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THE POTENTIATING EFFECT OF RHEUMATOID ARTHRITIS SERUM IN THE IMMEDIATE PHASE OF NEPHROTOXIC NEPHRITIS

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SUMMARY

Nephrotoxic nephritis induced in rats was employed as an experimental model to investigate the possible effects of rheumatoid factor on *in vivo* antigen–antibody reactions. Rats injected simultaneously with rheumatoid arthritis serum and rabbit nephrotoxic globulin showed a three-fold increase in immediate proteinuria compared with rats injected with nephrotoxic globulin alone. This potentiating effect of rheumatoid arthritis serum was evident even when the serum was injected 48 hr after the nephrotoxic globulin and was also apparent to a lesser extent in rats decomplemented by a prior injection of aggregated human IgG. Normal human serum had no effect on the proteinuria produced by a standard dose of nephrotoxic globulin while rheumatoid arthritis serum injected with normal rabbit globulin did not increase urinary protein excretion above baseline levels. In rats injected with rheumatoid arthritis serum and nephrotoxic globulin, human IgM (presumably rheumatoid factor) was detected by immunofluorescence on the glomerular basement membrane along with the nephrotoxic globulin and rat complement and persisted at this site for as long as 42 days after the initial injections. Rheumatoid factor activity was also recovered by elution from glomeruli isolated from rat kidneys 24 hr after the injection of rheumatoid arthritis serum and nephrotoxic globulin.

INTRODUCTION

During the last 20 years considerable information has accumulated concerning the physical and immunochemical properties of the rheumatoid factor group of antiglobulins which are consistently although not uniquely associated with rheumatoid arthritis. Thus it has been determined that the rheumatoid factors are mainly M-immunoglobulins with antibody specificity directed primarily against antigenic determinants exposed on denatured human IgG and with wide cross-reactivity with the G-immunoglobulins of other species. The biological significance of the rheumatoid factors, however, is still enigmatic.

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On clinical correlation, positive serological tests for rheumatoid factor are of bad prognostic significance in rheumatoid arthritis (Duthie *et al.*, 1957), and the systemic complications of the disease tend to occur more frequently in patients with the highest titres of rheumatoid factor (Vaughan, 1959). Nevertheless, despite the avidity of rheumatoid factor for denatured human IgG and antigen–antibody complexes in *in vitro* systems (Edelman, Kunkel & Franklin, 1958), until recently there was no evidence which might implicate the immunological reactivity of rheumatoid factor in the articular and systemic manifestations of rheumatoid arthritis. Thus, transfusion of healthy volunteers with rheumatoid plasma (Harris & Vaughan, 1961) or rheumatoid patients with normal human plasma and IgG as potential antigen (Waller *et al.*, 1965) produced no pathological effects in the recipients. In a study of immune haemolysis *in vivo*, Kaplan & Jandl (1963) found that the destruction of Rh-positive erythrocytes sensitized with incomplete anti-Rh antibody was not accelerated in rheumatoid recipients although the serum of the rheumatoid subjects was capable of agglutinating the sensitized cells *in vitro*. More recently, however, Restifo *et al.* (1965) demonstrated that the injection of autologous IgG into the unaffected joints of rheumatoid subjects produced an acute inflammatory response attributable to rheumatoid factor–IgG interaction and indicating that rheumatoid factor may not be as innocuous *in vivo* as was hitherto believed. The inflammatory potential of rheumatoid factor interactions is also suggested by the observations of Astorga & Bollet (1964) who found that phagocytosis of rheumatoid factor–IgG precipitates by polymorphonuclear leucocytes resulted in release of lysosomal enzymes.

On the other hand, there is also evidence that rheumatoid factor may have a protective rather than enhancing effect in certain complement-dependent systems *in vitro*. Schmid & Roitt (1965) found that the euglobulin fraction of rheumatoid arthritis serum protected human thyroid cells in tissue culture from the effects of autoimmune cytotoxic antibody and also inhibited complementary lysis of erythrocytes. Similar observations on inhibition of immune haemolysis *in vitro* have been reported by Romeyn & Bowman (1967). Zvaifler & Bloch (1962) showed that complement fixation by rabbit immune complexes was inhibited by rheumatoid arthritis serum in proportion to rheumatoid factor titre while Heimer, Levin & Kahn (1963) found that fixation of complement by latex particles coated with aggregated human IgG was blocked if the particles were pretreated with rheumatoid arthritis serum. Davis & Bollet (1964) have also reported that rheumatoid factor exerted a protective effect in a complement-sensitive mitochondrial enzyme system. Thus it appears that rheumatoid factor may assume a dual biological role, offering protection in some immune systems and potentiation in others.

The present investigation was undertaken to determine whether rheumatoid factor would exert a protective or enhancing effect during the immediate phase of nephrotoxic nephritis induced in rats by the injection of rabbit anti-rat glomerulus antibody. Previously it had been shown that fluorescein-labelled rheumatoid factor would react with rabbit anti-human glomerulus antibody bound on human kidney sections (McCormick, 1962), and it was anticipated that similar binding of rheumatoid factor might occur in a comparable *in vivo* system. Furthermore, it was shown by Unanue & Dixon (1964) that the immediate proteinuria produced in rats by moderate doses of rabbit nephrotoxic antibody was dependent upon the presence of complement. It seemed likely, therefore, that nephrotoxic nephritis would be an ideal situation in which to assess the possible effects of rheumatoid factor in a complement-dependent immune system *in vivo*.

MATERIALS AND METHODS

Rats

Female Wistar rats, 2–3 months old and weighing approximately 180 g, were used in all experiments in this investigation. For the collection of urine over 24-hr periods, the rats were housed in metabolism cages with access to food but without water. The urine obtained from each rat was centrifuged and the total volume recorded.

Estimation of urinary protein

The protein in 1·0 ml of appropriately diluted urine was precipitated with 2·0 ml of 3% salicylsulphonic acid and the turbidity was read at 623 μ in an EEL spectrophotometer. Protein concentration was calculated from a standard curve and recorded in mg/24 hr. The average spontaneous proteinuria in these rats was 5·5 mg/24 hr (range 1·5–9·0 mg).

Preparation of nephrotoxic antiserum

Glomeruli were isolated from pooled mature rat kidneys by the method of Krakower & Greenspon (1951), ground in a Griffiths' tube and suspended in a volume of isotonic saline calculated so that 1·0 ml suspension contained 20·0 mg crude glomerulus antigen (wet weight).

A group of seven New Zealand White rabbits were injected in multiple subcutaneous sites with a total of 4·0 ml antigen suspension incorporated in complete Freund's adjuvant given in four divided doses over a period of 1 month. After an interval of 4 weeks, each animal was injected intraperitoneally with 1·0 ml antigen suspension three times weekly for a further period of 8 weeks. The rabbits were bled out 7 days after the last injection.

The individual rabbit antisera were inactivated by heating at 56°C for 30 min and then absorbed exhaustively with rat erythrocytes to remove agglutinins and with small increments of rat serum until precipitin reactions with rat serum components were no longer detected by double diffusion in agar. In preliminary tests of nephrotoxic potency it was found that five of the seven rabbit antisera produced significant immediate proteinuria (i.e. >60 mg/24 hr) in at least two out of three rats when injected intravenously in doses of 1·0 ml. The five potent antisera were pooled, the crude globulin fraction was precipitated by 50% saturation with ammonium sulphate and the precipitate redissolved in a volume of isotonic saline equal to half that of the original serum pool. Ammonium sulphate was removed by dialysis against isotonic saline and the globulin solution was sterilized by filtration and stored at -20°C in 10-ml aliquots. The final protein concentration of this nephrotoxic globulin (NTG) was 42 mg/ml.

Other reagents

Sera with unusually high titres in the sensitized sheep cell agglutination test were obtained from a group of six patients with chronic rheumatoid arthritis. Two sera containing antinuclear factor were excluded, and the remainder were pooled, inactivated by heating at 56°C for 30 min, sterilized by filtration and stored at -20°C in 20-ml aliquots. The final sheep cell agglutinating titre of this material (RAS) was 1 : 32,000 and remained constant during the period of storage. No attempt was made to isolate rheumatoid factor from this pooled serum because of the difficulty in maintaining activity in purified preparations.

Pooled normal human serum (NHS) was obtained from laboratory personnel and was similarly inactivated, sterilized and stored at −20°C.

Normal rabbit globulin (NRG) was prepared from pooled rabbit serum by precipitation with ammonium sulphate. The final protein concentration of the globulin solution in isotonic saline was adjusted to 25 mg/ml.

Aggregated human γ-globulin (AHGG) was prepared by heating Cohn Fraction II at 56°C for 30 min followed by sodium sulphate precipitation (Christian, 1960). The protein concentration of this material was adjusted to 10 mg/ml for decomplementation experiments.

Induction of nephrotoxic nephritis

In preliminary experiments it was determined that the intravenous injection of 1·0–2·0 ml doses of the pooled NTG produced very marked immediate proteinuria (e.g. 350 mg/24 hr) in the majority of rats and that this was not obviously influenced by the simultaneous injection of 2·0–4·0 ml of RAS. This seemed to imply either that RAS had no biological effect in this system or that in the presence of maximal proteinuria any protective or potentiating effect of RAS could not be demonstrated with the volumes of serum which could be given intravenously without causing cardiac failure. Subsequently a dose of 0·4 ml NTG diluted to 1·0 ml with isotonic saline was found to induce a moderate but reproducible degree of immediate proteinuria and, therefore, this was the standard dose of NTG employed for all further experiments as outlined below.

Eight groups of rats were injected as follows:

Group A, thirty-seven rats injected with NTG alone;
Group B, thirty-five rats injected with NTG and 2·0 ml RAS;
Group C, eighteen rats injected with NTG and 2·0 ml NHS;
Group D, fifteen rats injected with 1·0 ml NRG and 2·0 ml RAS;
Group E, twelve rats injected with 2·0 ml RAS 48 hr after injection of NTG;
Group F, twelve rats injected with 2·0 ml NHS 48 hr after injection of NTG;
Group G, twelve rats injected with NTG alone, 2 hr after injection of 10 mg AHGG to remove complement; and
Group H, twelve rats injected with NTG and 2·0 ml RAS, 2 hr after injection of 10 mg AHGG to remove complement.

Urinary protein was measured in all rats during the first 24 hr following the initial injection (day 1). In addition, urinary protein was measured during day 3 and day 5 in all rats in groups E and F and in twenty-one and eighteen rats of groups A and B, respectively. The majority of rats were killed immediately after the last urine collection but the remaining rats in groups A and B were killed in pairs at intervals from 10 days until 3 months after the initial injections. One kidney from each rat was cut into four blocks which were quick-frozen in a dry ice–acetone mixture for immunofluorescence studies. The remaining kidneys from rats in groups A, B and D were stored at −20°C and employed subsequently for the elution procedure described later.

Immunofluorescence reagents and techniques

(1) Anti-human IgM antiserum was prepared in rabbits immunized with rheumatoid factor adsorbed on sensitized sheep cell stromata. This antiserum was absorbed with

sheep cells to remove agglutinins and with human group O Rh-positive erythrocytes sensitized with incomplete anti-D antibody to remove anti-IgG reactivity. The resulting antiserum was shown to be specific for IgM by immunoelectrophoresis.

(2) Anti-rabbit IgG antiserum was produced in guinea-pigs immunized with rabbit IgG prepared by DEAE-cellulose chromatography. On immunoelectrophoresis this antiserum was found to react weakly with IgA as well as with IgG but no further purification was attempted.

(3) Anti-rat complement antiserum was prepared in rabbits by the method employed by Unanue & Dixon (1964). On immunoelectrophoresis, the antiserum selected for subsequent use reacted with only two components in the β region, one of which was interpreted as β_{1C}-globulin.

Globulin fractions prepared from these antisera by precipitation with ammonium sulphate were conjugated with fluorescein isothiocyanate at a protein concentration of 10 mg/ml and a dye to protein ratio of 0·05/1·0 mg (Riggs *et al.*, 1958). Unconjugated dye was removed by passage over a column of Dowex anion exchange resin (1 × 10 cross-linked, 200–400 mesh), equilibrated in phosphate buffered saline (pH 7·2). Each conjugate was absorbed at least twice with pooled guinea-pig tissue powder to remove non-specific reactants as far as possible.

Sections of rat kidney were cut at 4 μ in a cryostat, dried with an electric fan for 30 min and then stained with the appropriate conjugate for 30–45 min. Unreacted conjugate was removed by thorough washing in phosphate buffered saline (pH 7·2) and the sections were mounted in 75% glycerol buffered to pH 8·0. Specificity of the immunofluorescence reactions was demonstrated by inhibition and absorption techniques. In the inhibition procedure, sections were pre-treated for 6 hr with one drop of unlabelled antiserum which was then mixed with one drop of the appropriate conjugate for a further 30 min. On control sections normal rabbit or guinea-pig serum was substituted for unlabelled antiserum. In the absorption procedure sections were treated with a sample of each conjugate which had been absorbed specifically with the antigen preparation used for immunization. By both techniques, specificity of the immunofluorescence reactions discussed later was confirmed by complete or substantial abolition of staining. The anti-rat complement and anti-human IgM conjugates reacted non-specifically with the juxtaglomerular apparatus and glomerular epithelial cells but this did not interfere with the interpretation of specific immunofluorescence.

Elution of rheumatoid factor from glomeruli

Whole kidneys removed from rats in groups A, B and D killed 24 hr after injection were combined in three separate pools. Each pool consisted of fifteen kidneys from which the glomeruli were isolated by the standard technique (Krakower & Greenspon, 1951). The ground glomeruli from the three pools were washed seven times with chilled isotonic saline and then finally washed for 4 hr at room temperature with 0·2 M acetate–acetic acid buffer (pH 4·0). The acidic eluates were dialysed against phosphate buffered saline (pH 7·2) and concentrated to a final volume of 1·5 ml by ultrafiltration. The concentrated eluates were absorbed twice with unsensitized sheep cells and then titrated against 1% sheep cells sensitized with 1 : 2 agglutinating dose of rabbit amboceptor. An aliquot of each eluate was also dialysed against 0·1 M-mercaptoethanol followed by 0·05 M-sodium iodoacetate in phosphate buffered saline (pH 7·2) before titration against sensitized sheep cells.

RESULTS

Proteinuria

The urinary protein excretions in groups A–F are shown in Table 1. It will be seen that the simultaneous injection of RAS with NTG (group B) produced a three-fold increase in

TABLE 1

Group	Injection	Proteinuria (mg/24 hr) Day 1 (mean/SD)	Day 3 (mean/SD)	Day 5 (mean/SD)
A	NTG alone	36·2/47·5 (37) *	16·4/15·0 (21) *	23·2/24·1 (21)
B	NTG+RAS	118·3/118·6 (35)	45·0/36·8 (18)	49·2/66·9 (18)
C	NTG+NHS	35·9/44·9 (18)	20·3/15·6 (7)	16·8/11·0 (7)
D	NRG+RAS	8·0/3·1 (15)	— **	— **
E	RAS 48 hr after NTG	42·5/46·2 (12)	77·8/38·9 (12)	106·7/39·3 (12)
F	NHS 48 hr after NTG	33·0/38·7 (12)	18·4/16·2 (12)	21·6/18·5 (12)

The figures in parentheses indicate the number of rats available. SD, Standard deviation; NTG, nephrotoxic globulin; RAS, rheumatoid arthritis serum; NHS, normal human serum; NRG, normal rabbit globulin.

* Proteinuria values increased significantly when compared with controls by *t*-test on the means (*$P = 0{\cdot}01$; **$P = 0{\cdot}001$).

TABLE 2

Group	No.	Injection	Immediate proteinuria (mg/24 hr) (mean/SD)
G	12	NTG alone 2 hr after AHGG	15·6/13·1 *
H	12	NTG+RAS 2 hr after AHGG	42·0/23·9

SD, Standard deviation; NTG, nephrotoxic globulin; AHGG, aggregated human γ-globulin; RAS, rheumatoid arthritis serum.

* Proteinuria is significantly higher than in group G ($P = 0{\cdot}01$: *t*-test on the means).

immediate proteinuria as compared with rats which received the NTG alone (group A). The difference between these groups was still significant 3 days after the initial injections.

The injection of NHS with NTG (group C) produced no effect on immediate or subsequent proteinuria while the injection of RAS with NRG did not increase proteinuria above normal baseline levels (group D). In the rats of group E which received 2·0 ml of RAS 48 hr after the initial injection of NTG there was an immediate increase in proteinuria on

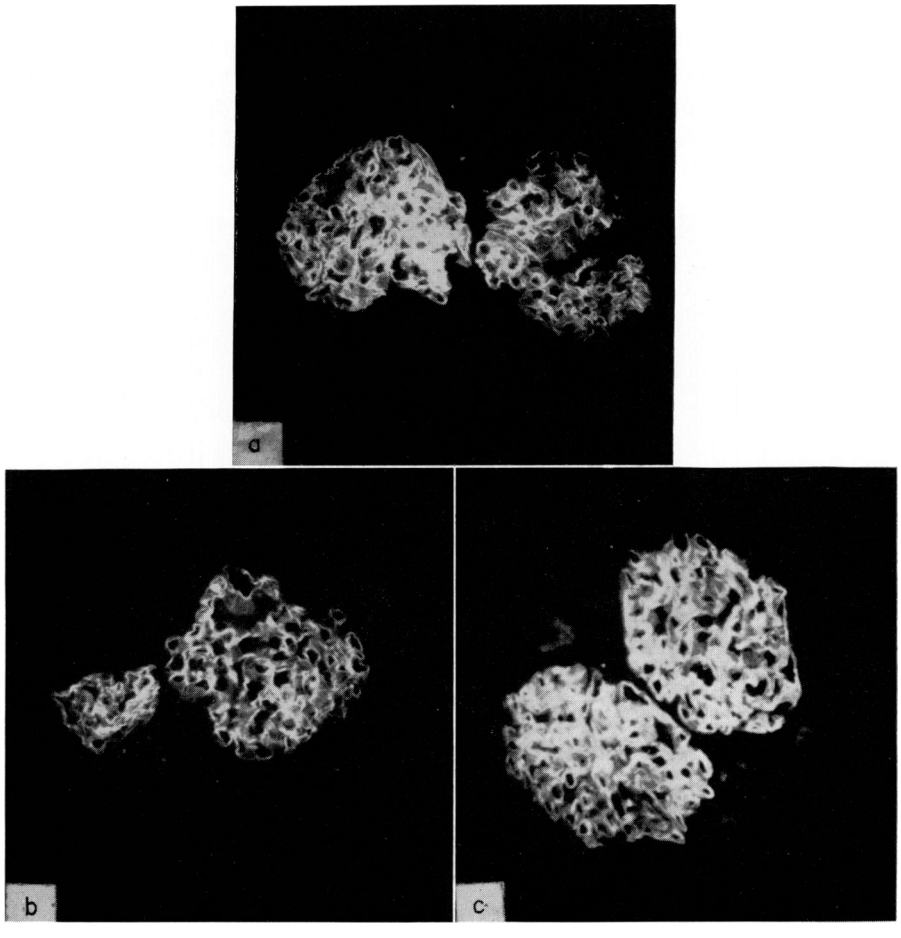

FIG. 1. Kidney sections from a rat 24 hr after injection of nephrotoxic globulin and rheumatoid arthritis serum. (a) Section stained with fluorescein-labelled anti-rabbit IgG antiserum showing fixation of rabbit IgG on glomerular basement membrane. (b) Section stained with fluorescein-labelled anti-rat complement antiserum shows similar capillary pattern of fluorescence. (c) Section stained with fluorescein-labelled anti-human IgM antiserum demonstrates fixation of human IgM (presumably rheumatoid factor) on glomerular basement membrane.

day 3 which was even more apparent 2 days later. A delayed injection of NHS, however, in the rats of group F had no effect on subsequent proteinuria.

The effect of decomplementation by the injection of 10 mg AHGG is shown in Table 2. In the rats of group G, the proteinuria produced by NTG alone was halved by prior decomplementation while in group H animals, the mixture of RAS and NTG produced

considerably less proteinuria than was observed in the non-decomplemented rats of group B. The average proteinuria in group H, however, is still within the range found in group A rats injected with NTG alone in the presence of circulating complement.

Immunofluorescence

In rats of group B killed 24 hr after the injection of NTG and RAS, human IgM was readily detected in a continuous linear distribution on the glomerular basement membrane corresponding to the pattern of fluorescence observed when companion sections were stained with the labelled anti-rabbit IgG antiserum. Human IgM was still discernible on the glomerular basement membrane in rats killed as long as 42 days after the initial injection

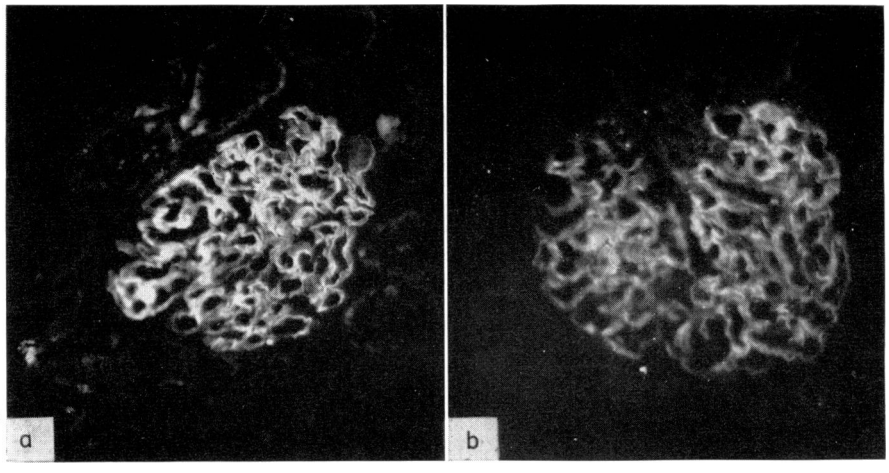

FIG. 2. Sections of kidney from a rat injected initially with nephrotoxic globulin and 48 hr later with rheumatoid arthritis serum. (a) Section stained with fluorescein-labelled anti-human IgM antiserum showing deposition of human IgM on glomerular basement membrane. (b) Section stained with fluorescein-labelled anti-rat complement antiserum showing linear distribution of rat complement in glomerular capillaries.

of RAS and NTG but the intensity of fluorescence was much diminished and some glomeruli were either unstained or showed only partial staining of the capillary tufts. Three months after the initial injection in group B rats human IgM could no longer be detected although the labelled anti-rabbit IgG antiserum demonstrated that NTG was still present on the glomerular basement membrane. In the control groups C and D, human IgM could not be detected in the glomeruli even 24 hr after the injection of NTG and NHS, or NRG and RAS.

Rat complement was also detected on the glomerular basement membrane in rats of groups A, B and C, killed 24 hr after the initial injections and was still present with rabbit IgG in a similar distribution in rats killed 3 months later. However, when sections from rats in these three groups killed at 24 hr were stained with the labelled anti-complement antiserum and examined in coded random order, it became apparent that in those rats injected with NTG and RAS, the intensity of complement-specific fluorescence in the glomeruli was reduced. Similarly, in the rats of group E which received 2·0 ml of RAS

48 hr after the initial injection of NTG and were killed on the 5th day of the experiment, human IgM was readily detectable but complement-specific fluorescence in glomeruli was

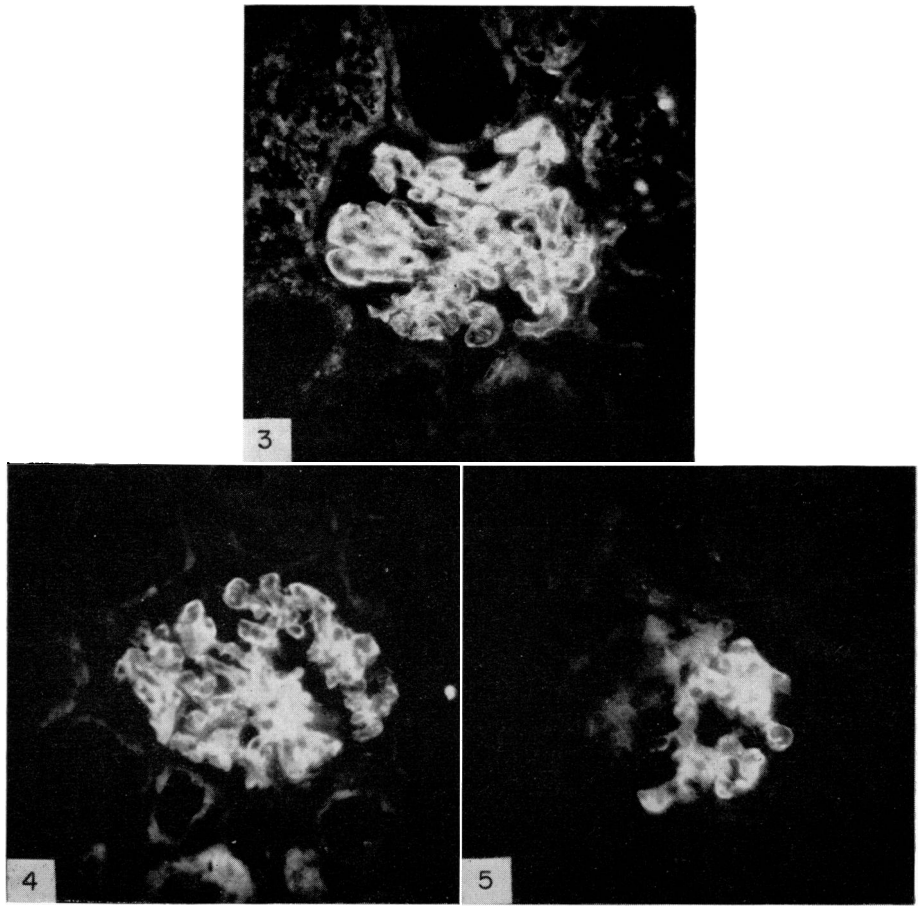

FIG. 3. Section of kidney from a rat 5 days after initial injection of nephrotoxic globulin without subsequent injection of rheumatoid arthritis serum. Section stained with fluorescein-labelled anti-rat complement antiserum and shows appreciably brighter complement-specific fluorescence in glomerulus than in Fig. 2(b).

FIG. 4. Section of rat kidney 18 days after injection of nephrotoxic globulin and rheumatoid arthritis serum. Section stained with fluorescein-labelled human IgM antiserum showing human IgM still present in a continuous linear distribution on the glomerular basement membrane.

FIG. 5. Section of kidney from a rat 42 days after injection of nephrotoxic globulin and rheumatoid arthritis serum. Section stained with fluorescein-labelled human IgM antiserum shows diminished and incomplete fluorescence reaction in the glomerular capillary tufts indicating partial removal of IgM 42 days after initial injection.

appreciably less than in rats killed 5 days after the initial injection of NTG without a subsequent injection of RAS. In the rats of groups G and H which were decomplemented by the injection of AHGG, traces of complement were still seen on the glomerular basement

and in the mesangium 24 hr later but the intensity of fluorescence was obviously less than in the other comparable groups examined. The intensity of human IgM-specific fluorescence, however, in decomplemented group H animals was apparently undiminished. In the control rats of group D injected with NRG and RAS, traces of complement were found only in the mesangium and there was no suggestion of deposition on the glomerular basement membrane.

Elution of rheumatoid factor from glomeruli

The results of sensitized sheep cell agglutination tests with the glomerular eluates are shown in Table 3. It will be seen that appreciable rheumatoid factor activity was present in the eluate from group B animals but only minimal activity was recovered in the group

TABLE 3

Glomerular eluate	Sensitized sheep cell agglutination titres
Group A	0
Group B	1/32 (0)
Group D	1/2 (0)

The titres following mercaptoethanol treatment are shown in parenthesis.

D eluate. All agglutinating activity in these eluates was lost following dialysis against 0·1 M-mercaptoethanol. The eluate from the group A animals injected with NTG alone did not agglutinate sensitized sheep cells, which seems to exclude the possibility that the agglutinating activity in the other eluates was attributable to early host response and the production of rat macroglobulin antibody against rabbit IgG.

DISCUSSION

It is apparent from the three-fold increase in proteinuria shown in Table 1 that rheumatoid arthritis serum potentiates the effect of rabbit anti-rat glomerulus antibody, and that potentiation can still occur when rheumatoid arthritis serum is injected 48 hr after the nephrotoxic globulin. This augmented proteinuria cannot be explained by increased excretion of foreign serum proteins in a damaged kidney since simultaneous or subsequent injections of normal human serum had no effect on the proteinuria produced by a standard dose of nephrotoxic globulin. It seems most likely that rheumatoid factor is the agent in rheumatoid arthritis serum responsible for this potentiation although the possibility that enhancement is mediated by some other serum factor cannot be entirely excluded. Nevertheless, the known affinity of rheumatoid factor for rabbit antibody–antigen complexes, the demonstration by immunofluorescence techniques that human IgM was bound on the glomerular basement membrane and the recovery of sensitized sheep cell agglutinating activity in the eluate from group B rat glomeruli at least imply rheumatoid factor participation in the potentiated nephrotoxic process.

The mechanism whereby this potentiation may occur is not clear. The participation of

complement in the immediate phase of nephrotoxic nephritis has been demonstrated by Unanue & Dixon (1964). This observation has been confirmed in the present investigation where the immediate response to a standard dose of nephrotoxic globulin was reduced in decomplemented animals. The level of proteinuria in rats injected with nephrotoxic globulin and rheumatoid arthritis serum was reduced even more dramatically by prior decomplementation although the resulting proteinuria was still higher than in decomplemented rats injected with nephrotoxic globulin alone. This might indicate that rheumatoid factor can substitute for complement in the immediate phase of nephrotoxic nephritis. Alternatively, any potentiating effect of rheumatoid factor may be wholly dependent on the presence of complement and perhaps directly proportional to complement activity since traces of complement were still detectable on the glomerular basement membrane in decomplemented animals.

On the other hand, it should be noted that in non-decomplemented rats, complement-specific fluorescence was demonstrably less in the glomeruli where human IgM was present, although proteinuria was increased. Although this might imply that rheumatoid factor competes with complement for combining sites on bound nephrotoxic globulin, the diminished fluorescence could also be due to steric hindrance by rheumatoid factor preventing access of the anti-complement antiserum to complement fixed at adjacent combining sites. In addition, the reduced complement-specific fluorescence seen at day 5 in the glomeruli of rats injected with rheumatoid arthritis serum 48 hr after the initial injection of nephrotoxic globulin could be explained by a similar mechanism of steric hindrance in the immunofluorescence system although displacement of bound complement by rheumatoid factor or prevention of further complement binding cannot be excluded. There was, however, no suggestion in this system that prior fixation of complement prevented subsequent specific binding of rheumatoid factor. Furthermore, the augmented proteinuria which occurred even when the injection of rheumatoid arthritis serum was delayed for 48 hr, suggests that the potentiating effect of rheumatoid arthritis serum is at least not dependent upon the initial kinetics of complement fixation and may imply an intrinsic deleterious effect of rheumatoid factor in its reaction with certain membrane-orientated antigen–antibody complexes.

Thus, in this particular system it has been demonstrated that rheumatoid factor can participate in an immune interaction *in vivo*, and that rheumatoid factor or some other agent in rheumatoid arthritis serum may potentiate immunologically determined tissue damage. Although the results of this study cannot implicate rheumatoid factor and other anti-globulins directly in the pathogenesis of human disease, the biological potential of such factors in immune systems warrants further investigation.

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