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Reduced complement-mediated immune complex solubilizing capacity and the presence of incompletely solubilized immune complexes in SLE sera

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SUMMARY

Reduced complement-mediated solubilization (CMS) of pre-formed immune complexes (IC) was demonstrated in sera from 11 out of 12 SLE patients. The presence of incompletely solubilized endogeneous IC in SLE sera was indicated by the following findings: (1) When IC positive SLE sera with reduced CMS capacity were mixed with normal donor sera they inhibited the CMS of the latter sera. (2) Resuspended PEG (2·75%) precipitates obtained from SLE sera inhibited the CMS of normal donor sera. (3) Non-solubilized or incompletely complement solubilized IC in SLE sera give a strong response in the PEG-CC assay for IC. The IC activity of SLE sera was clearly reduced in this assay when the endogeneous IC were solubilized prior to testing. In contrast, sera of 14 rheumatoid arthritis (RA) patients exhibited normal CMS. IC which could be further solubilized by complement were not demonstrable although all RA sera were IC positive.

Keywords complement solubilization immune complexes systemic lupus erythematosus

INTRODUCTION

The complement-mediated solubilization (CMS) of immune complexes (IC) has received increasing attention since the first reports by Miller & Nussenzweig (1975). It is by now well established that CMS is dependent on an intact alternative activation pathway (Czop & Nussenzweig, 1976; Takahashi, Takahashi & Hirose, 1980) and that the classical pathway has a facilitating effect due to the initial generation of C3b (Takahashi *et al.*, 1978). Solubilization leads to the formation of so called endstage IC, which according to Takahashi *et al.* (1980) react inefficiently with complement and therefore should have reduced phlogogenic potential (Takahashi *et al.*, 1976).

Reduced CMS capacity (CMSC) in sera from systemic lupus erythematosus (SLE) patients has recently been reported (Aguado *et al.*, 1981; Sakurai *et al.*, 1982; Schifferli *et al.*, 1981), while sera from rheumatoid arthritis (RA) patients exhibited normal CMSC (Schifferli *et al.*, 1981). These studies also indicated a correlation between CMSC and the serum concentration of various complement components and CH_{50}. The purpose of the present study was to investigate the CMSC of SLE and RA sera in a standardized assay (Baatrup *et al.*, 1983) and examine whether incompletely solubilized IC were demonstrable in the same sera.

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MATERIALS AND METHODS

Patients. Serum was obtained from 12 patients with SLE (ARA criteria) (11 females and one male, age 21–60, mean 31) and from 14 patients suffering from classical RA (nine females and five males, age 48–68, mean 62). All were in-patients at Odense University Hospital or Rigshospitalet, Copenhagen.

Normal donor sera. The normal range of CMS was determined on sera obtained from 24 healthy medical students (10 females and 14 males, age 22–32, mean 25). Two of the sera having a CMSC close to the mean, were used as normal controls throughout this study. Another group of 24 normal blood donors (10 females and 14 males, age 45–65, mean 52) showed a mean and standard deviation of CMS identical to that of the other reference group.

Negative control sera. Sera from the same bleedings which were used as normal controls, were deprived of their CMS activity by heating (56°C for 30 min) and stored at −70°C.

Serum handling. All blood samples collected in ordinary glass tubes were allowed to clot for 75 min at room temperature and centrifuged at 800*g* for 10 min. The sera were frozen in small aliquots at −70°C immediately after separation. Serum samples once thawed were not reused. All dilutions of serum (unless otherwise stated) were with phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (PBS^{++}).

Immune complexes. Antiserum to BSA, raised in rabbits as previously described (Baatrup *et al.*, 1983) was absorbed on a HSA–Sepharose 4B column and the serum complement was inactivated (Baatrup *et al.*, 1983). The anti-BSA IgG concentration after absorption was 1·63 mg/ml. ^{125}I-BSA (specific activity $3{\cdot}3 \times 10^7$ ct/min/μg) was prepared according to the iodogen method (Franker & Speck, 1978). IC were pre-formed at the point of equivalence (molar ab/ag ratio = 3·4). An IC stock suspension was prepared from 200 μl anti-BSA serum, 40 μg BSA and 0·8 μg ^{125}I-BSA, in 6·0 ml PBS. The suspension was washed twice with PBS by centrifugation at 3,000*g*, whirlmixed and dispensed by passage through a tuberculin needle (0·4 mm) and stored at −70°C in 1·2 ml aliquots.

CMS assay. The term 'initial kinetics of solubilization' (IKS) refers to the percentage increase in CMS per min between 10 and 20 min of incubation at 37°C. The term CMSC refers to CMS measured after 120 min of incubation of IC with serum.

The CMS assay has been described in detail in an earlier report (Baatrup *et al.*, 1983). In brief, the following procedure was used: test sera, control samples and IC suspension were thawed just before testing. One volume serum is diluted in one volume PBS^{++} and transferred to a thermostated 37°C waterbath and pre-incubated for 10 min. The IC suspension is whirlmixed and dispensed by passage through a tuberculin needle. Forty microlitres of IC suspension per ml 1:2 diluted serum is added, and samples (200 μl) drawn after 10, 20, 40, 60 and 120 min of incubation are transferred to 2·8 ml ice cold PBS containing 20 mM EDTA, to stop the reaction. The tubes are centrifuged at 3,000*g* for 10 min. The upper 1·0 ml supernatant is discarded, the next 1·0 ml (designated '1/3 supernatant') is transferred to another PVC tube. The remaining 1·0 ml is designated '1/3 supernatant + precipitate'. All tubes are counted in a gammacounter and CMS is calculated from the equation:

$$\frac{(3 \times 1/3 \text{ supernatant}) \times 100}{2 \times 1/3 \text{ supernatant} + (1/3 \text{ supernatant} + \text{precipitate})} = \%\text{CMS}.$$

PEG complement consumption assay. The assay has previously been described in detail (Brandslund *et al.*, 1981). Briefly, sera are subjected to PEG precipitation followed by washings in PEG (final concentrations of 2·75% and 3·5% PEG-6000 in borate buffer, pH 8·3), and the precipitate is resuspended in 0·15 M veronal buffer, pH 7·4 and mixed with guinea-pig serum absorbed with sheep erythrocytes. The amount of guinea-pig complement was adjusted to optimize sensitivity in each experiment. The complement consumption is measured as a reduction of haemolytic capacity after addition of sensitized sheep erythrocytes.

High pressure gel permeation chromatography (HPLC-GPC). The samples were centrifuged for 5 min at $10^4 g$ before the injection of 20 μl into the sample loop (U6K, Waters). The sample was by means of Waters 6000 A solvent delivery system pumped through a pre-column (7·5 × 75 mm) and a size separation column (7·5 × 300 mm, TSK 4000 SW, Toyo Soda, Japan) at a rate of 1 ml per min. The chromatography was in 100 mM Na_2SO_4, 20 mM phosphate, 7 mM NaN_3, pH 6·8. Fractions of 250 μl were collected onto 96 wells microplates (Immunoplate II, Nunc, Denmark) precoated by incubation with 200 μl of anti-gamma chain antibody (Dakopatts, Denmark) diluted 4×10^3 in 0·1 M bicarbonate, pH 9·6. Standard dilutions of IgG were applied to the last two rows of the plate. The plates were after incubation and wash developed with alkaline phosphatase-labelled goat anti-human IgG antibody (Sigma, USA).

Reagents and solutions. Bovine serum albumin, fration V and tetrasodium EDTA, from Sigma, St Louis, Missouri, USA. Polyethylene glycol mol. wt 6,000 (PEG-6000): Behringwerke AG, Marburg, FRG. Sepharose CL 4B, cyanogenbromide activated: Pharmacia Fine Chemicals, Uppsala, Sweden. Iodogen: Pierce Chemical Co., Rockford, Illinois, USA. PBS^{++}: PBS containing 15×10^{-5} M Ca^{2+} and 5×10^{-4} M Mg^{2+}, pH 7·4. EDTA-PBS: PBS containing 20 mM EDTA. Tween-20-PBS: PBS with 0·5 ml Tween 20 per l.

RESULTS

CMS capacity of sera from SLE and RA patients

Sera from 12 SLE and 14 rheumatoid arthritis patients were tested for CMS, in parallel with sera from healthy donors (NHS). The reference CMSC was from 32 to 60% (mean ± 2 s.d.), with a mean value of 46%. Only one SLE patient showed appreciable CMS activity (38%), the remaining having a CMSC clearly below the normal values. The mean CMSC for the 12 SLE sera was 21%. By contrast, the CMSC levels of all RA sera were within the normal range (mean CMSC, 44%) and the IKS of the sera was normal.

Sera from four SLE patients and three RA patients were selected for further investigations. The CMS of these sera, the reference CMS range and CMS of a negative control serum are shown in Fig. 1.

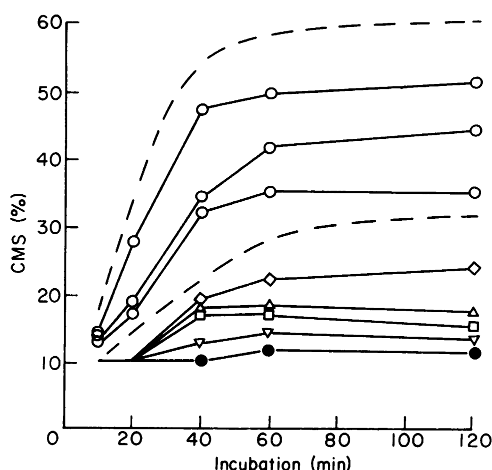

Fig. 1. Kinetics of CMS of four SLE sera (△, ▽, □, ◇), three RA sera (○) and a negative control serum (●). CMS was measured according to the standard procedure. Each point represents the mean of duplicate determinations (mean difference between duplicates was 2·4%). The area within the broken lines indicates the reference CMS (mean ± 2 s.d.). Each SLE patient is represented by the same symbol in all figures.

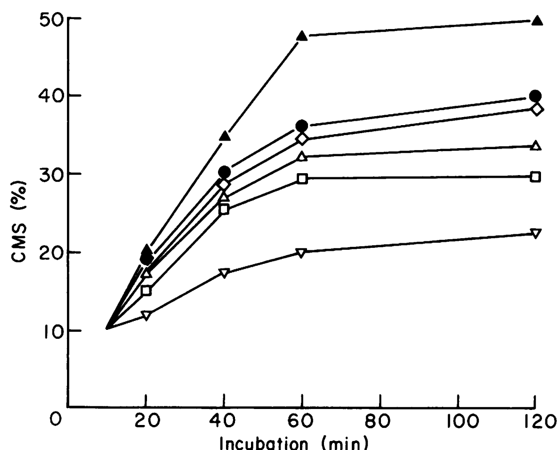

Fig. 2. Inhibitory effect of SLE sera on the CMS of normal donor serum. Two thousand one hundred microlitres of normal donor serum, diluted 1:2 in PBS^{++}, was added to 200 μl 1:2 diluted serum from another donor (▲) to 200 μl 1:2 diluted heat treated normal serum (●) and to 200 μl 1:2 diluted serum from the four SLE patients (△, ▽, □, ◇). The CMS of the serum mixtures was determined according to the standard procedure. Each point represents the mean of duplicate determinations (mean difference between duplicates 3·0%).

SLE sera inhibit CMS of normal donor serum

Normal donor serum (2,100 μl, 1:2) was mixed with 200 μl 1:2 diluted serum from (1) another normal donor, (2) heat treated serum from the same donor and (3) the four SLE sera, respectively, and incubated at 37°C for 20 min. ^{125}I-BSA–anti-BSA IC was added and the CMS of each serum mixture was determined. Fig. 2 shows that admixture of SLE sera to NHS reduced the CMS of the latter more than what could be accounted for by the addition of heat treated serum. The mixing of two NHS resulted in the expected CMS and no demonstrable inhibition.

As the RA sera exhibited normal CMSC, a comparison with heat treated serum would not be reasonable in inhibition studies. We examined these sera by comparing each of them with an NHS exhibiting the same level of CMSC. In such a system no inhibitory effect of RA sera was observed.

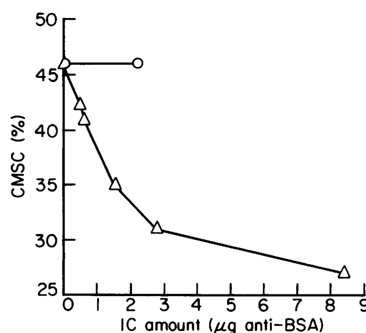

Fig. 3. Inhibition of CMS by resuspended PEG precipitates obtained from NHS containing pre-formed IC. Different amounts of BSA–anti-BSA complexes were added to 600 μl of normal donor serum at 4°C. The IC were precipitated by PEG-6000 (see text) and the precipitates were resuspended in 2·5 ml of a 1:2 diluted normal donor serum. The CMSC of the serum samples was measured by the standard procedure (△). PEG precipitates were also prepared from serum containing no pre-formed IC or a fixed amount of IC and which had been incubated at 37°C for 120 min. These precipitates were similarly resuspended in 2·5 ml of the 1:2 diluted normal serum and the CMSC of this serum was determined (○). Each point represents the mean of duplicate determinations.

PEG precipitates of sera with pre-formed unsolubilized IC inhibit CMS

Varying amounts of unlabelled BSA–anti-BSA IC were added to a normal donor serum at 4°C to prevent solubilization of the complexes. The serum samples were subjected to PEG (2·75%) precipitation to obtain an IC enriched fraction and the precipitates were resuspended in serum from another normal donor. The CMSC of this donor serum was then determined after addition of the standard amount (40 μl) of ^{125}I-labelled BSA–anti-BSA IC (see Materials and Methods). Fig. 3 shows the relation between the amount of unlabelled IC added to serum prior to PEG precipitation, and the inhibition of CMSC induced by the resuspended PEG precipitates.

Solubilized IC were prepared by adding unlabelled BSA–anti-BSA complexes (containing 2·2 μg anti-BSA per ml serum) to NHS and incubating the serum at 37°C for 2 h prior to PEG precipitation. A control serum, receiving no IC, was similarly incubated and PEG treated. In contrast to non-solubilized IC the solubilized IC were repeatedly found not to inhibit the CMSC of normal donor serum (Fig. 3). It is here pertinent to mention that the PEG precipitation of solubilized IC is less efficient than the precipitation of non-solubilized IC (see below).

Other experiments have shown that PEG by itself exerts a slight inhibitory effect on CMSC sufficient to explain the relatively low CMSC value obtained with the control serum containing no unlabelled IC (Fig. 3).

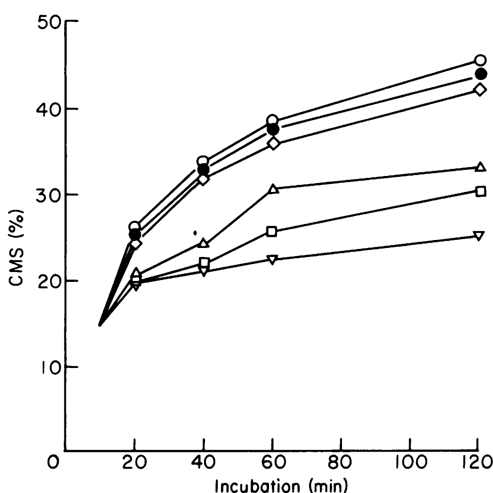

Fig. 4. Inhibition of CMS by PEG precipitated endogeneous IC from one RA serum (○), four SLE sera (△, ▽, □, ◇) and one normal donor serum (●). Six hundred microlitres of serum was used for precipitation and the precipitates were resuspended and tested for inhibition of CMS as described in the text. Each point represents the mean of duplicate determinations.

PEG precipitates from SLE sera inhibit CMS

PEG precipitates were obtained from 600 μl serum of each of the four SLE patients, one RA serum and one normal donor by the procedure described above. The precipitates were resuspended in 2·5 ml 1:2 diluted normal donor serum and the CMS of the serum was determined. Resuspended precipitates prepared from three of the SLE sera inhibited the solubilization (Fig. 4) and the degree of inhibition was approximately equal to that obtained with 200 μl whole serum from these patients (see Fig. 2). PEG precipitates from SLE patient No. 1 and the RA serum did not inhibit solubilization when compared to the normal donor control.

Precipitation of solubilized and non-solubilized IC by PEG

Hundred microlitres of ^{125}I-labelled BSA–anti-BSA suspension was added to 2·5 ml 1:2 diluted normal serum and 200 μl samples were drawn before incubation and after 10, 20, 60 and 120 min of

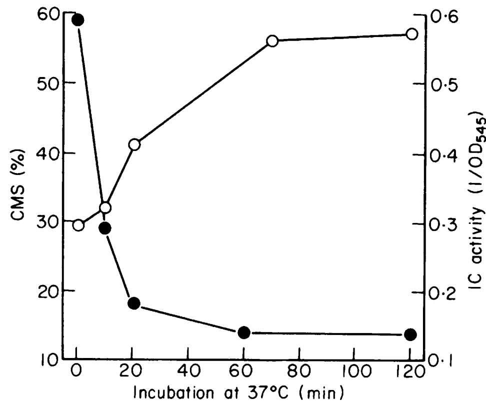

Fig. 5. The influence of the degree of solubilization of IC on the IC activity recorded in the PEG-CC assay. A BSA–anti-BSA suspension (100 μl) was solubilized in 2·5 ml (1:2) normal donor serum according to the standard procedure (○). Eighty microlitre samples were drawn from the same tubes and tested in the PEG-CC assay (●). Means of duplicate determinations.

incubation at 37°C. The degree of solubilization of the IC was determined for all samples. In addition, 80 μl samples collected after the same incubation times, were mixed with 120 μl cold borate buffer and subjected to the PEG precipitation procedure. The amount of IC precipitated was measured in a gamma counter before introduction into the complement consumption step of the PEG-CC assay. As shown in Fig. 5, the PEG-CC response is highly dependent upon the degree of solubilization of the IC. This is in part due to the fact that decreasing amounts of IC are precipitated as CMS proceeds. The average recovery of unsolubilized IC was 32% and of the IC solubilized to 60% (Fig. 5) 19% were recovered in the precipitate. The low recovery percentages are due to the extensive washing procedures in performing the PEG precipitation.

Solubilization of endogeneous IC in SLE sera

Endogeneous IC in SLE sera can be solubilized by addition of normal donor serum. Two hundred microlitres of 1:2 diluted SLE or RA serum was mixed with 400 μl 1:2 diluted normal donor serum at 4°C. One half of the sample was incubated for 60 min at 4°C or in the presence of EDTA, the other half for 60 min at 37°C to allow solubilization of the complexes. All samples were then tested in the PEG-CC assay. Solubilization of endogeneous IC resulted in a marked reduction of the activity recorded in the PEG-CC assay (Fig. 6). In contrast, similar treatment of the three RA sera—only

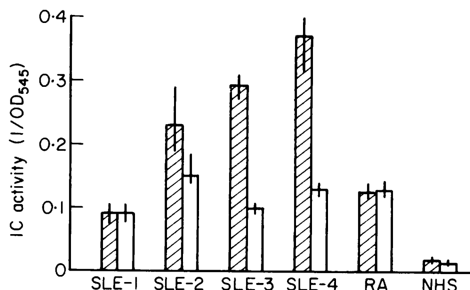

Fig. 6. Effect of solubilization of endogeneous IC in SLE and RA sera on the IC activity recorded in the PEG-CC assay. Two hundred microlitres SLE or RA serum, 1:2 in PBS^{++}, was added to 400 μl 1:2 diluted NHS at 4°C. The samples were divided into two aliquots; one was incubated for 60 min at 4°C (▨), the other for 60 min at 37°C (□). Vertical bars indicate the range of quadruplicate determinations.

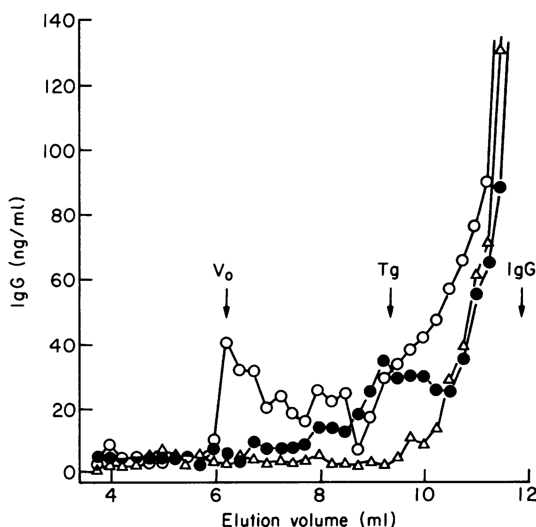

Fig. 7. Redistribution of macromolecular IgG in an SLE serum following solubilization of endogenous IC by addition of normal donor serum (NHS). Serum was fractionated on HPLC and the fractions analysed by IgG specific ELISA. V_0 = void volume; Tg = bovine thyroglobulin; IgG = monomeric IgG. SLE serum mixed with NHS (●), SLE serum mixed with heat treated NHS (○) and heat-inactivated NHS only (△).

one shown in Fig. 6—and serum from SLE patient No. 1 did not affect the IC activity measured in this assay. When the normal donor serum was incubated with heat treated serum or without admixture of other sera at 37°C for 1 h no significant decrease in IC activity was observed in the PEG-CC assay (Fig. 6).

In two preliminary studies SLE serum (50 μl) was incubated for 90 min at 37°C with either 100 μl 1:3 diluted normal donor serum or 100 μl 1:3 diluted and heat-inactivated donor serum. The control included donor sera incubated with PBS^{++} only. The samples were centrifuged at $10^4 g$ for 5 min and 20 μl were injected onto the HPLC column. Collected fractions were assayed for IgG by ELISA (Fig. 7). A reduction in the high molecular weight IgG in the SLE serum was observed upon incubation with normal donor serum but not after mixing with heat-inactivated serum.

DISCUSSION

We have investigated the CMSC of sera from 12 SLE and 14 RA patients and our results confirm the findings of reduced CMSC in SLE sera reported earlier (Schifferli *et al.*, 1981; Aguado *et al.*, 1981; Sakurai *et al.*, 1982).

This study indicates for the first time the presence of incompletely complement solubilized endogeneous IC in sera from SLE patients. This conclusion is based on the following findings: (1) SLE sera with reduced CMSC and high IC activity in the PEG-CC assay inhibit the CMS of normal donor serum to a greater extent than do IC positive RA sera and IC-negative control sera. (2) SLE sera exhibiting close to normal CMSC exert no such inhibitory effect. (3) CMS inhibitory factors in SLE sera were precipitable by a PEG-6000 concentration of 2·75%. (4) Endogeneous IC in SLE sera could be further solubilized by addition of normal serum resulting in a decrease of the IC activity in the PEG-CC assay which measures unsolubilized IC efficiently. This appears, in part, to be due to the PEG precipitation step, in which unsolubilized IC are precipitated more efficiently and partly to the complement consumption step in which solubilized 'end stage' complexes are less active. (5) Incubation of SLE serum with normal serum and subsequent analysis by HPLC-ELISA for macromolecular IgG indicated a reduction in high molecular weight IgG in the SLE serum. Such a

redistribution of macromolecular IgG in SLE serum was not seen after its incubation with heat-inactivated normal serum.

These data pointing to the existence of incompletely complement solubilized IC in SLE sera are in agreement with the experiments of Arroyave, Wilson & Tan (1976) showing that eight out of 10 SLE patients investigated exhibited a ⩾19S factor which contained IgG and which was capable of activating the alternative complement pathway. They are also in accordance with the data of Medof, Scarborough & Miller (1981) indicating that IC isolated from strongly IC positive SLE sera, by the Raji cell technique, could be released from the cells when incubated with normal donor serum. The release was interpreted to be due to a complement-mediated solubilization reaction.

A correlation between disease activity, especially renal involvement, and reduced levels of complement factors in the presence of circulating IC in SLE has been reported (Abrass *et al.*, 1980; Cano *et al.*, 1977; Lloyd & Schur, 1981). It is tempting to believe that the disease activity in SLE does not simply reflect high IC levels and high autoantibody titres, but the presence of incompletely solubilized phlogistic circulating IC, capable of activating complement when a fresh source of complement is supplied. These IC may be formed in situations when the serum concentration of certain complement factor(s) falls below a critical level (unpublished data) due to a high rate of and/or persisting IC formation. Both the postulated precipitation inhibition effect of classical pathway proteins (Schifferli, Woo & Peters, 1981) and the CMS phenomenon may be important for the prevention of accumulation of incompletely solubilized circulating IC. This may explain the low low levels of complement proteins of both pathways seen in severe cases of active SLE (Aguado *et al.*, 1980; Lloyd & Schur, 1981; Davis, Cumming & Verrier-Jones, 1977).

The RA sera investigated in this study exerted normal CMSC, caused no inhibition of the CMS of normal serum and no change in the PEG-CC assay was seen upon their incubation with normal donor serum. However, these sera were IC positive when tested in the PEG-CC assay, which means that they do contain complexes capable of consuming C factors. Apparently, the amount of incompletely solubilized IC precipitated by PEG from these sera was too low to cause inhibition of CMS in normal donor sera. Alternatively, the circulating IC may be stabilized by IgM rheumatoid factors, which was reported to inhibit CMS (Rodriguez *et al.*, 1982).

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