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Divalency of the monoclonal antibody 5-1-6 is required for induction of proteinuria in rats

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SUMMARY

A single i.v. injection of 3 mg of the $F(ab')_2$ fragment of MoAb 5-1-6 into rats induced immediate proteinuria ($128{\cdot}1 \pm 80{\cdot}7$ mg/24 h on day 1) which lasted 1–2 days. In contrast, rats administered 10 mg of the corresponding Fab fragment did not develop abnormal proteinuria even though an equivalent dose of the intact MoAb 5-1-6 far exceeded the nephritogenic dose. The total kidney binding of ^{125}I-Fab fragment was $209{\cdot}5 \pm 34{\cdot}3$ μg/2 kidneys. This exceeded that obtained by injection of 3 mg MoAb 5-1-6 IgG1 ($58{\cdot}9 \pm 12{\cdot}5$ μg/2 kidneys at 1 h) and was similar to that obtained following injection of 3 mg $F(ab')_2$ fragment ($235{\cdot}3 \pm 16{\cdot}9$ μg/2 kidneys). Immunofluorescence (IF) showed a linear pattern along the glomerular capillary wall at 1 h after the administration of MoAb 5-1-6 IgG1, $F(ab')_2$ or Fab fragment. On day 5, fine to coarse granules were observed scattered in $F(ab')_2$-injected rat glomeruli, whereas granules were densely localized in Fab-injected rat glomeruli. Complement-depleted rats injected with 3 mg of MoAb 5-1-6 IgG1 developed proteinuria with the same time course as non-depleted rats. This observation, together with the ability of $F(ab')_2$ to induce proteinuria, indicates that proteinuria induced by MoAb 5-1-6 is complement-independent. This study suggests that MoAb 5-1-6-induced proteinuria is initiated by cross-linking of the epitopes by divalent MoAb 5-1-6 and is independent of complement activity.

Keywords monoclonal antibody $F(ab')_2$ Fab cross-linking proteinuria

INTRODUCTION

Many approaches have been used to investigate the mechanisms of proteinuria [1–4], but details remain unclear. However the most important components controlling permselectivity of glomeruli have not yet been clarified. We have used a simple model of proteinuria induced by MoAb 5-1-6. This MoAb [5] induces massive, transient proteinuria in rats following a single i.v. injection, and binds only to the surface of glomerular epithelial foot processes. This proteinuria appears to be induced independently of complement activation and inflammatory cell infiltration [5]. Clarification of the mechanism of proteinuria induced by MoAb 5-1-6 may be obtained by focusing on ligand–receptor-like interaction. *In vitro* studies of membrane proteins, including the IgE receptor on basophils [6] and the insulin receptor [7], have indicated the importance of antibody-mediated antigen redistribution for cell biological reactions. Camussi *et al.* [8,9] proposed that in Heymann's glomerulonephritis, the animal model for human membranous glomerulonephritis which is characterized by *in situ* formation of immune complexes, formation of immune deposits may be initiated following the clustering of immune complexes on the epithelial cell surface. These may subsequently be shed from the podocyte of plasma membrane. The loss of the antigenic molecules recognized by MoAb 5-1-6 from cultured epithelial cells prevents the use of an *in vitro* system similar to those employed in the study of other ligand surface antigen interactions. Therefore, we examined the relationship between immunofluorescence (IF) pattern, which may reflect the antibody-mediated redistribution of cell surface antigens, and proteinuria induced by MoAb 5-1-6 $F(ab')_2$ and Fab fragment *in vivo*. In rats which received the MoAb 5-1-6 $F(ab')_2$ fragment, immediate proteinuria was observed with non-uniform and scattered granular IF pattern by day 5. In contrast, MoAb 5-1-6 Fab fragment failed to induce proteinuria, and gave rise to a densely granular IF pattern on day 5. This suggests that the induction of proteinuria by MoAb 5-1-6 is driven primarily by cross-linking and aggregation of the recognized epitope by the divalent MoAb.

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MATERIALS AND METHODS

Animals

Female Wistar rats (160–180 g) were obtained from Charles River, Japan, Inc. (Atsugi, Japan).

Preparation of MoAb 5-1-6 (IgG1) and production of F(ab')$_2$ and Fab fragment

MoAb 5-1-6 was prepared as described previously [5]. The MoAb was produced in ascitic fluid of BALB/c mice primed with 2,6,10,14-tetramethylpentadecane (Sigma Chemical Co., St Louis, MO). This fluid was subjected to 50% ammonium sulphate precipitation, and the immunoglobulin-rich fraction was dialysed against PBS for 2 days and stored at −70°C until use. The F(ab')$_2$ and Fab fragments were prepared from IgG1 principally as described by Parham [10]. The F(ab')$_2$ fragment was produced by digestion with pepsin. Thirty milligrams of MoAb 5-1-6 at 10 mg/ml were dialysed against 0·1 M citrate buffer, pH 4·0, and incubated at 37°C for 8 h with pepsin (Sigma) at a final concentration of 25 μg/ml. The reaction was terminated by raising the pH to > 7·0 with the addition of 3·0 M Tris HCl, pH 8·6. After dialysis against PBS to remove smaller fragments, the F(ab')$_2$ fragment was separated from undigested IgG1 by Sephadex G200 gel filtration chromatography (Pharmacia, Uppsala, Sweden). The Fab fragment was produced by papain digestion. Thirty milligrams of MoAb 5-1-6 were dialysed against 0·1 M sodium acetate, pH 5·5, at 10 mg/ml, and then incubated with papain (Elastin Products Co, Inc., Owensville, MO) at a protein:enzyme ratio of 100:1, at 37°C for 90 min. The 150 μl of 0·15 M iodoacetamide solution (Sigma) were added to terminate the reaction. Purification of the Fab fragment was completed by dialysis and Protein G Sepharose gel filtration chromatography (Pharmacia). Aliquots of each fraction were analysed by SDS–PAGE under non-reducing conditions and the appropriate fractions were collected and dialysed against distilled water. The F(ab')$_2$ and Fab fragments were freeze-dried and stored at 4°C. Purity was confirmed by SDS–PAGE loading the fragments prepared at the concentration used in the experiments. Reactivity of both fragments with glomeruli *in vitro* was assessed by indirect IF. Normal rat kidney cryostat sections were incubated with each fragment, followed by rabbit antibodies against mouse immunoglobulin Fab portion, and then stained with fluoresceinated goat antibodies against rabbit immunoglobulin (Dakopatts, Glostrup, Denmark) absorbed with rat whole serum. The relative reactivity of each fragment was as follows; IgG1:F(ab')$_2$:Fab = 1·0:1·0–1·5:0·25–0·5.

Endotoxin levels in aliquots of intact MoAb 5-1-6 and its enzymatically treated fragments checked by Endospecy method (Seikagakukogyo Co, Tokyo, Japan) were less than 150 pg/ml.

Experimental design

Experiment 1. Rats were injected intravenously with 3 mg of MoAb 5-1-6 F(ab')$_2$ (n=3) or 10 mg of MoAb 5-1-6 Fab (n=6), respectively. These doses were chosen to exceed the nephritogenic dose of intact MoAb 5-1-6 used in this experiment. (A single 3-mg i.v. injection of the intact MoAb 5-1-6 induced transient proteinuria of 113·7 ± 54·9 mg/24 h on day 5, n=7.) The rats were housed individually in metabolic cages with free access to water, and 24-h urine samples were collected on days 1, 3 and 5 after injection of each fragment of MoAb 5-1-6. All rats were killed under ether anaesthesia on day 5 and the kidneys were obtained for IF studies. Quantitative measurements of antibody binding to kidneys *in vivo* were performed by injecting trace amounts of the ^{125}I-labelled MoAb 5-1-6 IgG1, F(ab')$_2$ or the Fab fragment into three groups of rats: Group A, MoAb 5-1-6 3 mg, 2 mg, 1 mg (n=3 each); Group B, MoAb 5-1-6 F(ab')$_2$ 3 mg (n=9); Group C, MoAb 5-1-6 Fab 10 mg (n=6). Radioiodination was performed by the chloramine T method [11]. This gave labelled materials with specific activities of $1·1 \times 10^7$–$7·9 \times 10^7$ ct/min per mg protein, of which 89–96% was precipitable with 10% trichloroacetic acid. All rats of Group A and three rats of Group B and C were killed 1 h after the injection. Urine samples from the remaining rats were collected 1, 3 and 5 days after injection, and the rats were killed on day 5. Just before sacrifice, each rat was perfused with 500 ml of PBS from the left ventricle and visible blanching of the kidneys was observed. The kidneys were obtained to measure radioactivity, and some were subsequently processed for IF studies. A 0·5-ml blood sample was collected before perfusion, and the level of radioactivity was determined.

Experiment 2. Four rats were depleted of the complement (C3) with the 50-μg cobra venom factor (CVF) injected intraperitoneally 12 h before the i.v. injection of 3 mg of MoAb 5-1-6. Three rats were injected with PBS intraperitoneally as a control and received 3 mg of MoAb 5-1-6 intravenously 12 h later. Urine samples were collected as in experiment 1. Serum samples were obtained from the tail vein before and 12 h after i.p. injection of CVF, and also 1, 3 and 5 days after i.v. injection of MoAb 5-1-6. They were used to check the level of serum C3

Table 1. Urinary protein excretion of rats injected with MoAb 5-1-6 F(ab')$_2$ and Fab fragment

	Urinary protein excretion, mg/24 h			
	Day			
	Before	0–1	2–3	4–5
MoAb 5-1-6 F(ab')$_2$ 3 mg (n=9)*	0·6 ± 0·8	128·1 ± 80·7 †	6·43 ± 13·7	0·92 ± 1·0
MoAb 5-1-6 Fab 10 mg (n=9)*	0·7 ± 0·7	2·1 ± 2·0 †	0·8 ± 1·1	0·9 ± 1·1

Values are means ± s.d.
* n, Number of rats studied.
† P < 0·001.

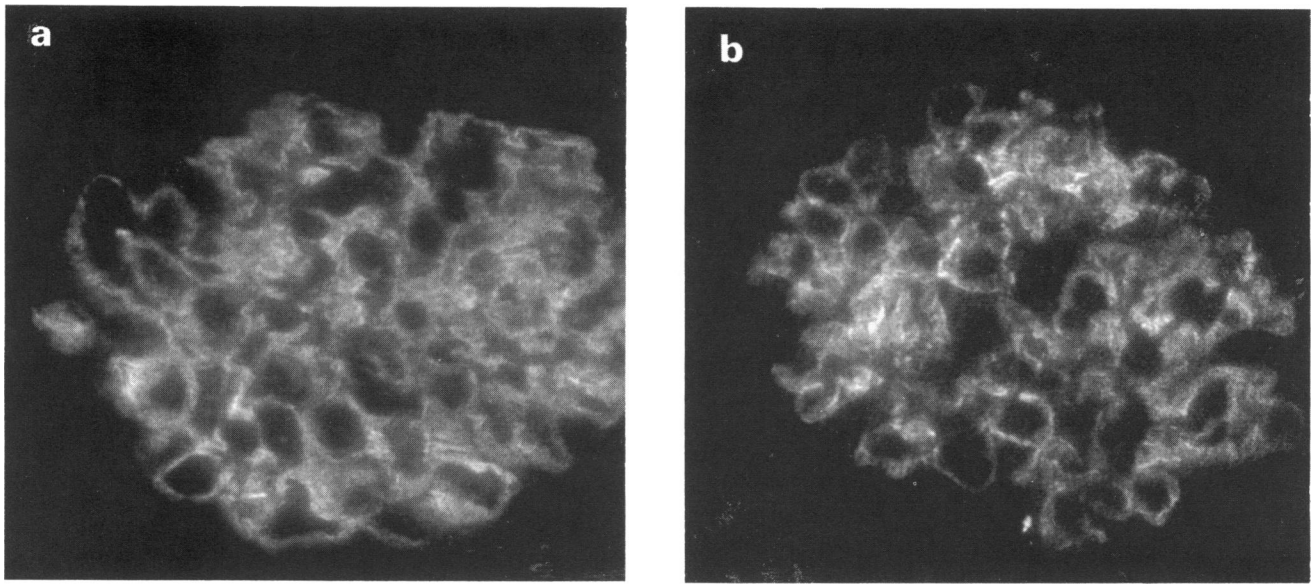

Fig. 1. Immunofluorescent (IF) pattern of the *in vivo* binding of the $F(ab')_2$ (a), and Fab fragments (b) of MoAb 5-1-6 1 h after administration, stained with rabbit anti-mouse IgG Fab portion followed by FITC-anti-rabbit immunoglobulin. Linear staining along the glomerular capillary wall is present (×400).

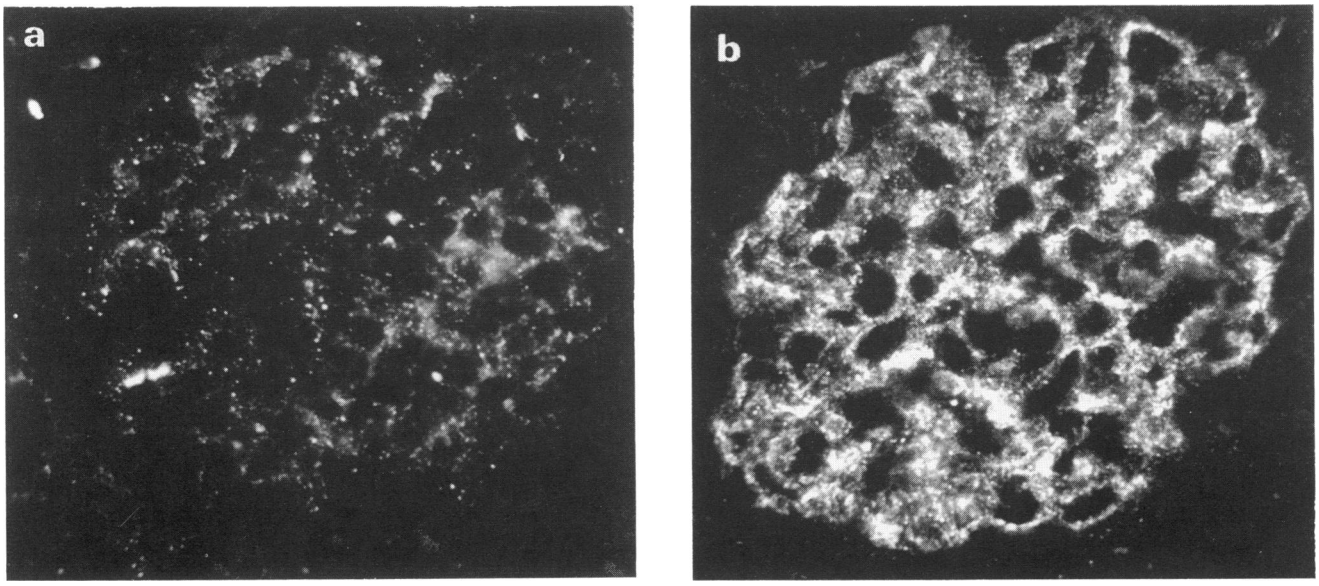

Fig. 2. Immunofluorescent (IF) pattern of the *in vivo* binding of the $F(ab')_2$ (a), and Fab fragments (b) of MoAb 5-1-6 5 days after administration, stained with rabbit anti-mouse IgG Fab portion followed by FITC-anti-rabbit immunoglobulin. Scattered fine to coarse granular staining is present in rats injected with $F(ab')_2$ (a) and dense granular staining is observed in rats injected with Fab (b) (×400).

using a single radial immunodiffusion test with anti-rat C3 (Cappel, West Chester, PA) in agar. All rats were killed on day 5, and kidneys were removed for IF studies. Quantitative measurements of the binding of 3 mg of MoAb 5-1-6 to kidneys *in vivo* following injection into complement-depleted rats ($n=2$) were carried out as in experiment 1.

Other methods

Urinary protein content was determined by the biuret method [12] Tissues were processed for IF according to the method of Kawamura [13], and the sections were observed with a Zeiss fluorescence microscope equipped with epi-illumination. Radioactivity was determined with an Autogamma Scintillation Photometer 5260 (Packard). Statistical analysis was performed by Student's *t*-test.

RESULTS

Experiment 1

Proteinuria. Urinary protein excretion on days 1, 3 and 5 after administration of the $F(ab')_2$ and Fab fragments of MoAb 5-1-6 to rats is shown in Table 1. The $F(ab')_2$ fragment induced massive proteinuria lasting 1–2 days, with the maximum effect occurring within 24 h of administration. In contrast, rats which had received the Fab fragment did not become proteinuric.

Table 2. Urinary protein excretion of rats injected with 3 mg of MoAb 5-1-6 IgG1 to complement-depleted and non-depleted rats

	Urinary protein excretion, mg/24 h				
	Day				
	Before	12 h	0–1	2–3	4–5
Complement-depleted rats ($n=4$)*	0±0·1 (100)†	(2·3±2·2)	104·4±76·8 (2·8±0·8)	123·3±59·1 (33±7·8)	124·7±78·3 (52·3±15·3)
Normal rats ($n=3$)	0·1±0·1 (100)	(106·5±5·6)	95·2±56·1 (107·3±15·1)	120·1±13·9 (102·3±23·9)	98·8±28·3 (97·0±5·1)

Values are means±s.d.

* n, Number of rats studied.

† The values in parentheses are the relative complement activity (%) at each time point. (The pre-experiment value was designated as 100%.)

Immunofluorescence. Linear staining along the glomerular capillary wall was observed 1 h after the administration of both $F(ab')_2$ (Fig. 1a) and Fab fragments (Fig. 1b). On day 5, the pattern in the glomeruli of rats injected with the $F(ab')_2$ fragment had changed to an irregularly granular pattern (Fig. 2a). Densely localized granules were observed on day 5 in glomeruli of rats which had received the Fab fragment (Fig. 2b).

Quantitative studies of kidney antibody binding. Contamination with unbound antibody was calculated as equivalent to 7% of the amount of antibody present in total blood of rats based on the results obtained with ^{125}I-labelled RVG1, a control murine IgG1 MoAb which was shown not to react with rat kidney [14]. The data below were corrected by subtracting the amount of calculated unbound antibody from the total count. One hour after injection of MoAb 5-1-6 IgG1, the $F(ab')_2$ fragment or the Fab fragment, the total amounts of kidney-binding antibody were: IgG1 1 mg, 12·6±1·3; IgG1 2 mg, 30·0±3·5; IgG1 3 mg, 58·9±12·5; $F(ab')_2$ 3 mg, 235·3±16·9; Fab 10 mg, 209·5±34·3 (μg/2 kidneys, $n=3$ respectively). The amounts of antibody bound to kidneys on day 5 were: $F(ab')_2$ 3 mg, 24·6±1·1 μg/2 kidneys ($n=6$); Fab 10 mg, 17·6±0·4 μg/2 kidneys ($n=3$).

Experiment 2

Proteinuria. Table 2 shows urinary protein and the activity of complement over the 5-day period. Massive proteinuria had been induced in both complement-depleted and control rats.

Immunofluorescence. A coarse granular pattern was observed along the glomerular capillary wall, and no difference in staining pattern was observed between complement-depleted and control rats on day 5.

Quantitative studies of kidney antibody binding. Total kidney binding of antibody was assessed as in experiment 1. The total amounts of antibody bound in complement-depleted rats ($n=2$) injected with 3 mg of MoAb 5-1-6 were 56·6 μg/2 kidneys and 56·3 μg/2 kidneys 1 h after injection.

DISCUSSION

The demonstration that the $F(ab')_2$ fragment of MoAb 5-1-6 induced proteinuria in rats confirmed that complement activity and/or inflammatory cell infiltration mediated by the Fc portion is not involved in the mechanism of proteinuria induced by this MoAb. Proteinuria was also induced in complement-depleted rats, again indicating the lack of complement involvement. In contrast, the Fab fragment failed to induce proteinuria (Table 1), though the total amount bound to kidney was much more than the amount of intact MoAb required to induce proteinuria. Proteinuria induced by intact MoAb 5-1-6 continued for several days after injection (Table 2). In contrast, rats injected with $F(ab')_2$ fragment showed a transient, but marked proteinuria within 24 h, followed by total remission by day 4. This rapid change may be due to the smaller size of the $F(ab')_2$ fragment than the intact MoAb, allowing easier access to the epithelial epitopes through the size barrier of the glomerular basement membrane. The amount of $F(ab')_2$ fragment bound to the kidneys of rats was about four times that of rats injected with an equivalent amount of intact MoAb. The amount of circulating $F(ab')_2$ was a quarter of that of intact MoAb 1 h after injection (data not shown). Rapid and sufficient binding to the epitopes on the epithelial cell surface for a brief time using Fab or $F(ab')_2$ fragments would give us useful information about the sequence of events leading to proteinuria. Our results revealed a close association between IF patterns and the induction of proteinuria following the injection of different MoAb preparations. Non-proteinuric rats injected with the Fab fragment showed a densely granular pattern, whereas highly proteinuric rats injected with the $F(ab')_2$ fragment showed a non-uniform and scattered granular pattern on day 5. These findings suggested that cross-linking and aggregation of the epitopes recognized by divalent MoAb 5-1-6 are associated with proteinuria. Subsequent internalization of the epitopes could occur, resulting in their temporary disappearance from the cell surface, together with bound divalent MoAb. Previous immunoelectron microscopic observations have shown that administered intact MoAb 5-1-6 was detected in multivesicular bodies of the glomerular epithelial cells on day 3 [5].

Many studies support the importance of divalent or polyvalent ligands in initiating a chain reaction by cross-linking of membrane proteins in cellular biological reactions [7, 15–19]. It has been proposed that the interaction between cross-linked receptors and cytoskeletal element such as microfilaments may be responsible for movement of receptors into patches and caps [20,21]. However, such movement has recently been reported to be associated with the interaction of receptors, and one of the

components of the membrane skeleton rather than with the cytoskeleton [6,22]. It is possible that in our model, the critical signalling event initiating the increase of glomerular permeability occurs during cross-linking and aggregation of the epitopes. If so, it would explain the failure of chlorpromazine, an inhibitor of cytoskeletal movement, to prevent proteinuria induced by intact MoAb 5-1-6 [23]. Although there is accumulating evidence for various signalling pathways [24–26], the precise role of the membrane skeleton and/or cytoskeleton in the signalling event in the present case is not clear. Thus identification of the epitope recognized by MoAb 5-1-6, now in progress, will help to resolve the exact mechanism of this MoAb-induced proteinuria. The importance of glomerular epithelial cells in the control of glomerular permeability is demonstrated by this model, which should help to clarify the complicated mechanisms of proteinuria in human disease.

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