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EFFECT OF ANTIBODY AVIDITY ON THE INDUCTION OF RENAL INJURY IN ANTI-GLOMERULAR BASEMENT MEMBRANE NEPHRITIS

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Summary.—Antibodies to glomerular basement membrane (GBM) of the rat could be fractionated according to their avidity by elution from trypsin-digested GBM (bound to Sepharose) with increasing concentrations of KSCN. The percentage of kidney-fixing antibody in each fraction and the degree of proteinuria induced as determined 24 h after injection increased with the avidity of the antibody fraction when equal doses were administered.

MASUGI (nephrotoxic serum) nephritis has often been used as an experimental model for human glomerulonephritis. It was shown by Unanue and Dixon (1965) that the kidney-fixing antibody had to occupy at least 45% of the glomerular antigenic sites in order to induce functional impairment of the kidney. In contrast to such a quantitative approach, very little is known about the quality of antibody and its biological effect in glomerulonephritis.

It is well known that antibodies show a variation in binding strength, even with simple antigenic determinants (Eisen and Siskind, 1964). In a separate paper (Shimizu *et al.*, 1978*b*) we showed that anti-2,4 dinitrophenyl (DNP) antibodies could be fractionated according to affinity by successive elution from 2,4 dinitrophenyl (DNP)-immunoadsorbent using increasing concentrations of thiocyanate as eluent. The validity of this method for multivalent systems, where haptens are not available, has also been demonstrated (Shimizu *et al.*, 1978*a*). Analogously, in this study anti-GBM antibodies were fractionated according to avidity by stepwise elution from a suitable immunoadsorbent, and furthermore the biological activity of the fractions were tested for their ability to induce nephritis.

MATERIALS AND METHODS

Male Wistar rats weighing about 120 g were used. Potassium iodide (0·1%) was added to the drinking water 24 h before the injection of the labelled antibody fractions.

Antisera.—GBM antigen was prepared from kidneys of Wistar rats according to the method of Krakower and Greenspon (1951) as modified by Spiro (1967). Anti-rat GBM antisera were raised in rabbits by injection of 5 mg of lyophilized rat GBM emulsified in 0·8 ml complete Freund's adjuvant into the footpads. The injection was repeated 3 times at biweekly intervals. The animals were bled out 2 weeks after the last injection.

A crude γ-globulin fraction was isolated from pooled anti-rat GBM sera by precipitation with 33% saturated ammonium sulphate. Before fractionation the pooled antisera were repeatedly absorbed with equal amounts of washed rat erythrocytes until agglutinins were removed. The globulin fraction was trace-labelled with ^{125}I as described by McConahey and Dixon (1966). Radioactivity was determined in a well-type scintillation counter (Friesecke and Höpfner, Erlangen, Germany).

Immunoadsorbent.—Pooled glomerular base-

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ment membrane of rat kidneys was digested with trypsin according to the method of Shibata *et al.* (1969); 700 mg of soluble trypsin-digested GBM was obtained. The (Tr-GBM) was coupled to 15 g CNBr activated sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden), with an efficiency of 40%.

Adsorption and elution of antibodies.—The ^{125}I labelled gamma globulin fraction of anti-rat GBM was incubated for 2 h at 37°C with an excess of GBM sepharose. Stepwise and direct elution as well as the re-elution procedure with potassium thiocyanate concentrations of 0·5, 2·0, 3·0 and 4·0M were performed as described previously (Shimizu *et al.*, 1978*b*).

Electrophoresis and electrofocusing.—These were carried out in the same manner as described previously (Shimizu *et al.*, 1978*a*).

In vitro *binding studies on the antibody fractions.*—The binding capacity of the antibody fractions eluted with successive concentrations of 0·5, 2·0, 3·0 and 4·0M KSCN were tested by mixing 0·5 ml of serial dilutions of the ^{125}I-labelled antibody fractions with 0·1 ml of suspended Tr-GBM sepharose.

After incubation for 30 min and centrifugation, the radioactivity in 0·4 ml of each supernatant was counted and related to the total radioactivity used in the test. The non-specific binding of ^{125}I-labelled normal rabbit IgG in parallel runs was less than 4%.

Binding studies of the antibody fractions in vivo.—Groups of 15–21 rats were injected i.v. with 0·5–3·0 mg of the labelled antibody fractions isolated by successive (stepwise) elution. Groups of 4 or 5 rats were killed on Days 1, 3, 7 and 21 respectively after the injection. The radioactivity in the kidney, spleen, liver and lung was determined. Control rats received equal amounts of normal rabbit IgG fractions treated with different KSCN concentrations as follows: fractions were dialysed overnight against 0·5–4·0 M KSCN with subsequent dialysis against PBS. For evaluation of the renal injury the total urinary protein was determined by using the Biuret method. Immunofluorescence studies were carried out with fluoresceinisothiocyanate-labelled rabbit anti-rat IgG antibody.

RESULT

Fractionation of anti-GBM antibodies and their electrophoretic properties

The percentage of antibodies eluted by the single step procedure is shown in Table I. Increasing amounts of antibodies could be eluted with increasing concentrations of KSCN. The amounts obtained by successive elution were in good agreement with those obtained from the corresponding KSCN concentrations applied in the single-step elution. The various fractions of the eluted antibodies showed essentially the same pattern in polyacrylamide gel electrophoresis as well as by isoelectrofocusing (Fig. 1).

TABLE I.—*Elution of Anti-GBM Antibodies from Tr-GBM–Sepharose with Different Concentrations of KSCN (Direct Elution)*

Eluent (M)	Eluted antibody (%)
0·5	28
2·0	60
3·0	70
4·0	75

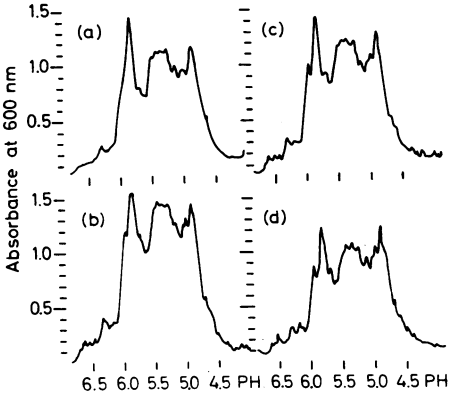

FIG. 1.—Electrofocusing densitometric scanning patterns of anti-GBM antibody fractions separated by stepwise elution with KSCN at concentrations of (a) 0·5M, (b) 2·0M, (c) 3·0M, and (d) 4·0M.

Binding properties of different fractions in vitro

Further experiments were designed to test the validity of the described fractionation with respect to the binding properties of the different fractions. First of all re-elution of the re-adsorbed fraction was examined. As shown in Fig. 2, the amount of re-eluted antibody depended on the molarity applied for the original elution, *i.e.* re-elution with 0·5M was much more effective with the fraction originally eluted

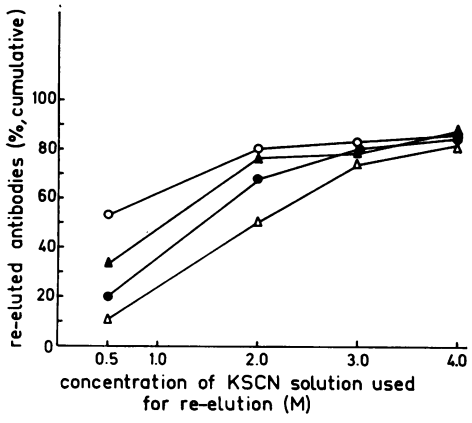

FIG. 2.—Re-elution of re-adsorbed anti-GBM antibody fractions originally stepwise eluted with (○) 0·5M, (▲) 2·0M, (●) 3·0M, and (△) 4·0M KSCN. In the abscissa the concentrations of KSCN used for re-elution are shown in the ordinate and the cumulative percentages of re-eluted antibodies are given.

with 0·5M (53%) than with the fraction originally eluted with 2·0M (33%) or 3·0M (20%) or 4·0 M (11%). The same held true for re-elution with 2·0 and 3·0M. Results of binding studies of serial dilutions of the antibody fraction to the immunoadsorbent are shown in Fig. 3. There is a shift in the equilibrium from free to bound antibody with increasing molarity of KSCN used for elution. This implies that the binding properties of the antibody fractions increase depending on the molarity applied for elution.

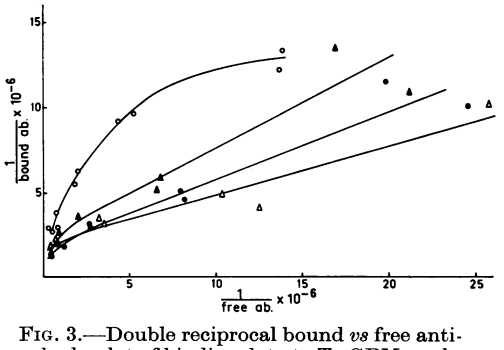

FIG. 3.—Double reciprocal bound *vs* free antibody plot of binding data to Tr-GBM–sepharose with the antibody fractions obtained by stepwise elution with (○) 0·5M, (▲) 2·0M, (●) 3·0M and (△) 4·0M KSCN.

Binding properties of the antibody fractions in vivo

As can be seen in Fig. 4, in comparison with antibodies eluted with lower KSCN molarities, a much higher percentage of

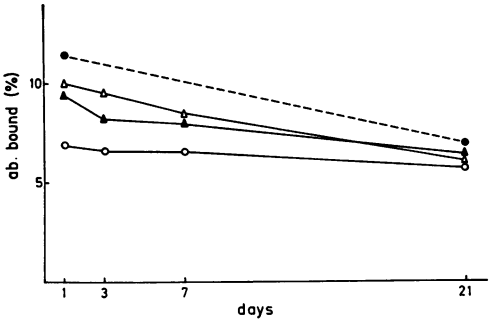

FIG. 4.—Fixation of the antibody fractions in the kidney and their persistence with time. (○) 0·5M, (▲) 2·0M, (●) 3·0M, and (△) 4·0M KSCN.

the 4·0M antibody was fixed to the kidney at Day 1. The differences seen initially in kidney-fixing ability of the different antibody fractions were no longer evident by Day 21, showing that the antibodies eluted with lower KSCN concentrations persisted relatively longer in the kidney than the fractions obtained with 3·0 or 4·0M KSCN. Initially nearly as much

TABLE II.—*Fixation of Anti-GBM Antibody Fractions in the Spleen, Liver, and Lung in Percent (per g Tissue)*

Days after injection	Eluent (M)	Spleen (%)	Liver (%)	Lung (%)
1	0·5	5·6	2·3	0·8
	2·0	8·8	6·2	1·0
	3·0	11·4	7·1	0·9
	4·0	10·5	5·9	0·4
3	0·5	3·7	1·9	1·0
	2·0	4·6	2·6	0·9
	3·0	n.d.	n.d.	n.d.
	4·0	5·3	2·8	0·3
7	0·5	3·5	1·3	0·4
	2·0	4·4	1·8	0·6
	3·0	n.d.	n.d.	n.d.
	4·0	4·8	2·3	0·5
21	0·5	2·5	0·8	0·7
	2·0	3·5	1·2	0·5
	3·0	3·1	1·1	0·3
	4·0	3·4	1·1	0·3

n.d.: not done

TABLE III.—*Effect of Anti-GBM Antibody Fractions on Renal Function as Measured by Proteinuria*

Antibody fraction injected (M)	Number of rats	Proteinuria per 24 h: mg proteinuria per mg ab injected	Proteinuria per 24 h: mg proteinuria per mg ab bound in kidney
0·5	6	24·6±13·1	303·0±152·0
2·0	10	31·0±15·0	347·0±174·0
4·0	12	44·7± 9·3	427·0± 84·0

antibody was localized in the spleen (per g tissue) as in the kidney. In the other organs much less was fixed. The activity in the spleen and liver decreased much more rapidly than in the lung or kidneys during the first 3 days. Thereafter the disappearance rates were similar (Table II). No significant difference in the fluorescent staining intensity for rabbit Ig was detectable after the i.v. injection of the different antibody fractions.

Injection of 2·5 mg of normal rabbit IgG treated with KSCN concentrations of 0·5–4·0M did not result in localization of globulin in the glomerular basement membrane or in the mesangial area.

Effect of the different antibody fractions on renal function

The capacity of different antibody fractions to induce renal lesions was deduced from the level of the resulting proteinuria. The results are listed in Table III. The amount of excreted protein was dependent on the quantity of antibody injected for each molarity fraction. A significant increase in protein excretion was seen on comparing the groups receiving 0·5M KSCN treated antibody and 4·0M ($P > 0·01$) as well as between the 2·0 and 4·0M groups; no significant difference was seen between 0·5–2·0M. No significant increase in protein excretion was seen in control rats receiveing 2·5 mg of rabbit IgG which had been treated with the various KSCN concentrations used.

DISCUSSION

In a separate paper (Shimizu *et al.*, 1978*b*), it could be shown that a fractionation of anti-DNP-antibodies with respect to their average intrinsic association constant could be achieved by desorption of antibodies from immunoadsorbent with thiocyanate at concentrations of 0·5–4·0 M as eluent. The results presented in this paper demonstrate that the same holds true for a complex system, namely the fractional elution of nephrotoxic antibodies from Tr-GBM–sepharose. The re-elution of readsorbed antibodies and the comparison of stepwise and direct elution provides, as well, evidence for a fractionation according to avidity, as shown from the binding data in Fig. 3, which imply an increase in binding properties of the antibody fractions dependent on the molarity applied for elution.

Though varying in binding strength the different antibody fractions, unlike anti-human serum albumin (HSA) antibodies (Shimizu *et al.*, 1978*a*), did not show noticeable differences in their isoelectric focusing (IEF) pattern. Anti-GBM antibodies are , apparently, in respect of their net surface charge much more heterogeneous than anti-HSA antibodies. In this connection it should be mentioned that the use of Tr-GBM–sepharose as immunoadsorbent preferentially selects anti-GBM antibodies reacting with the non-collagenous portion of the GBM.

The *in vivo* experiments showed that the capacity of the various antibody fractions to fix to the kidney increased with increasing antibody avidity. This would be consistent with the law of mass action, assuming a constant number of antigenic sites. When the same amount of each fraction was injected a higher level of

proteinuria could be induced by the antibody fraction eluted with 4·0M KSCN than with those antibodies fractionated with 0·5M KSCN or 2·0M. This suggests that higher-affinity antibody caused more renal injury. This does not exclude the possibility that larger amounts of low-affinity antibody could induce the same degree of renal injury as small amounts of high-affinity antibody. Similar effects were described with antibody fractions of different affinity in the passive cutaneous anaphylaxis reaction, in which antibodies of low affinity could induce injury of the same degree only when present in larger amounts (Hurlimann and Ovary, 1965; Siskind and Eisen, 1965).

In complex nephritis the situation concerning the antibody avidity is quite different. In this type of experimental model animals producing low-avidity antibodies show a significantly higher incidence of nephritis than animals producing high-avidity antibodies (Soothill and Steward, 1971; Steward, Katz and West, 1975). But the pathogenetic mechanisms leading to injury of the kidney are not comparable in complex nephritis and anti-GBM nephritis. One reason for the tissue-damaging capacity of low-avidity antibodies in complex nephritis may be that the immune complexes formed by high-avidity antibodies are taken up in higher quantitities by the cells of the RES or are, depending on their size, deposited predominantly in the mesangium. The latter mechanisms do not play a significant role in anti-GBM nephritis.

A relatively high percentage of the injected antibody fractions became localized in spleen and liver. In contrast to the kidney-fixing antibodies, the immunoglobulin molecules disappeared from these organs rapidly. Additionally larger quantities of the antibody fractions eluted with higher KSCN concentrations became localized in spleen and liver. These two observations suggest that the fraction obtained with higher concentration may have been predominantly taken up by the cells of the RES, possibly because the antibody molecules had been altered during elution causing aggregation. Relatively little activity was found in the lung, and any localization was probably due to the presence of cross-reacting BM antigens, as the antibodies fixed in the lung disappeared from this organ slowly.

The observation that the high-avidity antibody fractions disappeared from the kidney relatively faster than the antibody fraction eluted with 0·5M KSCN is not easy to explain. Perhaps it was due to partial alteration of the protein, leading to aggregation. It is known that the aggregated IgG can be taken up and rapidly cleared by the mesangium (Mauer *et al.*, 1972). In the experiments performed it was not possible to distinguish between antibody bound to the GBM or deposited in the mesangium. Additionally it must be borne in mind that in Masugi nephritis after 3–5 days the situation is complicated by the autologous phase owing to the response of the recipient animal to the injected foreign material.

Focusing our attention on the heterologous phase of Masugi nephritis, the data presented show that higher-avidity anti-Tr GBM antibody becomes localized to a higher extent in the kidney and causes more kidney damage than lower-avidity antibody, possibly by the activation of complement and other systems (Kashgarian, Hayslett and Spargo, 1977). In the natural situation of anti-GBM nephritis low-avidity antibodies will be produced inducing only slight alteration of kidney function. But in the course of maturation of the autoimmune response, antibodies of high avidity will cause severe alteration. Thus our finding that renal injury depends upon the avidity of anti-GBM antibodies could be of interest.

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