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THE INHIBITION OF COMPLEMENT-DEPENDENT LYMPHOCYTE ROSETTE FORMATION BY THE SERA OF CHILDREN WITH STEROID-SENSITIVE NEPHROTIC SYNDROME AND OTHER RENAL DISEASES

MARY D. SMITH, T. M. BARRATT, A. R. HAYWARD AND J. F. SOOTHILL

Department of Immunology, Institute of Child Health and Hospital for Sick Children, Great Ormond Street, London

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SUMMARY

Sera from patients with steroid-sensitive nephrotic syndrome (SSNS) in relapse, Henoch–Schönlein purpura with nephritis (HSP) and acute post-streptococcal glomerulonephritis inhibited EAC rosette formation by normal human lymphocytes; a similar effect was seen in some patients with focal glomerulosclerosis, but not in patients with congenital nephrotic syndrome. There was significantly less inhibition by sera of SSNS and HSP patients in remission. There were fewer EAC rosette-forming cells (EAC-RFC) in the blood of three patients with SSNS in relapse, suggesting that such blockade occurred *in vivo*. These findings, interpreted as evidence of circulating activated C3 in the sera, provide further indirect evidence of the immunopathogenesis of these diseases. Sera of healthy adults inhibited EAC rosette formation to a small extent which correlated inversely with the numbers of EAC-RFC in their blood. The EAC rosette inhibition test may be sensitive enough to detect normal variations of complement activation in healthy individuals.

INTRODUCTION

Immunological mechanisms have been implicated in a wide variety of renal diseases. Evidence for this usually includes detection of immunoglobulins and complement in the glomeruli, such as is found in many forms of proliferative glomerulonephritis. However in the steroid-sensitive nephrotic syndrome (SSNS) the glomeruli either appear normal or show only minimal changes on light microscopy (Blainey *et al.*, 1960) and most immunofluorescence studies have not detected immunoglobulins or complement in the glomeruli

Correspondence: Professor J. F. Soothill, Department of Immunology, Institute of Child Health, London, WC1N 1EH.

(Drummond *et al.*, 1966). The pathogenesis of this disease is not yet established, but some clinical associations suggest an immunological mechanism (Hardwicke *et al.*, 1959). The only consistent evidence of immunological disturbance however is the rise of immunoconglutinin during relapse (Ngu, Barratt & Soothill, 1970), pointing to complement activation.

Complement receptor lymphocytes (CRL) are a subpopulation of human B lymphocytes which form rosettes with erythrocytes coated with antibody and complement (EAC) because they have receptors for activated C3. In this paper CRL, which form EAC rosettes, are referred to as EAC rosette-forming cells (EAC-RFC). Activated fragments of the third component of complement (C3b and C3d) in solution may bind to these receptors on CRL and inhibit EAC rosette formation (Eden, Bianco & Nussenzweig, 1973). Ezer & Hayward (1974) demonstrated this inhibition by sera from patients with Crohn's disease, mesangiocapillary glomerulonephritis and lepromatous leprosy, all diseases in which either immune complexes or activated C3 fragments are present in the circulation. They suggested that the inhibitory effect could be due to activated C3 either in solution or bound to immune complexes. They also showed low numbers of circulating EAC-RFC in these patients, probably as a result of *in vivo* blockade of the complement receptors on the CRL.

We have therefore studied EAC rosette inhibition by sera of children with SSNS and other renal diseases seeking further evidence of complement activation during relapse, and have also measured the EAC-RFC count in the peripheral blood.

PATIENTS AND METHODS

Patients

Blood samples were obtained from ninety-six children with renal disease and twenty-three control subjects. Of the former, forty-eight fulfilled the criteria for SSNS, namely oedema, heavy highly selective proteinuria and a plasma albumin concentration less than 2·0 g/100 ml during relapse, in whom a remission (i.e. loss of proteinuria) was induced by corticosteroid therapy. Renal biopsies were taken in only a few of these patients, but where performed showed minimal histological changes on light microscopy. These patients were classified as being in relapse at the time of study if the urine albumin : urine creatinine concentration ratio exceeded 1·0 and remission if it did not (Barratt & Soothill, 1970). Fourteen samples were obtained during relapse and thirty-four during remission. Twelve of the fourteen patients studied in relapse and eleven of thirty-four studied in remission were on steroids. Six children were studied repeatedly during the course of relapse and remission of their nephrotic syndrome. Nineteen children with Henoch–Schönlein purpura (HSP) and nephritis were studied, nine during the acute phase (i.e. the first few weeks after the appearance of renal involvement whilst there were still extra-renal manifestations such as skin rash and arthralgia) and ten during the convalescent phase when the extra-renal symptoms had subsided. Seven children had acute post-streptococcal glomerulonephritis (AGN), twelve had steroid-resistant nephrotic syndrome with focal glomeruloscelerosis (FGS) and five had the congenital nephrotic syndrome (CNS).

Control subjects comprised thirteen healthy laboratory personnel and ten children hospitalized for elective minor procedures.

Blood samples were allowed to clot at room temperature. The serum was separated, stored at $-20°$ and tested in batches (which always included both patient and control sera).

Methods

Medium 199 (Wellcome Reagents, Beckenham) pH 7·4, buffered with 0·02 M HEPES was used throughout. For each assay lymphocytes were prepared from a single human adenoid within 1 hr of removal. The tissue was teased apart in medium 199 and the suspended cells were incubated with 200 mg of carbonyl iron for 30 min at 37°. Phagocytic cells and residual carbonyl iron were sedimented with a magnet and the supernatant was separated by centrifugation (20 min at 450 g) on a Ficoll–Triosil gradient (s.g. 1·083). The cells collected from the interface were washed and adjusted to a concentration of 2×10^6 cells per millilitre. Peripheral blood lymphocytes were obtained from 20 ml of defibrinated blood by the same method of separation. Erythrocytes coated with antibody and complement (EAC) were prepared as follows: sheep erythrocytes (Wellcome Reagents) less than 1 week old were washed three times and a 5% suspension was incubated with rabbit anti-sheep red cell serum (Wellcome Reagents) at a final concentration of 1:2000 for 30 min at 37°. The cells were washed and incubated with a subhaemolytic dilution (1:80–1:110) of fresh human serum for 30 min at 37°. The EAC were washed again and adjusted to 5×10^7 cells per millilitre. 1×10^7 EAC and 4×10^5 lymphocytes warmed to 37° were mixed in 7-mm plastic tubes. The cells were incubated at 37° for 1 hr and resuspended with a Vortex mixer. Trypan Blue was added and the preparation examined in a Neubauer counting chamber. If more than 10% of the cells failed to exclude Trypan Blue the preparation was rejected; this occurred only once. One hundred lymphocytes were observed and the number with three or more EAC attached counted. More than 90% of the EAC-RFC in our phagocyte-depleted adenoid lymphocyte preparations had cell surface associated immunoglobulin and so were presumably B lymphocytes. Less than 10% were monocytes as judged by peroxidase staining. Rosette formation was mediated by the C3 receptor on the lymphocyte, since under the conditions of our test, no rosettes were formed with erythrocytes coated with antibody only. It is unlikely that spontaneous rosette formation by T lymphocytes contributed to our results since no EAC-RFC were observed in human thymus lymphocyte preparations.

The percentage of lymphocytes forming EAC rosettes in the peripheral blood of three children with SSNS in relapse and thirteen healthy adults was determined directly.

To measure the inhibitory effect of sera on normal lymphocyte rosette formation, 4×10^5 packed lymphocytes prepared from adenoid tissue were incubated with 200 μl of serum or of medium 199 for 1 hr on a Matburn mixer in an incubator at 37°. The cells were washed three times and then incubated with EAC as above. One hundred lymphocytes were examined for EAC-RFC; between 17 and 30% of the lymphocytes which had been incubated in medium formed rosettes. Inhibition of EAC rosette formation by the serum incubation was expressed as: $\{100 - [(\text{percentage of lymphocytes forming EAC rosettes after serum preincubation})/(\text{percentage of lymphocytes forming EAC rosettes after medium preincubation})]\} \times 100$. Where the proportion of rosettes seen in the preparation incubated in the serum was similar to or greater than that in the preparation incubated in medium, the value of $<3\%$ inhibition was assigned.

RESULTS

The percentage EAC rosette inhibition by the sera of children with renal disease and control subjects is shown in Fig. 1. Results in the different groups were analysed by the Mann–Whitney *U* test. Inhibition significantly greater than that of healthy adult sera was found for sera of children with SSNS in relapse ($P = <0{\cdot}001$) HSP ($P = <0{\cdot}001$) and AGN ($P =$

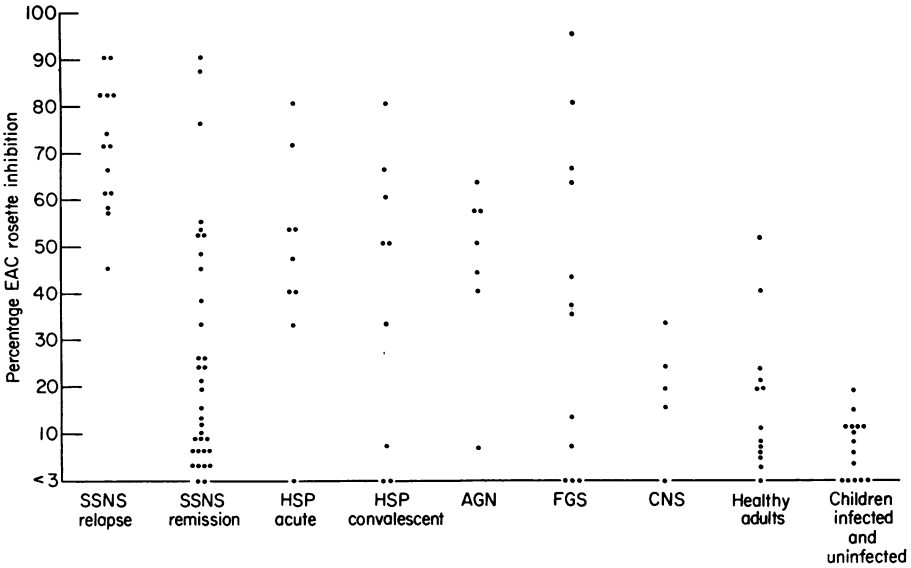

FIG. 1. Inhibition (%) by pretreatment with sera of children with various renal diseases of subsequent EAC rosette formation by human adenoid lymphocytes. SSNS = steroid-sensitive nephrotic syndrome; HSP = Henoch–Schönlein purpura; AGN = acute post streptococcal glomerulonephritis; FGS = focal glomerulosclerosis; CNS = congenital nephrotic syndrome.

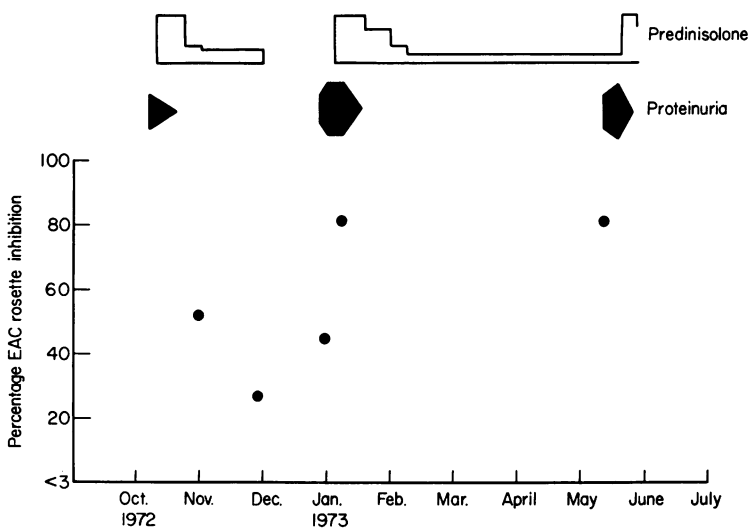

FIG. 2. EAC rosette inhibition (%) by the serum of a patient with steroid-sensitive nephrotic syndrome in relapse and steroid-induced remission.

0·03). It was not significantly greater for SSNS in remission ($P = 0·6$), FGS ($P = 0.6$), CNS ($P = 0·5$) and children with or without infections ($P = 0·2$). Some individuals who were in remission from SSNS and some with FGS gave values clearly higher than the healthy adults. All but two of the SSNS patients in relapse were on steroids. Some of the SSNS patients in

remission were also on steroids and both normal and raised inhibition of EAC rosettes was found with their sera.

In the HSP group EAC rosette inhibition by the sera of children in the acute stage of the disease was significantly greater than by serum from convalescent patients ($P = <0{\cdot}001$). Repeated values from one of the patients with SSNS in relapse and in remission is shown in Fig. 2. The serum taken during two relapses of the nephrotic syndrome gave greater inhibition of EAC rosette formation than those taken in remission. There was a progressive fall in the 6 weeks after the end of the first relapse and a rise in the first 2 weeks of the second relapse. Sequential studies in five other patients showed similarly higher values in relapse than in remission. Of special interest is a patient with seasonal nephrotic syndrome, associated with grass pollen sensitivity; he also had EAC rosette inhibition in relapse, but not while in remission (Table 1).

TABLE 1. Inhibition of EAC rosette formation by serum of patient T.T. (14 years, three seasonal attacks of SSNS, and positive prick tests) during the second and third relapses (whilst not on steroids) and during a steroid-induced remission

	Year	Month	Inhibition of EAC rosettes (%)	Steroids
Attack	1972	July	—	—
Relapse 1	1973	June	—	—
Remission	1973	January	<3	No
Relapse 2	1974	June	90	No
Remission	1974	June	4	Yes

TABLE 2. Peripheral blood EAC-RFC of three patients with steroid-sensitive nephrotic syndrome (SSNS) in relapse, and of thirteen healthy adults

Blood EAC-RFC (%)	EAC rosette inhibition by patients' sera (%)
SSNS	
9	82
18	74
8	66
Healthy adults	
25	<3
23	8
10	40
19	51
23	6
21	23
23	5
26	11
25	<3
23	14
20	21
22	7
21	19

The EAC-RFC count in thirteen healthy adults (Table 2) is inversely related to the inhibitory effect of their sera ($r_3 = 0{\cdot}86$, $P = <0{\cdot}001$). Two patients with SSNS had smaller proportions of EAC-RFC than any of the healthy adults, and a third, fewer than all but one adult control ($P = 0{\cdot}01$).

DISCUSSION

Ezer & Hayward (1974) reported inhibition of lymphocyte EAC rosette formation by the

sera of some patients with conditions associated with immune complex formation; they ascribed this phenomenon to the saturation of C3 receptors on the lymphocytes by activated complement components, either free or bound to immune complexes. The patients whose sera were inhibitory in this *in vitro* test also had fewer circulating EAC-RFC in their blood, suggesting that the C3 receptors were saturated *in vivo*. Consistent with this interpretation is the marked inhibition we have observed in acute post-streptococcal glomerulonephritis, another disease in which there is good evidence for immune complex formation (Zabriskie, 1971) and complement activation (Soothill, 1967).

We have studied by this technique four other renal diseases in which an immunological pathogenesis has been considered but for which the evidence is not conclusive. Of these we observed abnormal values in SSNS and HSP; there was a wide range of values in FGS, and normal values in CNS. The negative correlation in healthy subjects between the low levels of serum inhibition of EAC rosette formation and the EAC-RFC count suggests that complement activation occurs at all times. We are not aware of other direct evidence for this. It also suggests that the method is sensitive enough to measure normal variation in the activation of complement, perhaps both dependent on and independent of antigen–antibody reaction.

The inhibition of EAC rosette formation by the sera of children with SSNS in relapse and the low numbers of EAC-RFC in their peripheral blood provides evidence for complement activation in this disease; other studies of complement in SSNS have produced contradictory results. Complement components have not been detected in the kidney of such patients by immunofluorescent methods (Drummond *et al.*, 1966). Lange *et al.* (1951) reported that haemolytic complement may occasionally fall with relapse, and Lewis, Carpenter & Schur (1971) reported low C1q levels in the blood. However, Antilla, Barratt & Soothill (1974) analysed such data in terms of the amount of protein loss compared with patients with non-immunological renal disease and did not obtain clear evidence of complement abnormality in SSNS in excess of the likely effects of the urinary protein loss. Breakdown products of C3 were not detected by immunoelectrophoresis in patients with SSNS in relapse (Soothill, 1967) but immunoconglutinin, an antibody to activated complement, was (Ngu *et al.*, 1970). The present data are therefore the second piece of indirect evidence of complement activation in SSNS, a disease in which most direct studies by methods giving positive results in such clearly complement-mediated diseases as acute nephritis are negative. This discrepancy could be due to different sensitivities of the tests, or to activation of complement by different mechanisms.

The inhibition of EAC rosette formation we have observed might be part of the aetiological mechanisms of the diseases; equally it might be a characteristic of a person vulnerable to this disease or of its treatment, or it might be a coincidental and irrelevant effect of the precipitating event. Our data permit us to analyse these possibilities to some extent for SSNS. We obtained both normal and abnormal values for inhibition in the children in remission whether receiving prednisolone or not, so the effect is presumably not related to the drug. The normal values found in some children indicate that EAC rosette inhibition is not a characteristic inherent in those susceptible to SSNS. Abnormal values found in others might reflect a delay in achieving full remission. The inhibitory effect persists for some time after the proteinuria has disappeared (Fig. 2), providing evidence that proteinuria itself is not the cause. This is also suggested by the normal values in the five children with congenital nephrotic syndrome, though they are not an ideal group for the SSNS patients since they are

younger. The normal EAC rosette inhibition by the sera of control children with respiratory and other infections at least as severe as those associated with relapse of the SSNS suggest that the effect found in the SSNS children is not simply a consequence of infection. The abnormal values found in our atopic patient with seasonal nephrotic syndrome associated with grass pollen sensitivity (a form of SSNS of established immunological aetiology) (Hardwicke *et al.*, 1959) resemble those found in the non-atopic majority of SSNS patients and emphasize the similarity between these conditions. The EAC rosette inhibition by sera of patients with Henoch–Schönlein purpura associated with renal disease are consistent with our observation of C3 breakdown products and raised immunoconglutinin in this disease (Shulman, Barratt & Soothill, 1971).

We were surprised that only some of the results in focal glomerulosclerosis were abnormal and that the group as a whole did not differ significantly from the control group. Since some patients with this lesion respond to corticosteroid drugs and others do not, it is likely that this lesion is associated with a range of different aetiological entities, some of which may be related to SSNS. Hoyer *et al.* (1972) interpreted the immediate development of heavy proteinuria in a normal kidney grafted into a patient with FGS and renal failure as evidence against the immunopathogenesis of FGS. Our results suggest that there is hete rogeneity amongst the FGS patients and there is in any case no reason why a circulating mediator released as a result of an immunological reaction could not produce proteinuria rapidly. Evidence has been presented for a possible immunopathogenesis for some patients with congenital nephrotic syndrome (Kouvaleinen *et al.*, 1962), but it is not conclusive, and it is likely that there is more than one disease presenting as CNS. Our normal values in these patients do not support an immunopathogenic mechanism and provide control data for the SSNS patients. They are consistent with the previous negative studies in this syndrome for other evidence of complement activation (Shulman *et al.*, 1971).

The suggestion that SSNS might be an immunological disease was first made by Von Pirquet (1908) at the time that the immunopathogenesis of other forms of nephritis was being recognized. He linked this idea with the recovery of the disease, with measles and the immunosuppressive effect of measles. Besides the atopic aetiology in a few (Hardwicke *et al.*, 1959; Reeves *et al.*, 1975), the indirect evidence of complement activation (Ngu *et al.*, 1970) and our present data, the best evidence in favour of an immunopathogenesis comes from the success of treatments devised on an immunological hypothesis. Corticosteroids, cyclophosphamide, measles infections (Von Pirquet, 1908) and measles vaccine all induce remissions, and seem to have only immunosuppressive effects in common. It is difficult to see how the prevention of further attacks by the injection of antigen in the atopic form (Hardwicke *et al.*, 1959) or by giving cyclophosphamide during steroid-maintained remission in both the non-atopic (Barratt & Soothill, 1970) and the atopic (Reeves *et al.*, 1975) forms can work in non-immunological ways. It seems probable that they interfere with responsiveness to an environmental trigger. The frequent experience of relapse following upper respiratory tract infections suggest one form of trigger, in addition to the atopic one.

The case against an immunopathogenesis is that known mechanisms for glomerular damage (deposition of immunoglobulins, complement and fibrin with inflammation and proliferation) are not found in the kidney (Drummond *et al.*, 1966). The report by Gerber & Paronetto (1971) of IgE deposits in the kidneys of most patients with SSNS, who were not selected as being atopic, has not been confirmed in some later studies (Roy, Westberg & Michael, 1973). However, these techniques and the experimental data underlying them were

developed as a result of observations in other renal disease and it is possible that a completely different form of immunopathological injury occurs in SSNS.

The two laboratory studies providing evidence for immunological activation in SSNS patients, immunoconglutinin and our present data both involve complement but this does not necessarily mean that complement is directly involved in the glomerular injury. Contact with antigen usually activates many aspects of the immune response and it is possible that a circulating mediator could produce damage without lymphocytes, immunoglobulins or complement being localized in the kidney itself. However, the phenomena we describe could be involved directly if the mediators released by B cells which had bound complement (Wahl, Iverson & Oppenheim, 1974) injured the kidney.

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