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ORIGINAL ARTICLE

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Selective planting of cationized, haptenized ovalbumin on the rat tubular basement membrane

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Abstract We developed an experimental protocol for planting exogenous antigens with different molecular weights and charges on the constituents of the renal tubulointerstitium. The cationized antigens were injected selectively into the left renal arteries of Wistar rats. Antigen localization was documented by immunohistochemistry on frozen sections. Cationized bovine serum albumin (BSA; 68 kDa, isoelectric point =9.5) localized almost exclusively along the glomerular capillary wall. After application of highly cationic polyethyleneimine, cationized BSA given subsequently was found in a linear distribution along the glomerular capillary wall and along the peritubular capillaries. The fate of highly cationized ovalbumin conjugated with trinitrophenol (TNP-OA), subjected to gel filtration to obtain monomers (42 kDa, isoelectric point >10) differed; it was deposited in a linear pattern on the tubular basement membrane (TBM) and Bowman's capsule, and remained up to 36 h after injection. Noncationized, monomeric TNP-OA (42 kDa, isolectnic point =4.6) showed fine granular deposition in the tubular epithelium exclusively. These findings indicate that the barrier of the glomerular BM acts selectively on antigens with different molecular weights. They either settle on the peritubular capillaries, after passing the glomerular, or reach the urinary space, after which they are reabsorbed by the tubular epithelial cells to reach the TBM.

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Introduction

To investigate the pathogenesis of interstitial nephritis, recent research has focussed on identifying and purifying nephritogenic tubular basement membrane (TBM) antigens [29]. Serum from patients with tubulointerstitial nephritis (TIN) recognizes specific moieties of the human TBM [3, 8, 30]. Animals immunized with heterologous renal BM or TBM antigens develop TIN [9, 28], and the autoantibodies and complement localize exclusively on the TBM and Bowman's capsule, but not on the glomerular BM (GBM). Although the specific moieties of the TBM antigen which are recognized by these autoantibodies have been characterized and purified [2, 4], such experimental models do not directly demonstrate how an endogenous antigen can become immunogenic and induce an autoimmune response [19]. This process may depend on immunological modulation, because autoantibody production only occurs when TBM antigens derived from nonautologous animals are injected, and purified endogenous antigens are not immunogenic in syngeneic or autologous animals [15, 16].

Alternatively, TIN may develop in humans when exogenous antigens of infectious agents or drugs acting as haptens become planted on constituents of the tubulointerstitium and act as immunogens, or it may occur in hosts who are already sensitized against the planted antigens. To date, no appropriate experimental model in which deposition of exogenous antigen is confined to the TBM and Bowman's capsule has been developed [10, 28]. Such a model does not involve breakdown of the barrier against autoimmune induction. We focussed the present study on producing an experimental model with selective planting of antigens (haptens and/or carrier proteins) on the constituents of the tubulointerstitium.

Because both the GBM and TBM are negatively charged, positively charged antigens can be planted on them, however, selective planting on the TBM is difficult to achieve. To overcome the charge and filtrations barriers of the GBM, neutralizing its anionic site on the GBM with a polycationic agent may provide access for the cationic antigens to the tubulointerstitium via postglomerular flow, or cationic antigens with small molecular weights filtered through the GBM into the urinary space may be reabsorbed by the tubular epithelial cells and thus reach the TBM. Therefore, we used two cationic antigens, bovine serum albumin (BSA) and trinitrophenol-conjugated ovalbumin (TNP-OA) with molecular weights larger and smaller respectively than the size critical for the GBM.

Materials and methods

All experiments were performed on male Wistar rats weighing 150–180 g (Zentraltierzuechterei, Hannover, Germany).

In the preparation of antigens 2,4,6-trinitrobenzene sulphonic acid (TNBS) (Nakarai Chemicals, Kyoto, Japan) was initially conjugated to OA (Sigma, St. Louis, Mo, USA) by a method described previously [18]. In brief, 0.1% TNBS was added to ovalbumin (20 mg/ml) in 0.1 M phosphate-buffered saline (PBS; 1:10–1:20 w/w). After the solution was incubated for 90 min at 37° C, the remaining free TNBS was separated from TNP-OA on a Sephadex G 25 column (Pharmacia, Sweden). The average number of trinitrophenol groups per molecule of OA was 4.7 and 5.0, respectively, as calculated by the formula of Little and Eisen [18].

Cationization of antigens, $TNP_{4.7}$-OA and BSA was performed as previously described [20] by a modification of the method of Danon et al. [5] using l-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Sigma) as the activator and N-,N-dimethyl-1,3-propanediamine (DMPA; Eastman Kodak, Rochester, N.Y., USA) as the nucleophile to replace carboxyl groups. In brief, 0.4 ml of DMPA was added to 5 ml of distilled water, and the pH was adjusted to 6.5 with 1 N hydrochloric acid (HCl). BSA (1 g; Sigma) or $TNP_{4.7}$-OA (100 mg) and EDC (3 g) were then added and stirring. The pH was maintained at 6.5 for 4–6 h in a TTT 60 Titrator (Radiometer, Copenhagen, Denmark) using 0.2 N HCl. The reaction mixture was left to stand overnight at room temperature, dialyzed against six changes of cold PBS at 4° C, filtered through a millipore filter (0.45 μm) for sterilization, and stored at 4° C until required. The extent of cationization was estimated by isoelectric focussing (IEF) in a slab gel system (Desaga, Heidelberg, Germany). Cationized BSA, cationized $TNP_{4.7}$-OA, and native $TNP_{5.0}$-OA had isoelectric points (pI) of 9.5, >10.0, and 4.6, respectively.

The antigens were purified by gel filtration on a Sephadex G 100 superfine column (Pharmacia; 3×70 cm) equilibrated with 2 M saline to prevent nonspecific binding of cationic proteins to the gel. Protein samples were run in 2 M saline at a flow rate of 10 ml/h. To check for the presence of aggregated dimers or polymers, antigens were applied to 10% polyacrylamide gel using a basic gel system, and staining was performed with Coomasie blue.

To produce anti-TNP antibody, 2.0 mg/dl of TNP conjugated to human immunoglobulin G (TNP-human IgG), a carrier protein without cross reactivity with OA, was incorporated into complete Freund's adjuvant and used to immunize rabbits. IgG was then precipitated from the serum of the rabbits and labelled with fluorescein isothiocyanate (FITC) according to the method of Kawamura [14]. The haptenic group's specificity was checked by double immunodifusion in agarose IEF (Pharmacia) using different hapten-carrier systems, TNP-BSA, TNP-OA, and TNP-human IgG as described previously. No cross reactivity of this FITC-labelled anti-TNP antibody with rat IgG was determined by immunofluorescence in cryostat sections of glomeruli with discrete rat IgG deposition [12].

For renal perfusion via the left renal artery, rats were anaesthetized with ether, a 26-G needle was used to cannulate the aorta, and the aorta was ligated between the right and left renal arteries, just before the antigen was injected (0.5 ml/min) in a volume of 0.2 ml. Both before and after antigen injection, a low volume (0.25 ml) of PBS was perfused and the ligature was released. The duration of renal ischaemia ranged from 4 to 7 min.

For immunofluorescence studies, the kidneys were snap-frozen in *n*-hexane at −70° C. Frozen sections were cut and stained with FITC-conjugated anti-BSA (Cappel, N.C., USA), anti-ovalbumin (Cappel), and anti TNP-human IgG antibodies.

The experiment required that we attempted to overcome the charge barrier of the GBM by charge neutralization, and to allow this 100 μg of polyethyleneimine (PEI, molecular weight 40–60 kDa) (Polyscience, Pa., USA; 400 mOs; pH 7.4) was injected into the left renal arteries of three Wistar rats. This was followed by injection of 500 μg of cationized BSA by the same route. PEI concentrations of 5 or 1 mg/ml were also examined and an interval between BSA and PEI injection of 1 or 15 min was tested. Cationized BSA (500 μg) alone was injected into the left renal arteries of three Wistar rats, as described above. Three hours later, the rats were sacrificed for histological examination. To overcome the charge and filtration barriers of the GBM with small-sized cationized antigens, three groups of experiments were performed with the following antigens: crude, cationized $TNP_{4.7}$-OA: chromatographically purified, cationized $TNP_{4.7}$-OA; and chromatographically purified, noncationized $TNP_{5.0}$-OA. The antigens (300 μg in 200 μl) were injected into three Wistar rats each via their left renal arteries and the rats were sacrificed for histologic examination 24 h later. In the rats given purified, cationized $TNP_{4.7}$-OA, the disappearance kinetics of the antigen were additionally investigated. In this group, two rats each were sacrificed for histologic examination at 1, 36, and 72 h postinjection.

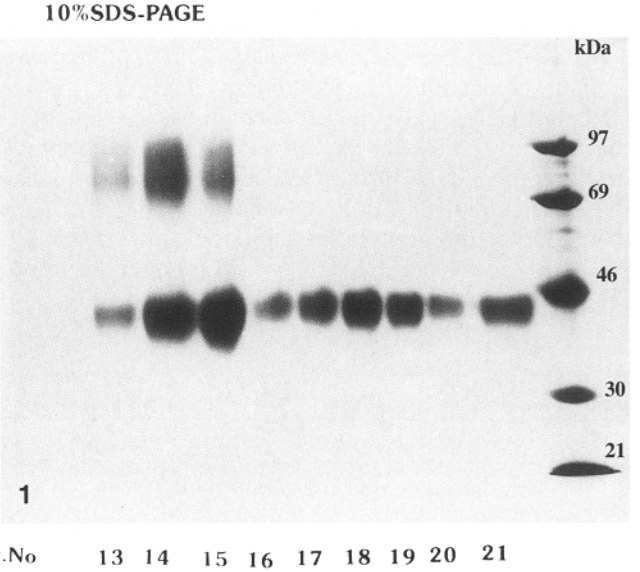

Fig. 1 Sodium dodecyl sulphate (10%)-polyacrylamide gel electrophoresis (SDS-PAGE) of cationized ovalbumin conjugated with trinitrophenol with 4.7 groups per molecule ($TNP_{4.7}$-OA) after purification on a Sephadex G100 superfine column. Fractions (*Fr*) 13–15 consisted of monomers (42 kDa) and dimers (84 kDa) of the antigens; Fr 16–21 contained only monomers (42 kDa) and were used for injection

Results

Crude, cationized $TNP_{4.7}$-OA was separated by gel filtration on a Sephadex G 100 superfine column (Pharmacia) into dimers (84 kDa, fractions 13–15) and monomers (42 kDa, fractions 16–21; Fig. 1). Crude, noncationized $TNP_{5.0}$-OA was also separated in the same manner. The antigenicity of monomeric cationized $TNP_{4.7}$-OA and monomeric noncationized $TNP_{5.0}$-OA was compared by gel diffusion using anti-TNP-human IgG antibody; both antigens were precipitated and showed a line of identity.

Immunofluorescence studies showed that cationized BSA localized exclusively along the glomerular capillary wall in a peripheral pattern at 3 hours after injection (Fig. 2a). By prior application of PEI, cationized BSA was found along the peritubular capillaries in the interstitium and along the glomerular capillary wall (Fig. 2b). Binding of cationized BSA to the glomerular capillary wall could not be prevented, although various PEI concentrations and intervals between the PEI and cationized BSA injections were tested. No deposition of cationized BSA on the TBM or on Bowman's capsule was found.

Monomeric cationized $TNP_{4.7}$-OA (fraction 18) was found in a homogeneous pattern exclusively along the TBM as well as along Bowman's capsule, but neither along the glomerular capillary wall nor in the cytoplasm of the tubular epithelium 24 h after antigen injection. Staining with anti-TNP antibody and anti-OA antibody showed the same pattern (Fig. 3a, b). The antigens were clearly stained at 36 h postinjection, but the intensity of immunofluorescence decreased markedly at 72 h. Crude cationized $TNP_{4.7}$-OA containing dimer forms localized not only on the TBM and Bowman's capsule but also along the glomerular capillary wall and the peritubular capillaries 24 h after injection (Fig. 4a). Monomeric native $TNP_{5.0}$-OA was found in a fine granular pattern exclusively in the cytoplasm of the tubular epithelium, but neither in the glomerulus nor along the TBM at 24 h postinjection (Fig. 4b).

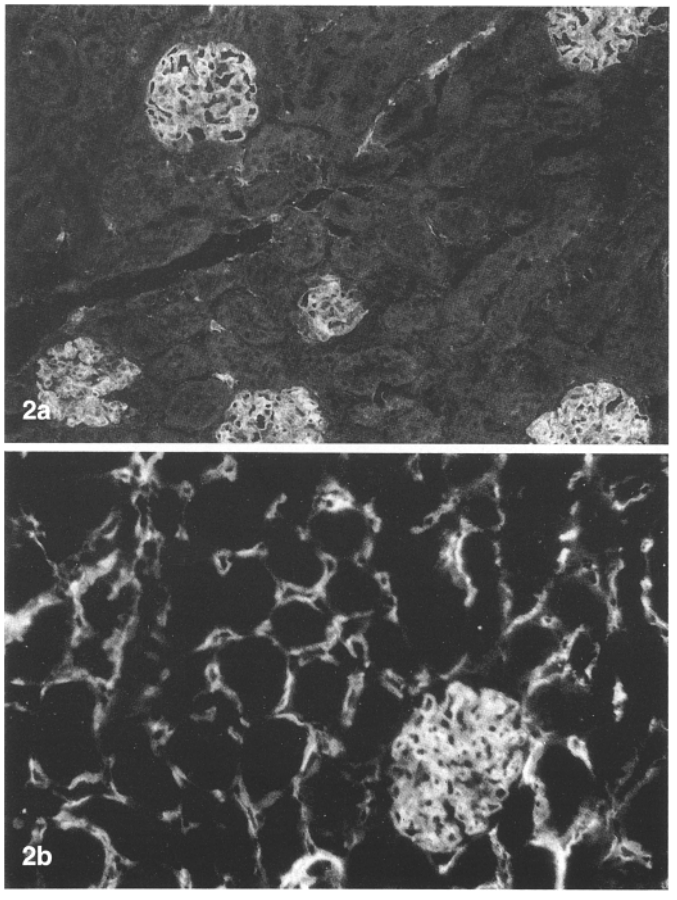

Fig. 2a, b Fluorescence micrographs: injection of cationized bovine serum albumin (BSA) without (**a**) and with (**b**) the preinjection of polyethylenimine. Immunofluorescence staining of frozen kidney sections using fluorescein isothiocyanate (FITC)-labelled anti-BSA antibody 3 h after antigen injection. **a** Glomerular capillary wall shows exclusively a linear deposition of BSA (×33). **b** The peritubular capillaries and the glomerular capillary wall show a linear deposition of BSA, whereas Bowman's capsule and the tubular basement membrane (TBM) are negative (×40)

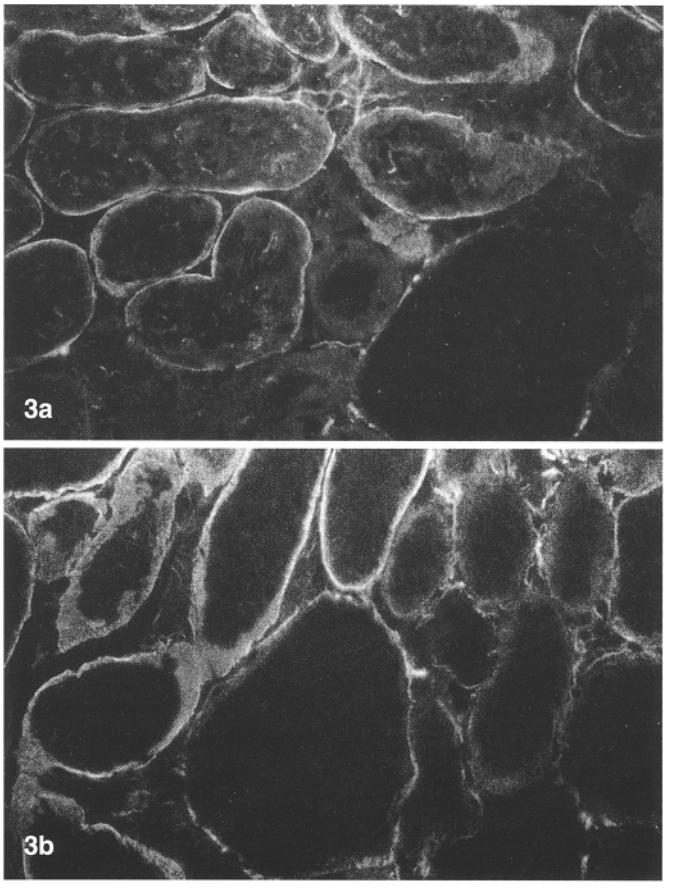

Fig. 3a, b Localization of purified, cationized TNP-OA. Purified cationized $TNP_{4.7}$-OA (Fr 18) is exclusively planted on the TBM and Bowman's capsule in a linear pattern. Immunofluorescence staining of frozen kidney sections using an FITC-labelled anti-TNP-human IgG antibody (**a**) and an anti-OA antibody (**b**; ×66) 24 h after antigen injection

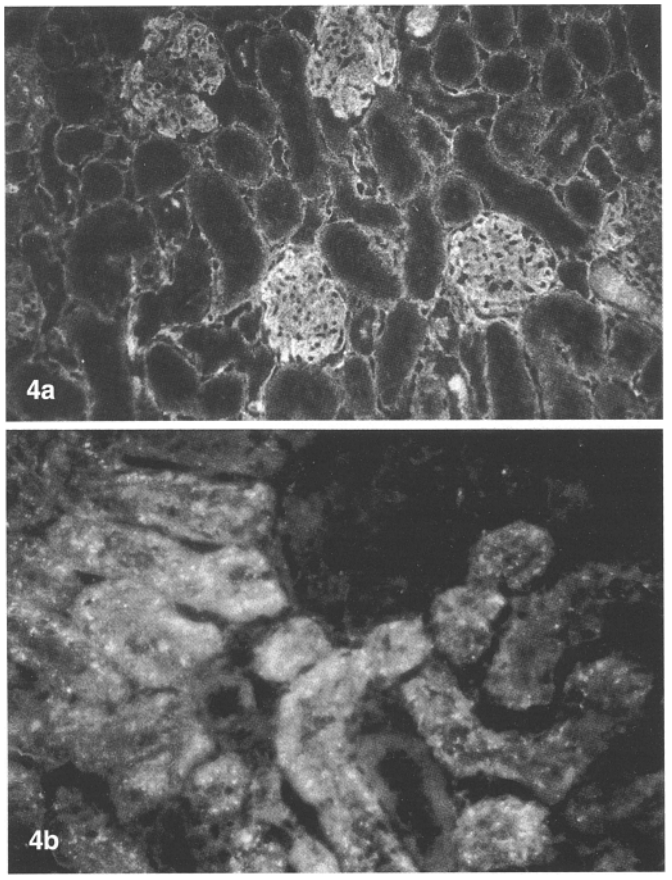

Fig. 4a, b Localization of crude, cationized TNP-OA and monomeric noncationized TNP-OA. Immunofluorescence staining of frozen kidney sections using an FITC-labelled anti-TNP-human IgG antibody 24 h after antigen injection. **a** Crude, cationized $TNP_{4.7}$-OA containing dimer forms shows a linear deposition on the TBM and Bowman's capsule as well as along the glomerular capillary wall and the peritubular capillaries (×33). **b** Purified, noncationized $TNP_{5.0}$-OA shows a fine granular pattern of deposition in the cytoplasm of the tubular epithelium (×51), but neither in the glomerulus nor along the TBM

Discussion

We have examined whether exogenous antigens with different molecular weights and charges travel from the blood stream to be deposited on such constituents of the interstitium as the TBM, Bowman's capsule, or the peritubular capillary bed. The selective permeability of the glomerular capillary wall has been extensively studied [6, 13]. Under physiological conditions, molecules larger than serum albumin (68 kDa) or with an effective radius of about 35.5 Å are virtually excluded from the filtration process [7]. Cationic antigens larger than OA (42 kDa) and smaller than human Ig M (900 kDa) with pI greater than 8.5 accumulate and persist on the GBM [25, 26]. Our previous study showed that monomeric lysozyme (14 kDa, PI =11) does not accumulate along the glomerular capillary wall, but the tetramer of lysozyme (57 kDa) made by crosslinking binds to the GBM and persists [25]. In the study reported here, we used two carrier proteins, cationized BSA (68 kDa) and cationized OA (42 kDa) with different molecular weights between which there may be the critical size for persistance on the GBM.

Cationized BSA localized exclusively along the glomerular capillary wall, as reported previously [22, 27]. Prior application of the highly cationic and relatively nonimmunogenic molecule, PEI, increased the binding of cationized BSA along the peritubular capillaries in addition to glomerular localization, but did not reach the TBM and Bowman's capsule. PEI has affinity for the anionic sites on the glomerular capillary wall, modulates GBM charge, and may reduce the binding of subsequently injected cationized BSA by a competitive binding to these anionic sites [1]. Our previous study showed that the binding of a cationized human IgG to glomerular anionic sites could be significantly, but not completely, prevented by a prior application of a polycation, protamine sulphate [21]. It is reasonable to assume that competitive binding of PEI allowed cationized BSA to enter the postglomerular flow, leading to localization on the peritubular capillary beds.

The fate of the highly cationized OA injected in monomeric form differed. The molecular weight of monomeric cationized TNP-OA was smaller than the exclusion limit of the GBM (60 kDa) and the pI was larger than 10. Monomeric native TNP-OA localized in the cytoplasm of tubular epithelium in a fine granular pattern. Monomeric cationized TNP-OA was probably filtered through the GBM to the urinary space and was reabsorbed by the tubular epithelial cells, because it was the same size as the native TNP-OA. However, there was a sharp contrast in localization between monomeric native and monomeric cationized TNP-OA 24 h after injection. Cationic TNP-OA was deposited in a linear pattern along the TBM as well as on Bowman's capsule, but did not accumulate along the glomerular capillary wall. As described above, the charge and molecular weight barriers of the GBM may distribute the exogenous antigens with different molecular weights and charges either into postglomerular flow to settle on the peritubular capillary beds or into urinary space to reach Bowman's capsule and the TBM. This possibility was supported by our finding that crude fraction containing cationic TNP-OA of dimeric forms was localized partially along the glomerular capillary wall and the peritubular capillary bed in addition to along the TBM and on Bowman's capsule. We do not know the mechanisms responsible for this deposition. Further study on the charge- and size-dependent handling by tubular epithelial cells, Bowman's capsule, and the TBM is needed.

In human TIN, the major tubulointerstitial lesions are located either on the peritubular capillaries or the TBM [17, 24]. The former type of TIN caused by bacterial infection arises in the interstitium by way of the peritubular vascular system [23, 31] and the latter type first presents with degeneration of the tubular epithelium and inflammation of the outer and inner side of the TBM as seen in drug-induced hypersensitivity nephritis [11]. We succeeded in localizing or planting haptenic TNP exclu-

sively on the TBM and Bowman's capsule by using cationic OA as a carrier protein. The pattern of the deposition was the same as the distribution of endogenous antigen expression in animals having TIN produced by immunization with nonautologous TBM antigen. Because cationic TNP-OA remained along the TBM and Bowman's capsule up to 36 h after injection, such haptenic groups may serve as targets for humoral and/or cellular immunity, as shown in the experimental glomerulonephritis models [22]. The present study provides some clues to clarify whether a humoral or a cellular immune response to a hapten is necessary for the induction of a tubulointerstitial lesion.

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