9 women and 1 male, aged 38 - 65 years, were studied.

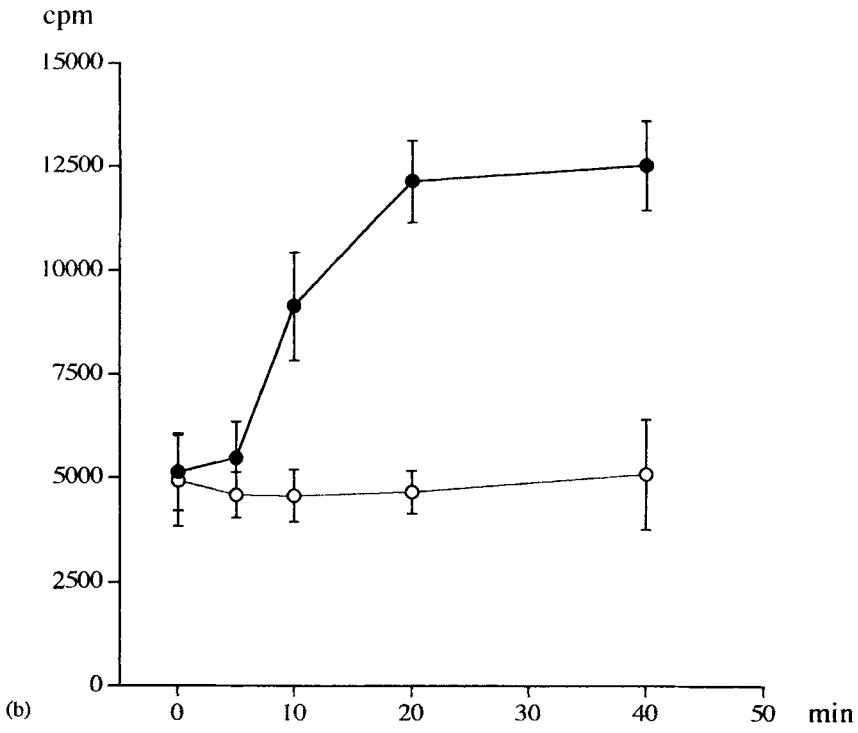

FIGURE 2b. Solubilization of BSA-anti-BSA complexes in serum from 10 healthy individuals, with (open symbols) and without (filled symbols) EDTA treatment. The mean and 2 standard deviations (2 SD) are given.

Serial serum samples from three patients were studied. Disease activity was assessed by the SLEDAI scoring (6). All patients had a multisystemic disease and all fulfilled 4 or more of the ACR criteria for classification of SLE (7).

Enzyme Immunoassay (EIA) for Immune Complex Solubilization

Horseradish peroxidase (Pe) (Type Sigma Chemicals Co., St Louis, MO, USA) and rabbit antiperoxidase (anti-Pe) (Dakopatts, Glostrup, Denmark) were reacted

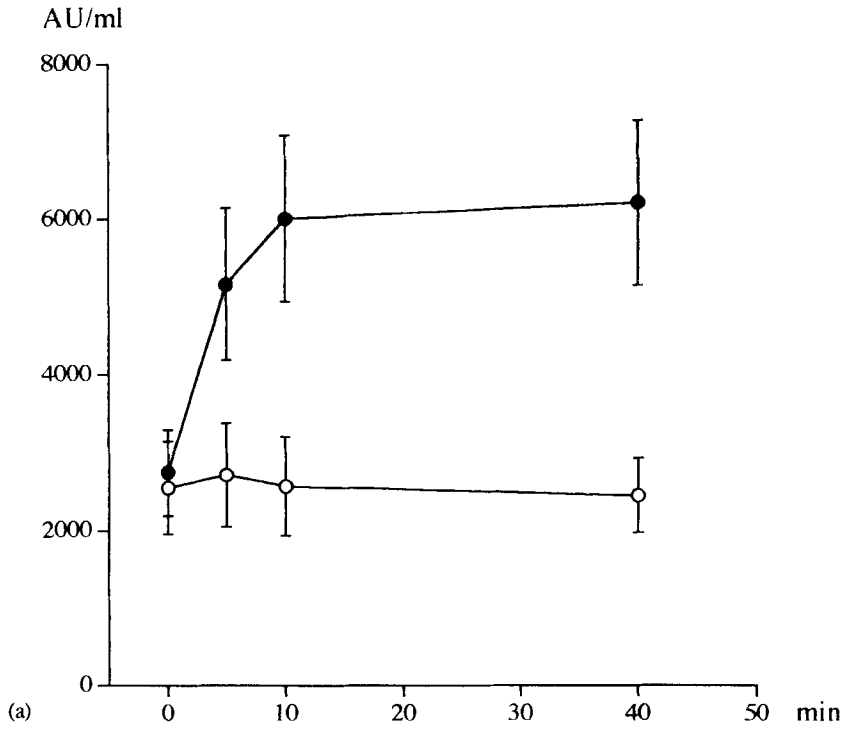

FIGURE 3a. Results from 21 repeated investigations of serum capacity to solubilize Pe-anti-Pe complexes. Serum from one healthy individual were used in all experiments and treated with (open symbols) and without (filled symbols) EDTA. The mean and 2 standard deviations (2 SD) are given.

at equivalence. Pe and anti-Pe were incubated for 1h at 37°C and then for 24h at 4°C. The Pe-anti-Pe complexes were prepared and used fresh at each experiment. The molar ratio giving maximal precipitation was obtained at a ratio of 1:55 (w/w) with the antibody preparation used (Fig 1). At this ratio, the difference between solubilization of Pe-anti-Pe in normal human serum (NHS) and NHS with EDTA (EDTA-NHS) was most pronounced.

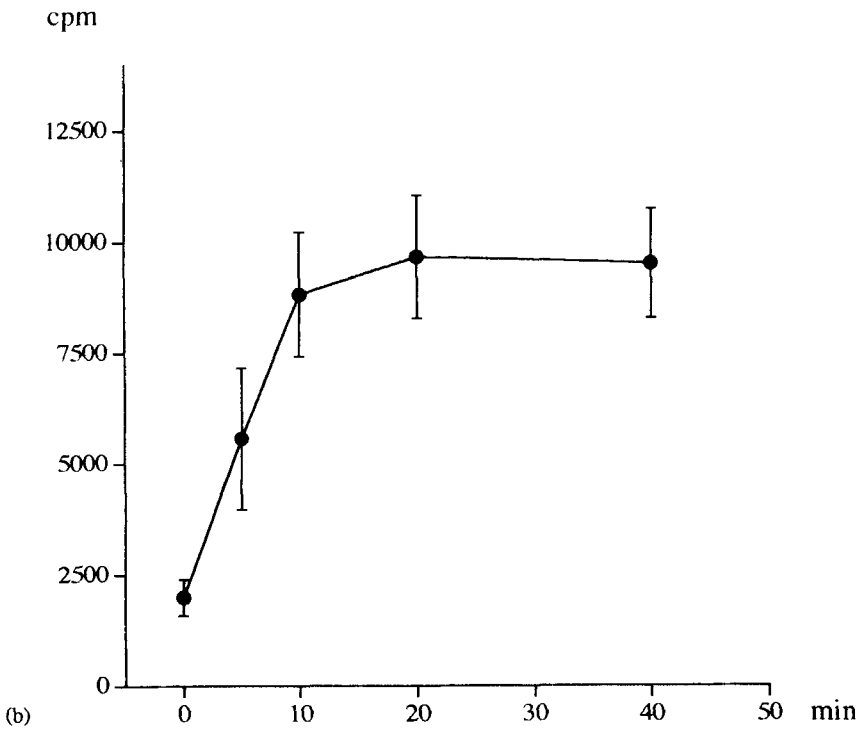

FIGURE 3b. Results from 12 repeated experiments with serum from one healthy individual. The mean and 2 standard deviations (2 SD) are given.

In the assay, 60 μl test serum and 40 μl VBS-CaMg were mixed and incubated for 10 minutes at 37°C. Ten μl Pe-anti-Pe was then added and the mixture incubated in aliquots for 5, 10, 20 and 40 minutes respectively at 37°C. We then added 280 μl ice cold VBS-EDTA. The mixture was centrifuged at 2,500xg for 15 minutes at 4°C. In all experiments, glass-tubes pre-coated with 1% gelatin solution were used. The supernatants were diluted 1/5 - 1/10 in PBS-Tween and 100 μl portions of each sample was transferred to a microtiter plate (Nunc,

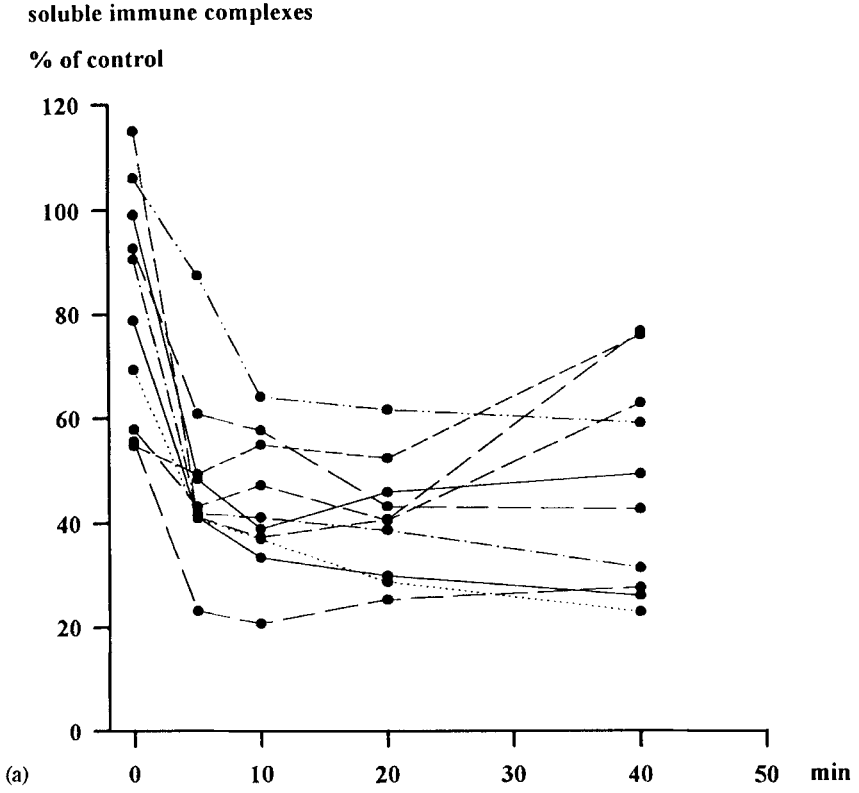

FIGURE 4a. Solubilization of preformed Pe-anti-Pe complexes in serum from 10 patients with active SLE (SLEDAI range 4 - 16) as estimated by EIA. The values are given in percent of a normal control.

Roskilde, Denmark). This was followed by addition of 100 μl Orthophenylenediamine (Dakopatts) in 0.1 mol/l citric acid phosphate buffer, pH 5.0. The microtiter plates were incubated for 15 minutes at room temperature. The reaction was thereafter stopped with 1 mol/l H_2SO_4, 100 μl added to each well. The absorbance at 492/620 nm was measured in a Multiscan filter photometer (Flow laboratories, USA).

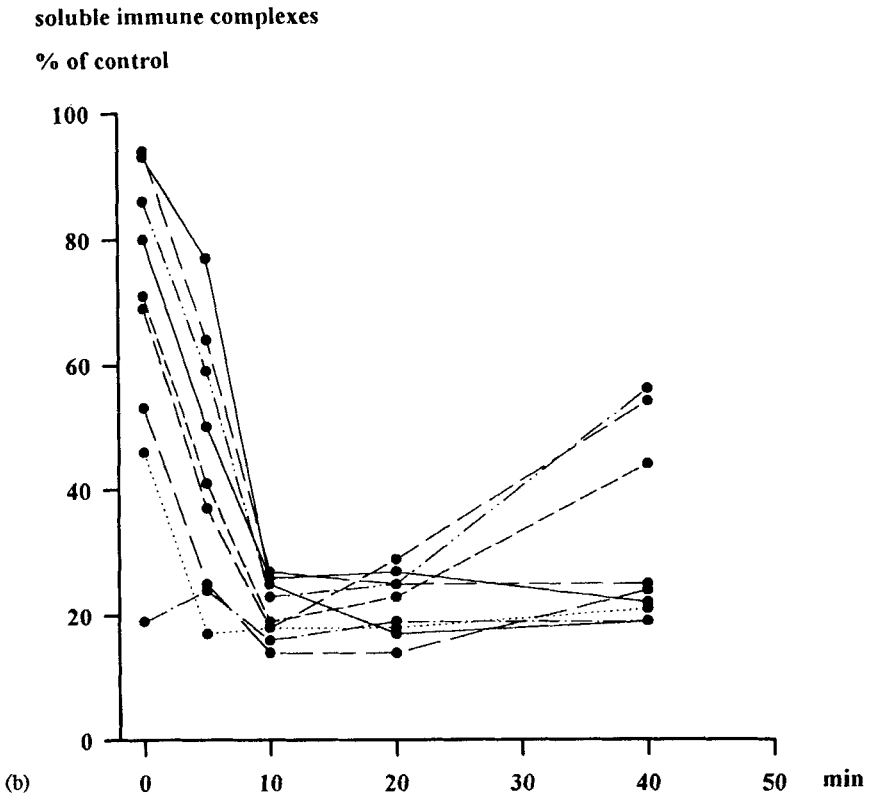

FIGURE 4b. Solubilization of preformed radiolabelled BSA-anti-BSA complexes in the same SLE sera as presented in Fig 4a. The values are given in percent of a normal control.

Radioimmunoassay (RIA) for Solubilization of Immune Complexes

The method has previously been described (8). In this assay, complexes between ^{125}I-bovine serum albumin (BSA, Sigma) and rabbit IgG anti-BSA (Dakopatts) were used. Immune precipitates were prepared at an antigen/antibody molar ratio that gave maximal precipitate formation. Precipitation was carried out for 1h at

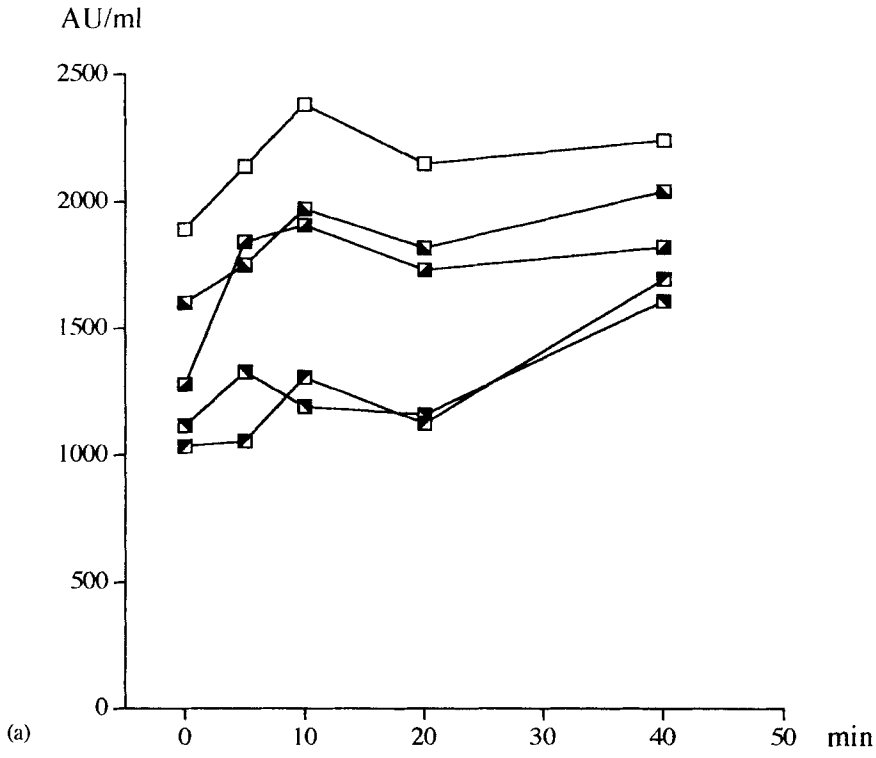

FIGURE 5-7. Serial analysis of solubilization of Pe-anti-Pe complexes (5a, 6a, 7a) and BSA-anti BSA complexes (5b, 6b, 7b) in SLE patients. One patient (5a-b) developed a flare with arthritis, alopecia, and systemic vasculitis with pancreatitis. The patient presented in 6a-b had a flare with arthritis, cutaneous vasculitis, minor cerebral infarction and glomerulonephritis. In 7a-b data are shown from a patient that developed glomerulonephritis. Prospectively collected samples were obtained 4 months before flare (◪), 2 months before flare (◪), at flare before start of treatment (◪) and at remission 2 - 4 months after start of treatment (◪). Normal control (□).

37°C and for 12 hours at 4°C in VBS-CaMg. Test serum, 60μl, was incubated with 40 μl VBS-CaMg for 10 minutes. Ten μl immune complex solution was then added after thorough dispersion and the mixture was incubated at 37°C during 0, 5, 10, or 40 minutes. The solubilization process was stopped by adding 20 mmol/l EDTA. The mixture was then centrifuged and the radioactivity in the precipitate and in the supernatant was measured in a gammacounter.

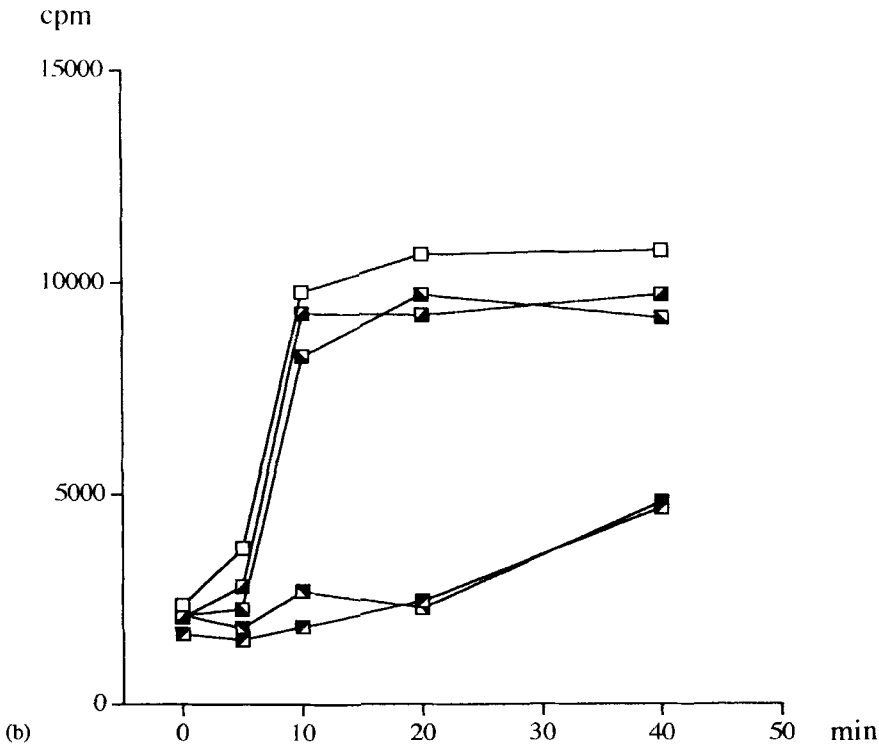

FIGURE 5b.

RESULTS

The capacity of sera from 10 healthy individuals to solubilize preformed immune complexes were investigated in the EIA and the RIA (Fig 2a and 2b). A significant difference between untreated and EDTA-treated serum was found after 5, 10, 20 and 40 minutes incubation at 37°C with both assays. The difference was most pronounced after 40 minutes. The standard deviation varied in the EIA between 12 and 18% of the mean.

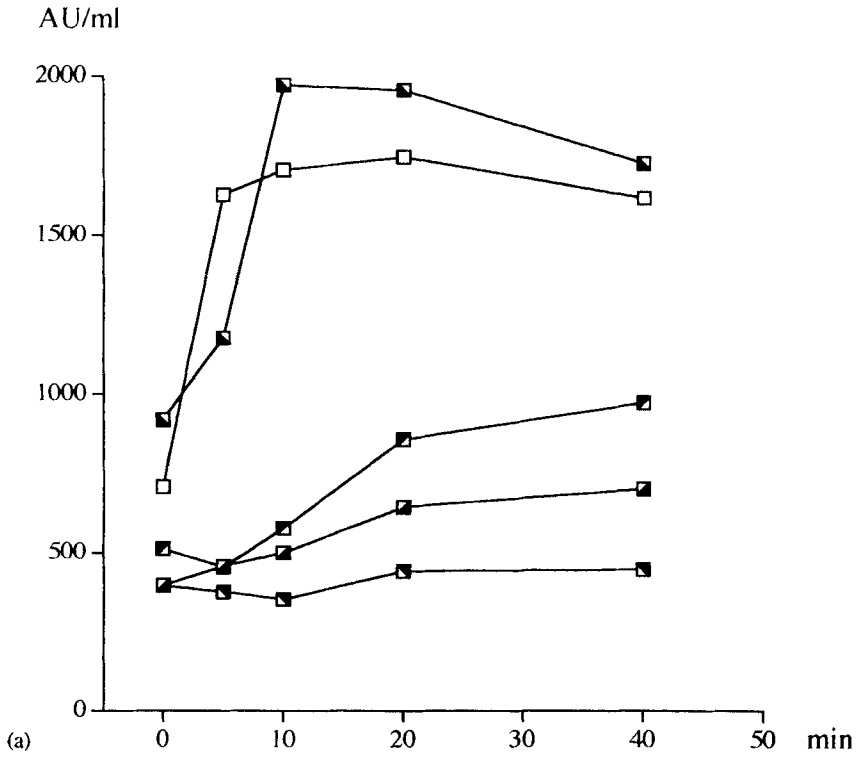

FIGURE 6a.

Repeated investigations of serum from one individual are shown in Fig 3a and 3b. The standard deviation was 18% of the mean with the EIA and 16% with the RIA.

Sera from 10 patients with active SLE were studied (Fig 4a and 4b). All sera showed a clearly reduced solubilization capacity after 10 minutes incubation at 37°C (>70% of the normal). As expected the lowest values were obtained among patients with severe disease and hypocomplementemia. The results were similar with the two assays.

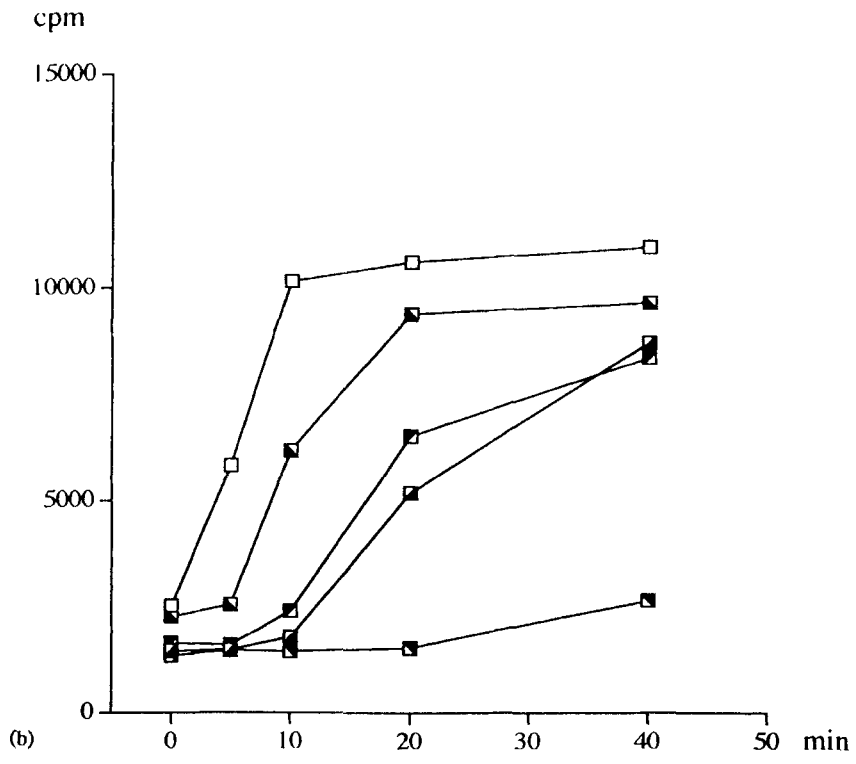

FIGURE 6b.

Serial samples from 3 patients with active SLE were investigated (Fig 5-7). The patients (Fig 5-7) had severe SLE manifestations with active glomerulonephritis (2 patients) and gastrointestinal vasculitis with pancreatitis (1 patient). The figures clearly show that the serum capacity to solubilize preformed immune complexes varies with disease activity and may predict flares in patients with SLE. However, during the pre-flare period the EIA appeared to be somewhat less sensitive indicator of disturbed immune complex solubilization. By the time of flare the results with the two assays were very similar.

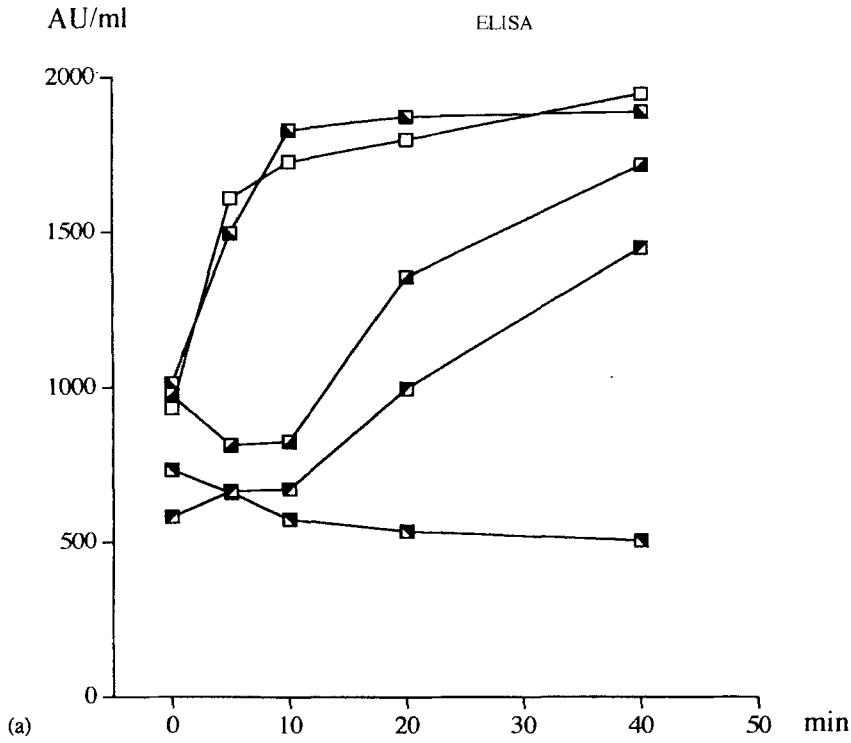

FIGURE 7a.

DISCUSSION

We developed an EIA for measurement of serum capacity to solubilize preformed immune complexes. The EIA was shown to be reliable and accurate. The advantage of using the tracer-enzyme as the antigen in the preformed complexes is obvious. Thus, the technical procedures in the assay are very simple, and the EIA could be considered for use in a routine laboratory setting.

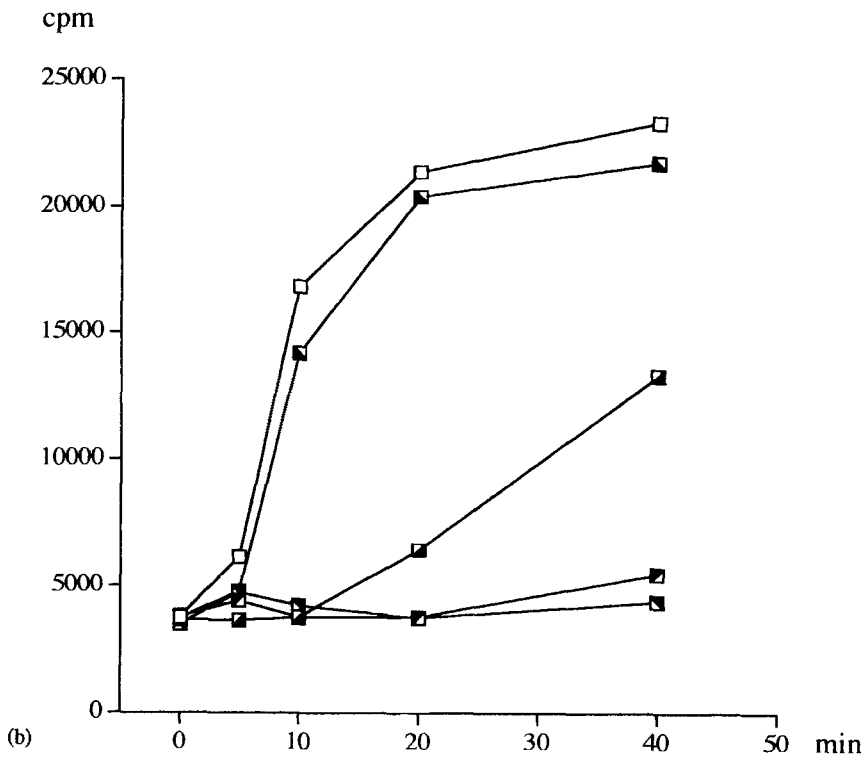

FIGURE 7b.

A close association was found between the SLEDAI index and the serum capacity to solubilize preformed immune complexes suggesting that measurements could be of clinical value (3,4). Interestingly, some discrepancies were found between results with the two assays used in the present study, especially in serial determinations in SLE patients. These differences are probably related to the physicochemical properties of Pe-anti-Pe complexes and BSA-anti-BSA complexes and also divergences in the test procedures. The molecular mass of the antigens

used for formation of immune complexes, BSA and Pe are slightly different, 66 and 44 kDa respectively. This difference as well as the affinity of the specific antibodies, might influence configuration and size distribution of the complexes. The accuracy and reproducibility of the methods appeared overall similar but the EIA has a clear advantage due to very simple and rapid test procedures. Furthermore, the EIA can easily be modified for estimation of serum capacity to inhibit immune precipitation by allowing serum to be present throughout the testing procedure.

In conclusion, we suggest that determination of the serum capacity to solubilize preformed immune complexes with EIA could be a useful test of complement function in diseases such as SLE.

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