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Examination of complement C3 metabolism in rheumatoid arthritis using 2-dimensional immunoelectrophoresis

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SUMMARY A previous report that C3 activation products are demonstrable in the plasma of subjects with rheumatoid arthritis was reinvestigated using 2-dimensional immunoelectrophoresis. Initially it seemed that results were in agreement, but the occurrence of similar patterns in normal sera and poor reproducibility of results suggested the findings were artefacts. When calcium and magnesium ions were removed with EDTA, C3 activation products were no longer demonstrable in plasma from normal subjects or from patients with rheumatoid arthritis. The results obtained without EDTA in the buffer showed a greater degree of complement activation in patients with rheumatoid arthritis, suggesting that in this condition the serum may have a greater potential ability to bring about complement activation.

Complement may be implicated in a disease process if levels of component proteins within the system are depressed, suggesting activation followed by degradation and excretion of the molecule. A better guide to the kinetics of complement metabolism is the demonstration of activation products of complement components in body fluids. The third component of complement (C3) is best used for this purpose since both it and its activation products are easily measured, and they can indicate activation of the system whether initiated by the classical or alternate pathway.

In active systemic lupus erythematosus (SLE) complement involvement in the disease process is implicated both by low CH50 (Vaughan *et al.*, 1951a; Townes, 1967; Schur and Sandson, 1968) and by the presence of C3 activation products in serum (Morse *et al.*, 1962; Lachman, 1963). In rheumatoid arthritis (RA) the role of complement is not clear. RA has similarities to SLE but is generally associated with normal or raised serum CH50 (Vaughan *et al.*, 1951b; Kellett, 1954; Ellis and Felix-Davies, 1959; Franco and Schur, 1971), although in the synovial fluid low CH50 and the presence of C3 activation products have been reported (Hedberg, 1963; Pekin and Zvaifler, 1964; Hedberg, 1967; Zvaifler, 1969). Versey *et al.* (1973) reported the presence of C3 activation products in rheumatoid plasma but Alper (1974) found no evidence for complement activation in rheumatoid serum.

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Patients and methods

PATIENTS

The investigation was carried out in two stages, with modification of the method for the second. Specimens were obtained from two groups of subjects, one for each stage. In both groups the normal subjects were healthy members of laboratory staff, and the rheumatoid patients (American Rheumatism Association, 1959) had active disease, judged clinically and by an ESR>30 mm/h. For stage 1 the normal subjects included 3 males and 4 females, age range 23–32 years, and the rheumatoid patients included 2 males and 9 females, age range 26–66 years. For stage 2 there were 9 normal males and 5 normal females, age range 23–51 years, with 6 rheumatoid males and 16 rheumatoid females, age range 23–68 years.

SPECIMENS

Venous blood samples from all subjects were collected into Sterilin KE 5 tubes containing EDTA, and the plasma was separated by centrifugation at 2000 rpm for 10 minutes within 3 hours of collection.

Specimens were examined immediately or stored in 1 ml aliquots at −70°C for periods up to 6 weeks without thawing.

STAGE 1 TECHNIQUE

Specimens were examined by 2-dimensional immunoelectrophoresis using a technique modified from Clarke and Freeman (1968). Veronal buffer (0·1 mol/l, pH 8·6) was used throughout, barbitone sodium and diethyl barbituric acid purchased from BDH Chemicals, Poole, England, and made up in a single distilled water. Agarose (Inubidose A37, L'Industrie Bilogique, Francaise SA) was made up as 1·5% solution in buffer and stored at 4°C in 20 ml aliquots. Electrophoresis was performed using PCD BES1 tanks connected to a PCD BES3 power unit (PCD, Farnborough, England).

Clean glass microscope slides 76 mm × 38 mm were covered evenly with 4 ml agarose, which was then cut into three strips 76 mm × 10 mm, the excess from each edge being discarded. The strips were separated from each other and left on the slide. A 2 mm diameter well was cut 10 mm from the cathodal end, near to one edge of the strip. 2 μl samples were applied to the wells and the first dimension electrophoresis was performed for 2½ hours at 6·6 mA constant current per strip on a water-cooled glass plate. Absorbent lint wicks (Veron-Canis, England) connected the strips to the buffer tanks.

After completion of the first dimension electrophoresis, each strip was moved onto a clean microscope slide with the specimen well innermost. The remainder of the slide was covered with 3 ml agarose, containing antiserum as noted below, ensuring contact with the first dimension strip. The antiserum was sheep antihuman C3c (Department of Experimental Pathology, University of Birmingham). It was not monospecific and contained an additional antibody which was not identified. The mixture of antiserum and agarose was prepared at 47°C in the following proportions: 1·5% agarose 2·0 ml, buffer 0·8 ml, antiserum 0·2 ml. This gives 6·6% antiserum in 1% agarose.

The second dimension electrophoresis was performed at 90° to the first, but in similar fashion, with the first dimension strip at the cathodal end. A constant current of 7·5 mA per slide was passed for 16 hours. Cooling was not used. Slides were dried beneath weighted wads of filter paper, then in a stream of warm air. The dried gel was overstained for 1 minute with naphthalene black and excess stain removed in several changes of stain solvent.

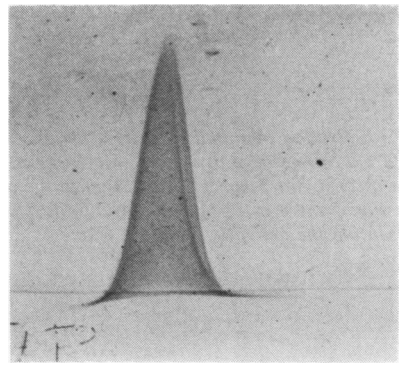
STAGE 2 TECHNIQUE

This was similar to stage 1 technique, the only modification being that 2·02 g/l EDTA (BDH) was added to the buffer used in the electrophoresis tanks and for the first dimension gel. EDTA was not included in the second dimension gel as calcium ions are essential for complete precipitation of complexes. (Laurell, 1972).

Results

PRELIMINARY EXPERIMENTS

A fresh plasma specimen subjected to 2-dimensional immunoelectrophoresis (IE), with activation of complement inhibited by EDTA, produced a single complement precipitation peak corresponding to native C3 (Fig. 1). The unidentified antibody present



Figs. 1–8: *2-dimensional immunoelectrophoresis using anti-C3c antiserum.*

Fig. 1 *C3 activation inhibited by EDTA buffer. Normal EDTA plasma showing a single C3 precipitation peak. The densely stained peak is C3 and the less dense peak lying behind it is due to an impurity in the antiserum.*

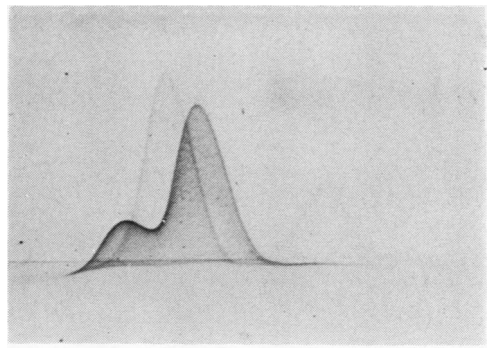


Fig. 2 *C3 activation inhibited by EDTA buffer. Normal EDTA plasma after storage at −20°C for 2 weeks. Two peaks were produced by C3; a slow moving peak, probably due to complexing of some of the C3, and the native C3 peak.*

in our antiserum gave the less distinct peak seen in the figures lying behind the native C3 peak. This protein had constant electrophoretic mobility and served as a marker to distinguish between C3 and the degradation product (C3c) if only one peak was present. Storage at −20°C for 2 weeks produced a second complement peak with slower electrophoretic mobility (Fig. 2). Storage at −70°C did not give rise to the slow component. Incubation to induce activation of the complement (Laurell and Lundh 1967), after storage at −20°C, produced a third complement peak with faster electrophoretic mobility (Fig. 3).

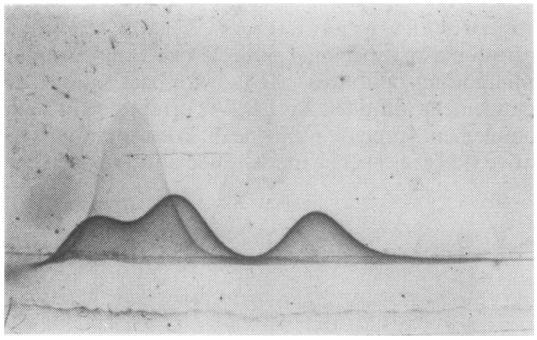


Fig. 3 *C3 activation inhibited by EDTA buffer. Normal EDTA plasma after storge at −20°C for 2 weeks, then incubation at 37°C for 3 hours. In addition to the peaks present in Fig. 2 there is a peak produced by fast moving C3, representing the activation product C3c.*

STAGE 1 METHOD

Fig. 4 shows typical results obtained using plasma from normal and rheumatoid subjects. Both the slower and faster moving components of C3 were present, although these runs were performed on fresh plasma, without storage at −20°C or incubation. In all the specimens examined, both normal and rheumatoid subjects showed apparent C3 activation but there was a difference in pattern, with a greater amount of fast moving component present in rheumatoid specimens. The distinction between the peaks was not clear and this made accurate measurement impossible.

The reproducibility obtained from stage 1 method was confirmed by running the same specimen at the same time in a single electrophoresis tank (Fig. 5). When reproducibility was examined using four separate electrophoresis tanks connected to the same power pack, each containing veronal buffer used over

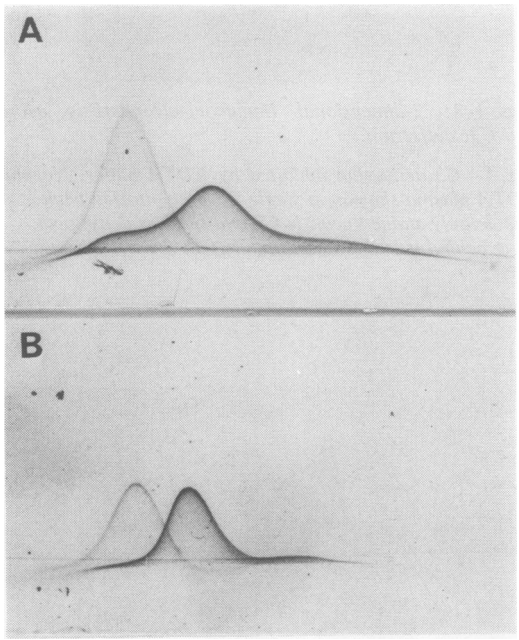


Fig. 4 *Buffer without EDTA. Typical results using EDTA plasma from rheumatoid (A) and normal (B) subjects. Although neither was stored nor incubated, both show slow and fast moving components of C3. The RA specimen shows a greater degree of activation than the normal.*

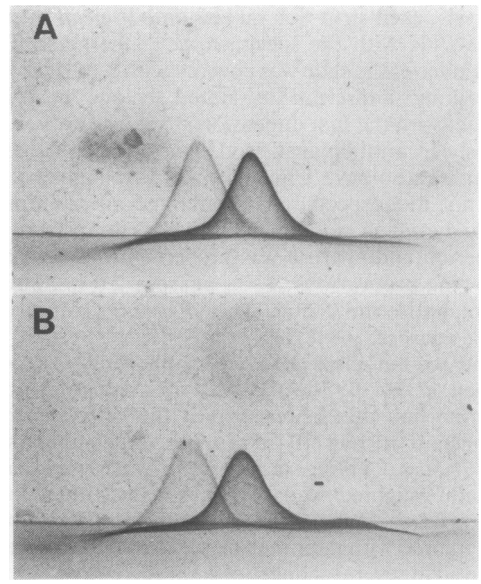


Fig. 5 *Buffer without EDTA. Two aliquots of a single EDTA plasma specimen examined at the same time in a single buffer tank. The C3 precipitation patterns are similar.*

an equal period, completely different patterns resulted (Fig. 6).

STAGE 2 METHOD

The reproducibility of this method was examined in the same way as stage 1 and was maintained when using different tanks of buffer, all subjected to routine use (Fig. 7). Examination of normal and rheumatoid plasma specimens by this method showed no evidence of activation products in either (Fig. 8).

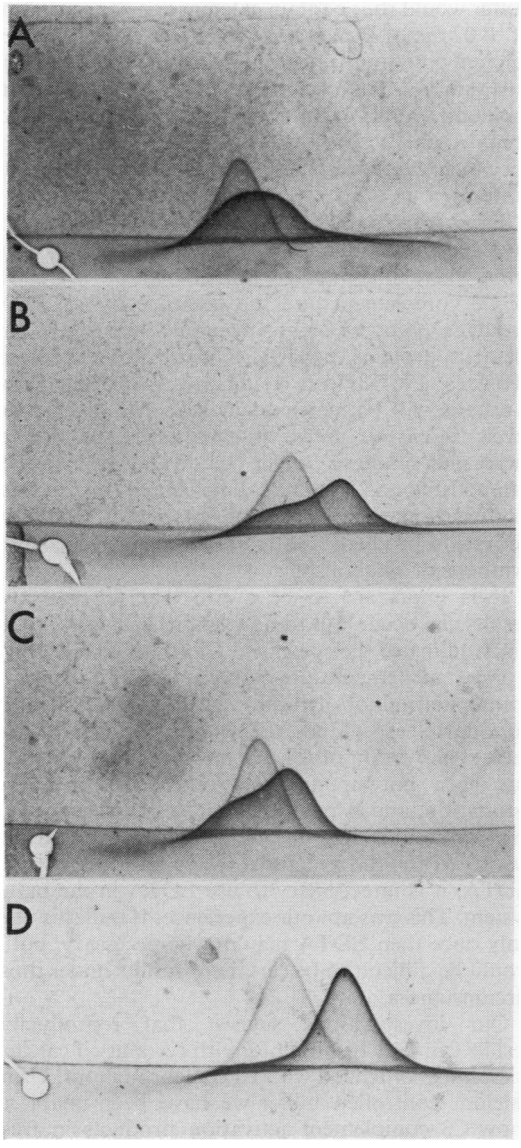


Fig. 6 *Buffer without EDTA. Four aliquots of a single EDTA plasma specimen examined at the same time in four separate buffer tanks (A–D) connected to the same power pack. The C3 precipitation curves vary in configuration, showing greater or lesser C3 activation depending upon the tank used.*

Discussion

The positive results which were obtained without EDTA in the buffer are almost certainly due to activation of C3 complement occurring during electrophoresis. This is suggested by the lack of

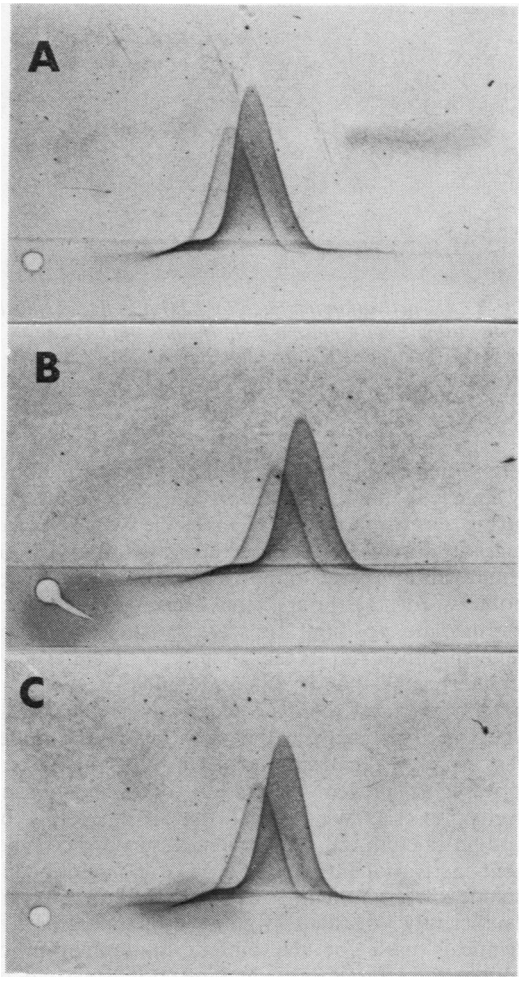


Fig. 7 *C3 activation inhibited by EDTA buffer. Three aliquots of a single EDTA plasma specimen examined at the same time in three separate buffer tanks connected to the same power pack. The C3 precipitation curves are similar, showing no fast component and little slow component.*

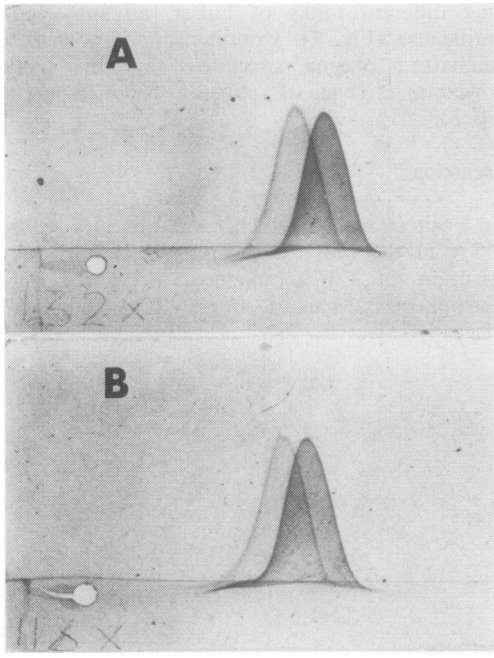


Fig. 8 *C3 activation inhibited by EDTA buffer. Typical results using EDTA plasma from rheumatoid (A) and normal (B) subjects. Neither shows evidence of C3 activation.*

definition between peaks, probably brought about by change in mobility of the protein at variable times during electrophoresis. A molecule which began migration as C3 and became activated after 1 hour would run at slower mobility for 1 hour and faster mobility for 1½ hours, thus terminating in an intermediate position. In stage 1, aliquots of the same specimen run in the same bath of buffer gave similar results, but if different baths of the same buffer were used then the patterns were markedly different. This suggests that a factor in the buffer was involved. Inclusion of EDTA in the system appears to prevent activation occurring during electrophoresis. We suggest that it does this by binding calcium ion, which is necessary for complement activation. Calcium was not intentionally included in the system, but could have entered from insufficiently distilled water, contamination in chemicals used for the buffer, contamination of equipment by tap water, a leaking cooling system, or from the antiserum in the second phase gel. This latter would be particularly important if the automated system of 2-dimensional IE was used, since in that technique the antiserum is already on the plate while the first dimension electrophoresis is being performed and diffusion of calcium can occur into the first dimension gel. BDH state that their barbitone sodium contains calcium at a concentration of 18 ppm. Our distilled water contained no detectable calcium when analysed on a system which will detect 0·01 mmol/l (approx 0·5 ppm). A solution of BDH barbitone salt in the concentration used for our buffer would thus contain calcium at a concentration of 0·02 mmol/l. Analysis of used buffer yielded calcium concentrations ranging from 0·02 mmol/l to 0·08 mmol/l. The occurrence of this further contamination would explain the variation in degree of complement activation which occurred when the specimen was examined using different baths of buffer.

There can be little doubt that complement is involved in the inflammatory process in RA. In the joint, where inflammation is most marked, low levels of C3 complement and the presence of activation products point to complement involvement. Complement proteins behave as acute phase proteins (Townes, 1967) and it would thus be expected that synthesis will be increased in RA. The high serum levels found in active disease could be due to increased synthesis which could mask increased catabolism or activation. 2-dimensional IE is an ideal method for assessing activation since it will show activation products and is independent of increased synthesis or catabolism.

It is important to be aware that false-positive results can occur. Up to now emphasis has been laid on handling of the specimen. Clotting (Krøll, 1969), storage at temperatures above −70°C, bacterial contamination, or disruption of cells with lysosomal enzyme release (Alper, 1974) will all lead to artefactual activation of C3 complement. Less emphasis has been put upon activation occurring during estimation, and Alper (1974) suggested that providing the initial few ml of withdrawn blood are discarded and the remainder mixed immediately with EDTA, it is unnecessary to use EDTA in the buffer system. This was not our experience. If buffer is used only once then EDTA may not be necessary, but it would be difficult to be certain of results under those circumstances.

Our investigations suggest that reproducible results can only be obtained with certainty if calcium is actively controlled with EDTA throughout. Using calcium controlled buffer we have been unable to show C3 complement activation products in fresh plasma from patients with RA.

If one accepts that the activation products shown by Versey *et al.* (1973), and by ourselves before controlling calcium, were artefactual, the fact that both investigations showed a higher degree of activation occurring during electrophoresis in RA

plasma, compared with normal plasma, remains unexplained. This suggests that RA serum, or plasma to which calcium is added, has a greater than normal potential to undergo C3 complement activation. It is possible that activation occurs *in vivo* but the products do not reach sufficiently high concentrations to be demonstrable by 2-dimensional IE; C3 activation products are cleared from the circulation five times more rapidly than the parent molecule (Alper *et al.*, 1967). We are undertaking further studies to examine the potential of rheumatoid serum to initiate complement activation.

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