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Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice

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Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice. We investigated the capacity of five human monoclonal IgG anti-DNA antibodies derived from lupus patients to produce glomerular immune deposits. The hybridomas secreting these antibodies were administered intraperitoneally to severe combined immunodeficiency (SCID) mice. Three of the five antibodies (B3, 35.21, 33.C9) were detected in the kidneys, but only one (33.C9) deposited exclusively in the glomeruli in the mesangium and capillary wall, whereas the other two antibodies bound to nuclei both in the kidney and in other organs. The antibodies were tested against a variety of autoantigens by ELISA, the only unique feature of 33.C9 was that it also bound strongly to histones. There were no particular amino acid motif that was related to immunoglobulin deposition in the kidney. All the mice that had immunoglobulin deposited in the kidney, either extracellularly or intranuclearly developed 2 to 3+ proteinuria, whereas the other mice had only trace amounts of proteinuria. This study demonstrates that some human monoclonal IgG anti-dsDNA antibodies are capable of binding to the glomerulus while others can penetrate cells and bind to nuclei *in vivo*. Although no abnormal pathology was observed, proteinuria was detected, perhaps representing an early phase of disease. These results indicate that the affinity for dsDNA is not the sole determining factor governing the biological properties of human anti-DNA antibodies *in vivo*.

Anti-DNA antibodies are found in the sera of 50 to 75% of most published series of patients with SLE, but only about 30 to 50% of patients develop renal disease. One report which correlated serum anti-DNA antibodies with disease exacerbations concluded that there was no qualitative change in these antibodies during flares using three different assays, and that anti-DNA antibodies were an epiphenomenon not directly linked to pathogenesis [1]. In contrast, the association between anti-DNA antibodies and renal disease appears much stronger in those murine models of SLE where the relationship between anti-DNA antibodies and nephritis has been studied most extensively. In one study almost 100% of the lupus prone mice developed nephritis and had anti-DNA antibodies [2]. Similarly, whereas there is little direct evidence that anti-DNA antibodies play a role in human lupus nephritis, in elegant experiments using mouse monoclonal anti-DNA antibodies, Vlahakos et al demonstrated that only a proportion of exogenously administered murine monoclonal anti-DNA antibodies deposited in the glomeruli and induced nephritis in non-autoimmune mice [3]. Studies using human polyclonal affinity-purified anti-DNA antibodies suggest that this specificity is related to the pathogenesis of nephritis [4], but also illustrate the weakness of drawing conclusions from serological studies or using polyclonal preparations in which precise correlations between specificity and function cannot be made. In the case of the human disease where the serological association between the presence of anti-DNA antibodies and nephritis is weaker, there are no data to indicate which, if any, of the anti-DNA antibody population is pathogenic. This study attempts to provide direct evidence for the pathogenic role of human anti-DNA antibodies.

Murine monoclonal IgG anti-DNA antibodies are generated consistently and easily from lupus mice with nephritis. We have previously shown that the equivalent human antibodies have been produced from only a minority of patients with active renal disease [5, 6]. This may be due to the technical difficulties with human hybridoma technology but, alternatively, may be a reflection of the different role anti-DNA antibodies play in the human disease and in the animal model.

It is not possible to analyze the effects of these monoclonal antibodies in normal individuals. Therefore we have made use of the severe combined immunodeficiency (SCID) mouse as the non-autoimmune recipient to study the effects of human anti-DNA antibodies *in vivo*. This strain of mouse will not reject foreign cells and has been used for ten years to support the growth of monoclonal antibodies from different species [7, 8]. We have injected the hybridomas secreting the human IgG anti-DNA antibodies into SCID mice to observe and document their binding capacity *in vivo* and any ensuing pathological effects.

Methods

Animals

Nine-week-old CB-17-scid/scid (SCID) mice were purchased from Charles River (Margate, Kent, UK). The SCID mice were kept in sterile boxes covered by a filter and fed sterile water and food without any antibiotics. The mice were bled before the experiment to check for the presence of endogenous murine antibodies (leakiness). Only those with no detectable murine immunoglobulin were used for this study.

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Table 1. The isotype and binding properties of monoclonal IgG anti-DNA antibodies derived from two SLE patients

Clone	Isotype	Anti-DNA reactivity ELISA: ssDNA	dsDNA	Crithidia	Farr
B3	IgG1, lambda	+	++	+	Not tested
D5	IgG1, kappa	++	+	−	Not tested
32.B9	IgG3, lambda	+	++	+	+
33.C9	IgG2, kappa	+	++	+	−
35.21	IgG2, lambda	−	++	+	+

Clone	Nucleosomes	Histones	Heparan sulphate	Chondroitin sulphate	Laminin	Collagen type IV
B3	++	+	−	−	−	−
D5	+	−	−	−	−	−
32.B9	++	−	−	−	−	−
33.C9	++	++	−	−	−	−
35.21	+	−	−	−	−	−

Each antibody was titrated to each antigen starting at 1 μg/ml. Scale ++ represents OD > 1 at 250 ng/ml, + OD > 0.25 at 1 μg/ml and − for OD < (blank + 3 SD) at 1 μg/ml. No difference was found in the binding to the antigens when the supernatant was treated with DNAase except for the binding of B3 to histones which was abolished when DNAase was added to the supernatant. Further information on the binding of 32.B9, 33.C9 and 35.21 to other antigens (and their affinities to DNA) can be found in [9]. The affinities of B3 and D5 to DNA can be found in [10].

Table 2. Time to development of ascites (increase of weight by 20%), serum and ascites immunoglobulin concentration and proteinuria as recorded by Albustix® (Bayer Diagnostics, Berkshire, UK) for the mice that received the hybridomas secreting anti-DNA antibodies detailed in Table 1.

Number of mice	Hybridoma	Time to ascites *days*	Human Ig ascites mean ± SD, *μg/ml*	Human Ig serum mean ± SD, *μg/ml*	Proteinuria (dipstick) *mean/range*
6	B3	45 ± 10	122 ± 56	124 ± 50	2.4/2-3+
6	D5	37 ± 11	15 ± 6	14 ± 5	0.2/Trace-1+
5	33.21	30 ± 5	556 ± 181	612 ± 174	2.2/2-3+
6	33.C9	41 ± 8	386 ± 121	314 ± 104	2.4/2-3+
5	32.B9	29 ± 7	409 ± 92	422 ± 77	0.8/1-2+
4	CB-F7	28 ± 8	0	0	Trace
4	—	—	0	0	Trace

Production of human monoclonal IgG anti-DNA antibodies

Five human monoclonal IgG anti-DNA antibodies were produced from two patients with active SLE by fusion of the patient's lymphocytes with the heteromyeloma cell line CB/F7 as described previously [5, 6]. One of these patients had active nephritis, the other active arthritis. The designation of these antibodies and their characteristics are given in Table 1. The B3 and D5 monoclonal antibodies (derived from the patient with active arthritis) were purified from hybridoma supernatant by affinity chromatography using a protein G column (Pharmacia, Hertfordshire, UK) before they were injected intravenously.

Administration of human hybridomas and purified mAb to SCID mice

The SCID mice were treated with 0.5 ml sterile pristane [4,6,10,14-tetramethylpentadecane] (Sigma, Poole, Dorset) 10 days prior to intraperitoneal (i.p.) injection of hybridoma cells. Hybridoma cells producing the monoclonal antibodies were harvested from midlog phase cultures and resuspended in 0.5 ml RPMI so that the total number of cells was 1×10^6 cells. The original cell lines had been subcloned three times, and were recloned once immediately prior to injection. The cells were injected into the peritoneum under sterile conditions using a 21 gauge needle. Five or six mice were used for each antibody (injected at different times, with different batches of the same cell line) and four uninjected pristane primed mice and four mice injected with the non-secreting fusion partner cell line were used as controls.

Ascites developed in all the mice injected with cells. The mice were sacrificed either when their body wt increased by 20% due to the ascites, or if the mice appeared unwell. The urine was checked for proteinuria using a dipstick (Albustix) and a blood sample was taken. The peritoneum was opened and the ascites collected. The kidney, spleen, liver and skin were sampled and part was snap frozen or fixed in 4% formaldehyde. The ascites and serum were assayed for the presence of human immunoglobulin and anti-DNA activity by ELISA as described previously [9, 10].

Intravenous injection of the purified antibody into SCID mice: 1 mg of purified monoclonal antibody (B3 or D5) in 0.2 ml of PBS was injected into the tail vein of the mouse (4 mice/antibody). Each mouse was sacrificed six hours after the injection and the same organs were taken as described above. One milligram of purified human IgG antibodies (Sigma) was injected as a control.

ELISAs for the analysis of polyreactivity

Anti-histone activity was detected using an ELISA method as described elsewhere [11]. Briefly, histone type IIS (Sigma) was coated overnight at a concentration of 10 μg/ml at 4°C in PBS. The plate was then blocked with 2% casein and any DNA contaminating the histone was removed by treatment with

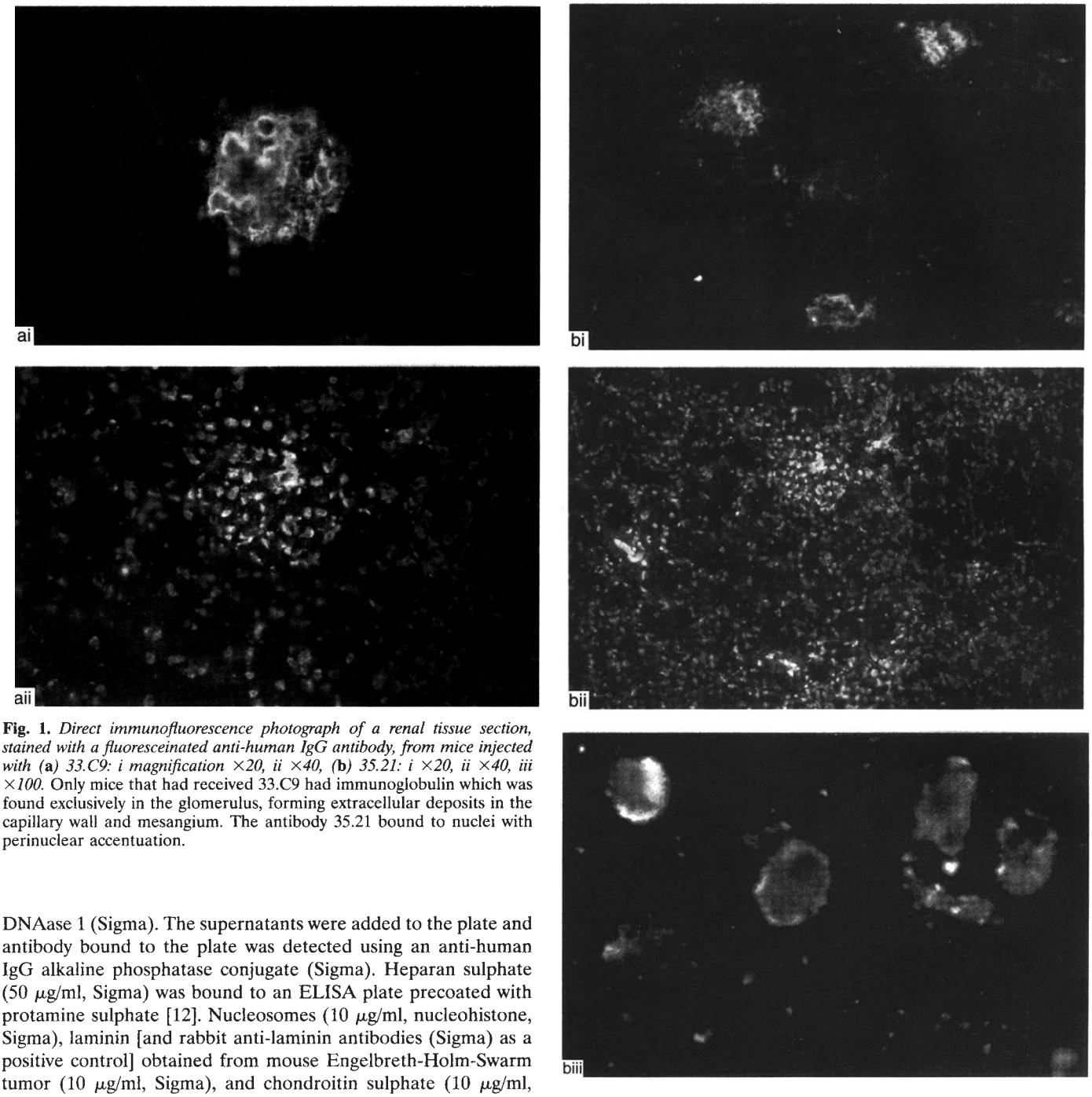

Fig. 1. *Direct immunofluorescence photograph of a renal tissue section, stained with a fluoresceinated anti-human IgG antibody, from mice injected with* (**a**) *33.C9: i magnification ×20, ii ×40,* (**b**) *35.21: i ×20, ii ×40, iii ×100.* Only mice that had received 33.C9 had immunoglobulin which was found exclusively in the glomerulus, forming extracellular deposits in the capillary wall and mesangium. The antibody 35.21 bound to nuclei with perinuclear accentuation.

DNAase 1 (Sigma). The supernatants were added to the plate and antibody bound to the plate was detected using an anti-human IgG alkaline phosphatase conjugate (Sigma). Heparan sulphate (50 μg/ml, Sigma) was bound to an ELISA plate precoated with protamine sulphate [12]. Nucleosomes (10 μg/ml, nucleohistone, Sigma), laminin [and rabbit anti-laminin antibodies (Sigma) as a positive control] obtained from mouse Engelbreth-Holm-Swarm tumor (10 μg/ml, Sigma), and chondroitin sulphate (10 μg/ml, Sigma) were directly bound to the plate diluted in PBS. The plates were then blocked with 2% casein and after the supernatants were added, antibody bound was detected as for the histone ELISA. Each supernatant was tested for reactivity with an antigen uncoated well in the same plate (or in the case of heparan sulphate, protamine sulphate) and any binding was subtracted against the binding to the test antigen. The supernatants were also tested for antiglomerular basement membrane activity using an ELISA kit which utilizes the M2 subunit from type IV collagen as substrate (Bio-diagnostics, Upton, Warwickshire, UK). Antibody containing supernatants were tested with or without pretreatment with DNAase 1 as previously described [6].

Immunohistochemical studies

Human immunoglobulin was detected on frozen sections using goat anti-human IgG fluoresceinated conjugate (1:50; Southern Biotechnology, Birmingham, AL, USA) which was incubated on the slides for 30 minutes at room temperature. In addition, counterstaining with propidium iodide was performed on sections stained with the goat anti-human IgG fluoresceinated conjugate, derived from mice that had received hybridomas secreting 35.21, and viewed using a 630 nm wavelength filter to demonstrate

nuclear localization. The monoclonal antibodies were also incubated *in vitro* on sections of untreated SCID mice and binding was detected using the same conjugate as above. The slides were washed in phosphate buffered saline and viewed under UV light (filter set at 530 nm) using an MRC 600 scanning system connected to image analysis software (BioRad, Hertfordshire, UK) or using a Nikon microscope attached to a camera. The formalin fixed material was paraffin embedded for light microscopy and epoxy resin embedded for transmission electron microscopy. The paraffin sections were stained in routine fashion with hematoxylin and eosin.

Results

The binding of the antibodies to DNA and other autoantigens is shown in Table 1. All the antibodies bound to DNA and nucleosomes with varying affinities, 33.C9 bound strongly to histones. The antibodies did not bind to any other antigen tested. Injection of the hybridomas intraperitoneally into the SCID mice resulted in ascites in all cases. The ascites became clinically apparent between three to seven weeks after administration. The human immunoglobulin levels in the ascites and serum were similar in individual mice. There was a wide range of immunoglobulin levels between the mice that had received different cell lines. Table 2 indicates the time to ascites formation, the serum and peritoneal immunoglobulin level and the degree of proteinuria.

Mice that had received three of the five antibodies (B3, 35.21, 33.C9) showed evidence of immunoglobulin deposition in tissue. All the mice that had received the same antibody showed similar staining patterns (5 or 6 mice per antibody). One antibody, 33.C9, localized exclusively to the glomeruli forming extracellular deposits in the capillary wall and mesangium (Fig. 1a). 33.C9 was not found in any of the other tissues examined. Two antibodies (B3 and 35.21) showed binding to nuclear structures with accentuation of IgG deposition around the nuclear envelope. All the antibodies were able to bind to the nuclei when they were incubated *in vitro* on fixed kidney sections from untreated SCID mice (data not shown), but only two exhibited this pattern *in vivo* indicating the specificity of the phenomenon. Figure 1b shows the distribution of antibody 35.21 in the kidney, the high power view reveals some heterogeneity in nuclear binding with accentuation of the perinuclear areas. These two antibodies also stained cell nuclei at other sites, such as liver, spleen and skin. Propidium iodide, which binds to nuclei, was used as a counterstain on sections derived from mice that had received the hybridoma secreting 35.21 already stained with an anti-human IgG fluoresceinated conjugate. The propidium iodide produced a similar pattern to that seen for immunoglobulin distribution indicating the intranuclear localization of the antibody (Fig. 2). Intravenous injection of B3 gave an identical pattern to that seen with the hybridomas secreting B3. Administration of the monoclonal antibodies 32.B9 or D5 (as hybridomas or intravenously) did not lead to immunoglobulin deposition, either extracellular or intranuclear, in any organ. The architecture of the kidneys (Fig. 3, 33.C9 antibody) and other organs from all the mice appeared normal. Electron microscopy of the kidney sections did not show any electron dense deposits and there was no significant ultrastructural morphological abnormality (data not shown). However, the mice that had antibodies deposited in the kidneys all developed significantly more (between 2 to 3+) proteinuria compared to the mice that had received secreting hybridomas, but did not have immunoglobulin deposited in tissues ($P < 0.001$).

Discussion

This study represents the first attempt to transfer human monoclonal IgG anti-DNA antibodies *in vivo* and to study their effects, particularly in terms of antibody deposition. The SCID mouse provides a unique tool for the analysis of human monoclonal antibodies; by growing antibody secreting hybridomas in SCID mice, high levels of immunoglobulin are achieved over a relatively long period. There was a similar concentration of immunoglobulin in the ascites and the blood, suggesting that the human immunoglobulin was able to penetrate into the blood. Some of the serum immunoglobulin could have derived from hybridoma cells that had migrated to the blood compartment. The immunoglobulin levels varied depending on which antibody was injected but there was no correlation between the immunoglobulin concentration and its presence in tissues.

Only one of the five antibodies (33.C9) bound exclusively to glomeruli. This demonstrates that human IgG anti-DNA antibodies are capable of binding to glomeruli *in vivo*. However, it also demonstrates that not all IgG anti-DNA antibodies are potentially pathogenic and that the affinity to DNA is not correlated to renal deposition. Moreover, whereas three of the antibodies bound well to nucleosomes by ELISA, only one of these deposited in the glomerulus. Thus the concept that an antibody that can bind to nucleosomes will also necessarily bind to glomerular structures is disputed for the human disease. The only difference between 33.C9 and the other clones was its strong binding to histones. Histones can bind to the glomerulus directly via a charge interaction with heparan sulphate and enhance the deposition of antibodies. Recent work has demonstrated that histones present in human and murine SLE kidneys were only detected by the use of antibodies against histones rather than whole nucleosomes [reviewed in 13]. Thus antibodies that bind histones specifically may deposit preferentially in the kidney. It is also possible that an unidentified antigen may be responsible for the binding of 33.C9 to the glomerulus. A mouse monoclonal anti-DNA antibody that binds to the glomerulus was shown to cross-react with a novel protein which had sequence homology to the family of SPARC extracellular matrix proteins [14].

An analysis of the amino acids thought to be important in binding to DNA and histones is shown in Table 3 (data from [15–17]). In contrast to the murine data the small number of human antibodies studied here precludes any conclusions about distinct pathogenic variable genes. The two antibodies that were able to penetrate cells and bind to nuclei *in vivo* are not from the same germ line gene. The only difference between 33.C9 which deposited in the glomerulus and the other antibodies is the smaller number of arginines. In terms of somatic mutation 33.C9 is the only antibody out of the five studied to gain negative charge and lose arginine residues. In contrast, a recent report found that there were a higher number of positively and negatively charged amino acids in the CDRs of the heavy chain of murine pathogenic anti-DNA antibodies compared to non-pathogenic anti-DNA antibodies [18]. Arginines were found to be absent in the CDRs of the heavy chains of these murine non-pathogenic antibodies but present in the pathogens. Arginines are known to be the most versatile amino acid for binding to DNA, whereas negatively-charged amino acids are thought to be important in binding to

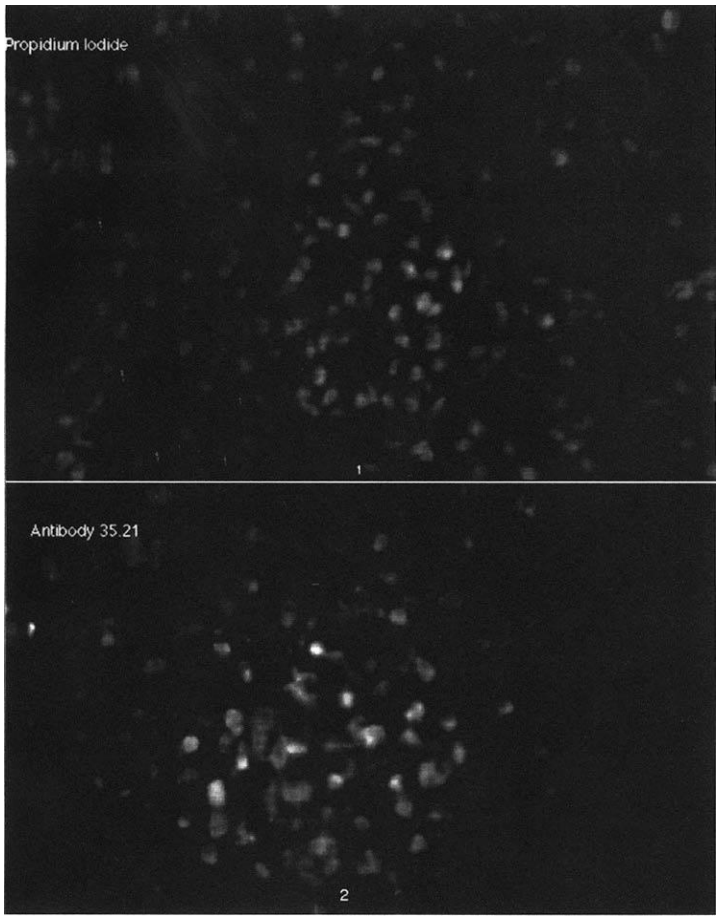

Fig. 2. *A kidney section derived from a mouse that had received hybridoma cells secreting 35.21.* The section has been labeled with both propidium iodide (viewed using a 630 nm filter) which binds to the nucleus, and a fluoresceinated anti-human IgG antibody (viewed using a 530 nm filter), demonstrating that the localization of propidium iodide [1] and of immunoglobulin [2] was similar and suggesting that the antibody bound to nuclear structures (magnification ×40).

histones [19]. Future studies will need to concentrate on which amino acids are accessible for antigen binding and in elucidating the important antigens that determine the exclusive binding to glomeruli.

In contrast to the murine monoclonal anti-DNA antibodies that induced histological evidence of nephritis in normal mice [3], none of the human antibodies used in this study did so. However, significant proteinuria was detected in those mice in which immunoglobulin was deposited. This may be due to the disruption of anionic sites in the basement membrane which can occur without any overt histological changes [20]. Fab fragments of antibodies can induce proteinuria without inducing nephritis, implying a direct toxic effect of the antibodies. The lack of immune complexes may be due to the antibody binding directly to glomerular structures and not forming immune complexes. The absence of nephritis may be explained by the failure of the human antibodies to interact with murine complement [21]. This may be more relevant than the fact that functional B and T cells are not present in the SCID mouse, since, in a recent report, murine monoclonal anti-DNA antibodies that induced nephritis in normal mice also produced an identical picture in SCID mice [22]. Moreover, in the experiments by Raz and coworkers using the isolated rat kidney, only when complement was added to the human polyclonal anti-DNA antibodies was there any significant change in renal function or proteinuria [4].

Two of the antibodies appeared to bind to the nuclei within cells as has been observed with a subset of murine monoclonal antibodies [23]. This phenomenon was unlikely to be due to a staining artifact as only a proportion of the antibodies had this property. The nuclear localization was not cell specific as it occurred in a variety of organs. Since all the antibodies were able to bind to the nucleus in fixed sections of mouse kidney (data not shown), the capacity of the antibody to show intranuclear localization *in vivo* must be related to its binding to cell surface and subsequent internalization. One previous report showed that murine anti-DNA antibodies did bind to cell surface proteins present on cells derived from a number of tissues *in vitro* and that the binding was not changed by the addition of DNAase [24]. Intranuclear localization has been observed in the kidneys and other organs of lupus patients [25, 26], but this is the first report of a human monoclonal antibody directly penetrating cells *in vivo*. The proteinuria associated with this phenomenon could be due to a variety of different causes as discussed for murine monoclonal antibodies [23], perhaps linked to an interaction with a number of intracellular processes.

In summary, this study represents the first attempt to investigate the pathogenicity of human anti-DNA antibodies *in vivo*. One anti-DNA antibody bound exclusively to glomeruli forming extracellular deposits, whereas two others were apparently able to penetrate cells and bind to nuclei in a variety of different organs. None of the mice developed pathological evidence of glomerulonephritis though proteinuria was a feature in some of the mice.

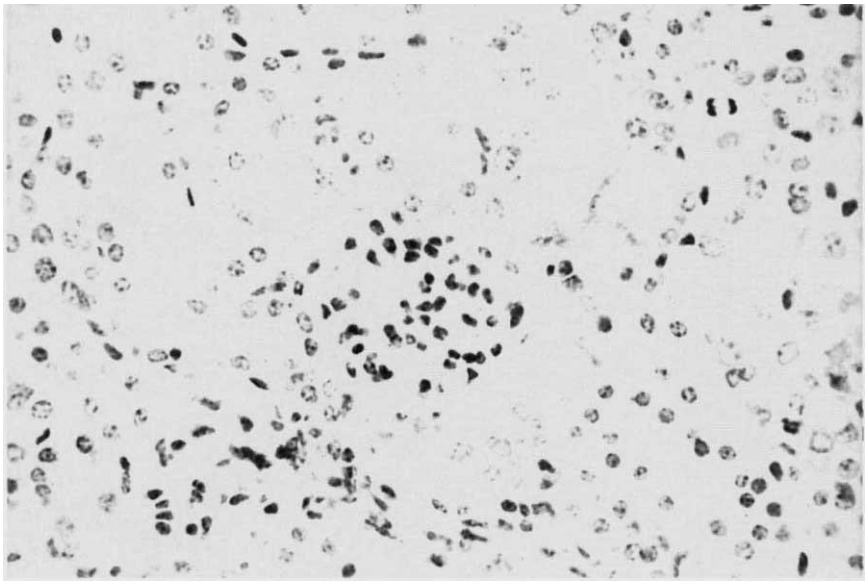

Fig. 3. *A kidney section (stained with hematoxylin and eosin) from a mouse that had received hybridoma cells secreting 33.C9 is shown viewed under light microscopy (×40).* There were no histopathological changes in any of the mice.

Table 3. The number of amino acids thought to be important in binding to DNA and histones in the CDR regions of the various antibodies

	Positive	Negative	Asparagine	Arginine
32.B9	4	1	3	3
B3	5	3	2	5
D5	6	1	5	4
35.21	5	2	3	4
33.C9	5	2	2	1

The amino acids are categorized into negatively and positively charged, and the number of asparagines (neutral) and arginines (positive) are shown individually. Note that the light chain sequence of 35.21 has not been determined. Sequence data are from [15–17].

These results indicate the importance of utilizing monoclonal antibodies in determining specificity-function relationships, and that the affinity for dsDNA is not the sole determining factor governing the biological properties of these antibodies *in vivo*.

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