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The treatment of systemic lupus erythematosus (SLE) in NZB/W F_1 hybrid mice; studies with recombinant murine DNase and with dexamethasone

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SUMMARY

The effects of recombinant mouse DNase on a well established murine model of spontaneous SLE have been evaluated. Daily intraperitoneal injections of DNase were given to female NZB/NZW F_1 mice during the period of disease development from 4 to 7 months of age or at the height of disease activity from the age of 7 months for 3 weeks. This treatment was compared with the injections of diluent and with an immunosuppressive dose of dexamethasone. The effects of treatment were evaluated using the immunological parameters of disease activity (antinucleoprotein antibody, immune complexes, serum immunoglobulins, anti-cardiolipin antibodies), proteinuria, serum creatinine and renal histopathology (light microscopy, immunofluorescence and electron microscopy). The dose of dexamethasone used (1 mg/kg per day from the age of 4 months) was sufficient to suppress the development of lupus entirely. Treatment with DNase starting at the age of 4 months postponed the development of the disease by about 1 month and extended the period from the onset of disease to death by about 30%. Mice treated for 3 weeks during the most active phase of the disease at 7 months of age showed more dramatic effects. Proteinuria and serum creatinine were significantly reduced and renal histopathology was strikingly less severe than in the control group. Immune complexes involving DNA-containing antigens are believed to play a crucial role in the pathogenesis of SLE. DNA-nucleoprotein, even in immune complexes, can be destroyed by DNase. This enzyme therefore provides a rational way to interfere with the disease process. The results reported here encourage a trial of recombinant human DNase in human SLE and lupus nephritis.

Keywords systemic lupus erythematosus DNase dexamethasone immune complexes anti-DNA antibodies NZB/W F_1 mice

INTRODUCTION

The description of the lupus erythematosus (LE) cell by Hargraves *et al*. [1] as a characteristic laboratory finding in SLE precipitated the discovery that this disease was autoimmune in nature and was the beginning of the modern era in the study of autoimmune phenomena. Haserick *et al*. [2] showed that LE cells could be formed by incubating normal bone marrow with lupus serum and Miescher & Fauconnet [3] demonstrated that the capacity of SLE sera to form LE cells could be absorbed out with cell nuclei and concluded that an antinuclear antibody produced the reaction. Thereafter, it was rapidly shown that lupus sera contained many antinuclear antibodies, including the initially surprising finding that there were antibodies to DNA itself [4–6]. the LE cell factor itself was found to be an antibody to native DNA-nucleoprotein (what would now be called a polynucleosome) [7]. The activity of both pure DNA and of native DNA-nucleoprotein as antigens was destroyed by treatment with DNase 1.

At around the same time it was recognized that immune complexes were likely to play an important pathogenic role in SLE. Dixon *et al*. [8] had shown that immune complexes were responsible for experimental serum sickness, and it was shown as early as 1959 that antinuclear antibodies could be eluted from the glomeruli in the case of lupus nephritis [9]. By 1961, therefore, it was already regarded as entirely plausible that immune complexes involving DNA-containing antigens might be important in SLE [10].

Some years earlier, Johnson *et al*. [11] had injected crystalline bovine pancreatic DNase into patients with bronchiectasis and into normal human volunteers without ill-effect, indeed with clinical benefit in liquifying sputum. It was subsequently given intrathecally in pneumococcal meningitis [12] and subcutaneously in gout [13]. For these reasons, a trial of bovine pancreatic DNase given subcutaneously in human SLE was carried out in the early 1960s [10,14]. The initial results were encouraging, there being some

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clinical response and, over a number of weeks, the loss of antibodies to DNA-containing antigens. Unfortunately, the enzyme proved itself to be antigenic and after a few weeks of administration gave rise to antibody formation and to Arthus reactions; and the treatment therefore had to be discontinued. Only eight patients in all were treated, of whom four had clinical improvement, two dubious clinical improvement and two patients with only lupus nephritis showed no improvement.

In recent years the importance of the nucleosome as an antigen in SLE has come to the fore again. It has been recognized that nucleosomes are released from cells undergoing apoptosis [15]; that there are nucleosomes present in the blood [16,17] and that there are receptors for nucleosomes on cells and in the kidney [18–20]. It has been demonstrated that nucleosomes bound to the kidney basement membrane and there reacting with antibodies give rise to glomerulonephritis [21–23]. The thinking underlying the experiments in the early 1960s has therefore been fully justified by subsequent findings.

Since the 1960s it has also become clear that homozygous deficiencies of the early components of the classical complement pathway C1 and C4, and to a lesser extent C2, are a powerful genetic predisposing cause for the development of SLE in man. Indeed, deficiencies of C1q, C1r and C1s and C4 are close to being sufficient causes for the development of SLE before middle life [24]. A pathogenic scheme has been proposed to explain the development of SLE on the basis that the essential abnormality is a failure to handle immune complexes properly. Instead of their permanent and effective removal in the reticuloendothelial system, especially the liver, the immune complexes are removed across endothelia, where they give rise to endothelial cell activation and inflammatory reactions which will in turn give rise to feedback antibody formation to nucleosomes (and splicosomes) which are so typical of SLE. There is substantial evidence to support such a schema. Studies of a C2-deficient lupus patient before and after reconstitution of her complement with fresh frozen plasma [25] clearly demonstrated the abnormality of immune complex handling that is associated with complement deficiency and its correction by the restoration of normal complement function. The activation of endothelial cells with C5a in lupus has been demonstrated by Belmont *et al.* [26].

Three implications of this scheme are important when considering the treatment of SLE with DNase:

1 It implies that the antibody response in SLE is antigen-driven. Evidence for this can be obtained from a variety of sources. The antibody response in SLE is a mature polyclonal response with antibodies of multiple IgG subclasses; and where antibodies have been sequenced they show mutation from the germ-line sequence. The observation that antibodies are found both to the native nucleosome structure and to all its components, DNA, histone and non-histone proteins, defies explanation except on the basis of antigen drive. The earlier studies on treating patients with DNase also demonstrated a loss specifically in antibodies to DNA-containing antigens [10].
2 The requirement in the pathogenic scheme that the initial formation of these antibodies is not intrinsically abnormal, and indeed it is known that low titres of antinuclear antibodies can be made following various forms of tissue damage [27].
3 Finally, the role of actin acting as a DNase inhibitor is postulated as being of importance in allowing the persistence of nucleosomes at inflammatory sites sufficiently long for them to be immunogenic. The presence of heat-labile DNase inhibitor (which was shown to be actin by Lazarides & Lindberg [28]) in the serum of patients with SLE [28a] and of New Zealand black/white hybrid mice [29] when they develop antinuclear antibodies has previously been documented and it has been shown that this material is likely to derive from the lysis of platelets. Inhibition of DNase is a property of G actin and requires ATP. Not all species of DNase are inhibited by actin. Mouse DNase and human DNase are inhibited, whereas rat DNase is not [30]. The possibility that this correlates with the capacity of mice and men, but not rats, to develop SLE, is an attractive speculation.

This work was taken no further until the cloning of human DNase 1 at Genentech [31] gave rise to the opportunity of re-investigating the phenomenon. However, it was felt that before embarking on any further clinical studies it would be wise to try DNase in a model of lupus in experimental animals. For this purpose the well established murine model of spontaneous SLE occurring in female NZB/NZW F_1 hybrid mice was used and mouse DNase 1 was cloned and expressed at Genentech especially for these experiments—which consumed all the available enzyme.

MATERIALS AND METHODS

The time of appearance and changes in the concentration of serum anti-DNA antibodies with age in NZB/W F_1 hybrid mice were measured in a series of preliminary experiments.

Two separate experiments on the treatment of murine lupus in NZB/NZW F_1 mice with DNase have been performed. In the first experiment the DNase was given from the age of 4 months—when antinuclear antibodies first begin to appear—and continued for 9 months. In the second experiment DNase was given from the age of 7 months, i.e. at the height of the anti-DNA antibody response; the injections were given for 3 weeks.

Treatment of murine lupus in NZB/W F_1 hybrid mice with DNase from the age of 4 months

Female New Zealand White × New Zealand Black F_1 hybrids of 4 months of age and 30 g in weight were used. The experimental mice were injected intraperitoneally daily with diluent ($n = 14$), DNase ($n = 14$) or dexamethasone ($n = 14$) from the age of 4 months until the end of the experiment, as follows: diluent, an aqueous solution containing 0·15 mg/ml $CaCl_2$ and 8·77 mg/ml NaCl; 0·3 ml was injected to each mouse; murine DNase (MuDNase), at concentration 0·5 mg/ml; 150 μg (0·3 ml) was given on each occasion. This corresponds to approximately 7·5 mg/kg; dexamethasone (dexamethasone sodium phosphate) at 0·1 mg/ml solution in sterile saline; 30 μg (0·3 ml) was given on each occasion. This corresponds approximately to 1 mg/kg.

Before the start of the injections (age 4 months) and at monthly intervals (5, 6 and 7 months of age) all animals were bled by cardiac puncture (T0, T1, T2 and T3). Approximately 0·2 ml of blood was taken, left to clot at room temperature for 2 h, centrifuged at 3000 rev/min for 5 min, and the serum collected and stored at -70°C.

Animals that died as a result of cardiac puncture were autopsied. The remaining animals were followed until death and post-mortem examination. The animals still alive at the age of 12 months were killed and autopsied. The following organs and tissues were taken for light, fluorescence and electron microscopy: kidneys, lungs, liver, spleen, brain, heart, joints and lymph nodes.

Measurements of DNase activity were performed by two techniques: end-labelled oligonucleotide assay and single radial enzyme diffusion method.

End-labelled oligonucleotide assay. DNase activity in murine serum was determined with a synthetic oligonucleotide substrate. Two complementary 25 base pair oligonucleotides were synthesized with different labels on their 5′ ends. One oligonucleotide was labelled with biotin and the other was labelled with dinitrophenol. Hybridization of the two strands produced a double-stranded substrate for DNase with different labels on each end.

For pharmacodynamic experiments, 1 μl of recombinant mouse DNase (rmDNase) at varying concentrations and 1 μl of double-stranded oligonucleotide substrate at a concentration of 8 μg/ml were mixed with 18 μl of undiluted serum samples in microcentrifuge tubes. The mixture was incubated at room temperature for 1 h. Limited digestion of the oligonucleotide substrate by DNase produced a mixture of reaction products that were labelled at either one or both ends. After stopping the reactions by addition of 1 μl of 0·5 M EDTA, the concentration of unhydrolysed substrate was determined by an ELISA. Briefly, microtitre plates were coated with affinity-purified antibody to dinitrophenol. After blocking the plates, samples or standards containing known concentrations of the substrate were incubated in the wells of the plates for 2 h at room temperature with agitation. The buffer used to dilute the samples in microtitre plates contained 25 mM HEPES, 1 mg/ml bovine serum albumin (BSA), 0·05% Polysorbate 20, 0·01% Thimerosal pH 7·5. After washing the wells, a streptavidin–horseradish peroxidase (HRP) conjugate was added and incubated for 2 h at room temperature with agitation. The wells were washed again before addition of a chromogenic substrate (orthophenylenediamine) for HRP. The peroxidase reaction was stopped after 8–12 min of incubation at room temperature by the addition of 4·5 N H_2SO_4 and the optical density (OD) of the solution in each well was measured at 492–405 nm. Oligonucleotide standards between 0·1 and 25 ng/ml typically produced standard curves with ODs that ranged between 0 and 2 absorbance units. Standard curves were generated by fitting the data to a four-parameter logistic function and the concentration of unhydrolysed substrate in samples was determined by interpolation from the curves. The concentration of hydrolysed substrate was calculated by subtraction from the total concentration of oligonucleotide added to the serum samples.

For pharmacokinetic experiments, DNase concentration was estimated using the activity assay described above. Serial dilutions of the serum samples were prepared in 25 mM HEPES, 2·5 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml BSA, 0·5% Polysorbate 20, 0·01% Thimerosal pH 7·5 before addition of the end-labelled oligonucleotide substrate. Standards of rmDNase from 0·16 to 10 ng/ml were prepared in the same buffer. The remainder of the procedure was identical to the assay described for determination of DNase activity.

Measurement of DNase activity in mouse serum by single radial enzyme diffusion method. Calf thymus DNA (953 μl) dissolved in distilled water at 5 mg/ml, 47 μl ethidium bromide dissolved in distilled water at 10 mg/ml, 8·96 ml 0·05 M Tris–HCl pH 7·2 with 0·05 M $MgCl_2$ and 40 μl 1 M $CaCl_2$ were added to 10 ml boiled 2% agarose (ultra pure, electrophoresis grade, high melting point, dissolved in distilled water). Plates (10 × 10 cm) were poured giving a 2 mm thick final gel. Wells (1 mm diameter) were cut and filled with 2 μl of the samples. Samples: (i) standards: double dilutions of murine DNase from 500 ng/ml to 15·6 ng/ml; (ii) 2 μl of serum. These were incubated for 15 h at 37°C, overlaid with 0·1 M EDTA and photographed on an UV transilluminator (UVP Inc, San Gabriel, CA). The dark circular area of hydrolysed DNA was scanned on an Optomax Image analyser (UV micromeasurements) and a standard curve constructed by plotting the log DNase concentration against the log value of hydrolysed DNA area using a Microplate manager computer program. Controls: (i) 0·05 M EDTA instead of Ca and Mg in Tris buffer; (ii) rabbit G-actin 5·5–0·087 mg/ml pre-incubated with DNase at 0·5 μg/ml in Tris/Ca/Mg buffer containing ATP 0·001 M; (iii) anti-DNA antibody-positive NZB/W mouse serum followed by DNase standards.

Anti-DNA-nucleoprotein antibody tests. Anti-nucleoprotein antibodies were measured by indirect immunofluorescence and anti-DNA ELISA. Preliminary tests to demonstrate the presence of antinuclear and anti-DNA antibodies in the sera of NZB/NZW F_1 hybrids were done by immunofluorescence assay using HEP-2 cells and *Crithidia lucidae* as antigens, respectively.

Antinuclear factor (ANF) was measured by indirect immunofluorescence. Cryostat sections of rat liver (5 μm) were air-dried, fixed in cold acetone, washed and incubated with test sera diluted 1 : 5 for 60 min. After further washings, the slides were incubated for 60 min with FITC anti-mouse IgG + IgA + IgM (diluted 1 : 20), washed again and mounted in 90% glycerol. Antinuclear antibodies were scored on a scale from 0 (no fluorescence) to 4 (very strong fluorescence).

Anti-double stranded (ds)DNA was measured by ELISA. Calf thymus DNA was coated onto ELISA plates (100 μl/well) at 5 μg/ml concentration in 1 mM EDTA/PBS. After overnight incubation the plates were blocked with 3% BSA for 30 min at room temperature. The plates were then washed × 3 with 1 mM EDTA/PBS/0·05% Tween 20. Mouse sera were diluted 1 : 200 in washing buffer (urines for urinary anti-DNA diluted 1 : 2) and incubated for 60 min at room temperature. After washing, the plates were incubated with peroxidase-labelled goat anti-mouse IgG (1 : 1000 dilution), washed again and then revealed with 2,2′-azinobis(3-ethylbenzthizolinesulfonic acid) (ABTS) in ABTS buffer + H_2O_2. The plates were read at dual wavelength measurements, 405 and 490 nm in a BioRad plate reader (Model 3550; Hemel Hempstead, UK). The results were expressed as units equal to 100 multiplied by the ratio of the sample absorbance divided by the absorbance obtained with a pool of 9-month-old NZB/W F_1 sera.

Serum immune complexes (IC) were measured by conglutinin binding in an ELISA assay. Bovine conglutinin at a concentration of 2 μg/ml in 0·0125 M borate buffer saline + 0·01 M $CaCl_2$ (BBS + Ca) was coated onto ELISA plates (100 μl/well). After overnight incubation the plates were blocked with 2% BSA for 30 min at room temperature. The plates were then washed × 3 with BBS + Ca/0·05% Tween 20. Mouse sera were diluted 1 : 100 in BBS + Ca/0·05% Tween 20 and incubated for 60 min at room temperature. After washing, the plates were incubated with peroxidase-labelled goat anti-mouse IgG (1 : 1000 dilution), washed again and then developed with ABTS in ABTS buffer + H_2O_2. The plates were read at 405 and 490 nm in a BioRad plate reader. The results were read from a standard curve made with serial dilutions of alexinated human aggregated IgG starting from 100 mg/ml. Bound aggregated human IgG was measured using lactoperoxidase conjugated anti-human IgG.

Anti-cardiolipin (CL) ELISA was carried out using an analogous technique. In plate of conglutinin, the plates were coated with cardiolipin (Sigma, Poole, UK) at concentration of 12·5 μg/ml in absolute alcohol and allowed to evaporate overnight. The results were expressed as percentage of a strongly positive serum pool from 9·2-month-old NZB/W F_1 hybrids.

Anti-rmDNase ELISA was carried out using an analogous technique. The plates were coated with rmDNase at 0·5 μg/ml. Antibody titre was defined as the logarithm of the serum dilution for which the absorbance was equal to twice the absorbance of a 100-fold dilution of the negative control. Titre values $<2{\cdot}0$ were defined as negative since all samples were minimally diluted 100-fold.

Proteinuria was measured using Pierce BCA Protein Assay (Rockford, IL).

Serum IgG and urinary IgG were measured by ELISA. The assays were analogous to the conglutinin binding ELISA. Plates were coated with anti-mouse IgG at 2 μg/ml. Sera were tested at dilutions of 1 : 10 000 and urine, 1 : 100. Mouse IgG was used as a standard.

Histopathology

Light microscopy. Standard stains (haematoxylin and eosin, periodic acid-Schiff, Jones's methanamine silver) were done on paraffin sections of the kidneys, liver, spleen, lungs, brain, joints and lymph nodes. The changes were scored from no significant abnormalities (NSA) to severe.

Direct immunofluorescence. Cryostat sections of the kidneys were incubated with FITC-conjugated anti-mouse IgG and anti-mouse C3. The intensity of immune deposits was scored from 0 (no deposits) to 4.

Evaluations were done by a pathologist who was unaware of the treatment group from which samples were obtained.

Treatment of murine lupus in NZB/W F_1 hybrid mice with DNase from the age of 7 months

In the second experiment a different protocol was used. Two groups (10 NZB/W F_1 hybrids, 28 weeks old in each group) were treated with DNase (dosage identical to the previous experiment) or with diluent daily for 3 weeks. Animals were bled before treatment (0·2 ml blood taken by cardiac puncture) and again, after 3 weeks of therapy. The following tests were performed: serum anti-DNA antibody, serum IgG and serum creatinine. Proteinuria was tested at weekly intervals. After 3 weeks all the animals were killed and their kidneys were removed and examined by light and fluorescence microscopy.

Statistical analysis

Statistical analysis was done by StatView statistical analysis program.

Experiment 1. To investigate possible survival differences among treatment groups, the log-rank test for homogeneity among the three treatment groups was performed on days from the start of the treatment until death. Kaplan–Meier survival curves were plotted for each treatment group. The Cochran–Mantel–Haenszel ANOVA statistic was calculated at each time point to test for differences in serum ANF titre among treatment groups.

Experiment 2. A two-sided *t*-test was performed to test for the difference between diluent- and DNase-treated animals for serum creatinine after 3 weeks of treatment. The Cochran–Mantel–Haenszel ANOVA statistic was calculated to test for a difference in the intensity of histological lesions between groups.

RESULTS

NZB/W F_1 hybrids develop anti-DNA antibodies after the age of 3 months, with the peak of anti-DNA antibody response occurring at 7 months. Subsequently, the serum concentration of anti-DNA antibody declines—presumably in consequence of progressive uraemia.

Treatment of murine lupus in NZB/W F_1 hybrid mice with DNase from the age of 4 months

Pharmacokinetics and pharmacodynamics of rmDNase in mice. The pharmacodynamics of rmDNase was studied in murine serum (Fig. 1) using the end-labelled oligonucleotide assay. These data indicate that rmDNase concentrations between 0·1 and 1 μg/ml were necessary to produce detectable nuclease activity in serum. The absence of detectable nuclease activity at lower concentrations is consistent with earlier reports on the existence of DNase inhibitors in plasma [28].

The pharmacokinetics of rmDNase following i.v. and i.p. dosing were studied in male New Zealand White mice. The mice were given rmDNase at 0·5 mg/kg and 5 mg/kg for i.v. dosing and at 5 mg/kg for i.p. dosing. The mice were bled up to 1–480 min after dosing and the concentration of rmDNase in serum was determined by the end-labelled oligonucleotide assay. A bioexponential model was fitted to rmDNase serum concentration *versus* time data following i.v. administration, giving an initial half-life of approximately 4·5 min and a terminal half-life of approximately 105 min. Following i.p. administration at 5 mg/kg, rmDNase levels in excess of 1 μg/ml were maintained for approximately 5 h (Fig. 2). The bioavailability of rmDNase, estimated from these data using a truncated under the curve algorithm, was 44·5%.

Measurement of DNase concentrations in mouse serum by radial enzyme diffusion method. Concentrations of serum DNase in the range 15–500 ng could be easily measured by the radial enzyme diffusion method. Hydrolysis of DNA by DNase was inhibited by G-actin or when EDTA was used instead of Ca and Mg in Tris buffer. DNA hydrolysis was not affected when anti-DNA-positive NZB/W serum was added to wells before DNase.

Six individual normal mice were injected intraperitoneally with 150 μg DNase and bled 24 h after injection. The active enzyme was detected in blood 24 h after injection.

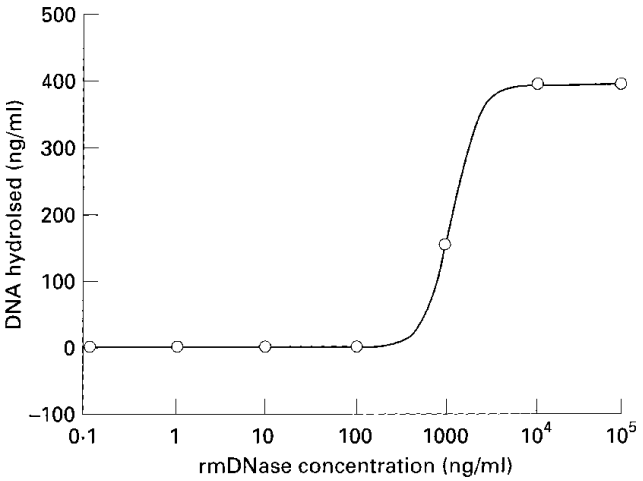

Fig. 1. The pharmacodynamics of rmDNase in male NZW mice using the end-labelled oligonucleotide assay. rmDNase concentrations between 0·1 and 1 μg/ml are necessary to produce detectable nuclease activity in the serum.

Mortality

Survival curves are shown in Fig. 3. Those animals which died due to cardiac puncture were excluded (eight animals in diluent group, six DNase and nine dexamethasone treated). It can be seen that the animals treated with 30 μg of dexamethasone daily survived throughout the period of observation with no deaths other than those attributable to cardiac puncture. The control mice all died during the period of observation and 50% of them were dead at 250 days. While most of the DNase-treated mice also died, their survival was prolonged, 50% of them surviving at about 280 days, i.e. a month later than controls. The first serological manifestations of the disease occurred at about 5 months (150 days), so the mean survival in the control mice was about 100 days from the onset while that of the DNase-treated mice was 130 days, an increase of 30%. There was therefore a substantial slowing of the progress of the disease. Statistical analysis of Kaplan–Meier survival curves showed that DNase and diluent group differed significantly ($P < 0{\cdot}04$).

Serological findings

Antinuclear antibodies. These were measured monthly and were scored by one observer on a scale from 0 (no fluorescence) to 4 (very strong fluorescence). The results are shown in Fig. 4a. This shows that the development of a positive ANF is much slower in the DNase-treated mice, their medium titre at 7 months being lower than that of the diluent group at 6 months.

Anti-dsDNA antibodies. These are shown in Fig. 4b. Even though these levels were measured quantitatively, their distribution was not normal and, for this reason, medians have been given rather than means. It can be seen that anti-DNA levels rose progressively as the disease developed, and again the DNase-treated animals acquired raised anti-DNA titres approximately 1 month after the diluent animals. No significant rise was seen in steroid-treated animals.

By contrast, antibody levels to a non-DNA-containing antigen, cardiolipin, are shown in Fig. 5a. Rises in antibody levels to cardiolipin were smaller than those to DNA, the peak value being little more than twice the normal level, and these levels tended to fall off later in the disease. However, there was no significant difference between the DNase-treated group and the diluent-treated group. The steroid-treated group again failed to show any rise. Similarly, total serum IgG levels rose during the disease (Fig. 5b) and showed the same response pattern as that seen with the anti-cardiolipin antibodies. The rise was similar in the control and DNase-treated groups, and the level in the dexamethasone-treated group did not rise at all.

Immune complex levels in serum are shown in Fig. 4c. All the NZB/NZW F_1 mice had a somewhat elevated IC level before the start of treatment. In the dexamethasone-treated animals they did not rise any further, whereas in both the other groups they rose to nearly double their previous level. The rise was somewhat smaller in the DNase-treated animals. In both groups the IC level was at its maximum after 1 month of treatment, with some diminution thereafter.

Serum antibodies to rmDNase. These were detected in all mice treated with rmDNase beginning 1 month after the start of injections (Table 1). Antibody titres were low throughout the duration of the treatment period. With a single exception, no antibodies to rmDNase were detected in the animals treated with diluent or dexamethasone. One mouse in the diluent group developed a low titre (log titre = 2·4) at 5 and 6 months of age. Serum from this mouse at 7 months of age was negative for antibodies to rmDNase.

Urinary findings. Urinary IgG (Fig. 6a) and anti-DNA antibodies (Fig. 6b) were measured. At 5 months of age, urinary IgG levels were increased in both the diluent- and DNase-treated groups, and to a lesser extent in the steroid-treated mice. It is interesting that at certain times there was more IgG in the urine of DNase-treated mice than in the diluent-treated animals. Similarly, anti-DNA antibodies were found in urine at a significantly enhanced level in the DNase-treated mice. These observations may show that there is enzymatic breakdown of DNA/anti-DNA IC already lodged in the kidney and that this leads to the secretion of the antibody from the IC in the urine. The difficulty of collecting urine from individual mice on a timed basis makes this observation difficult to pursue. However, it should certainly be pursued in human studies, where the secretion of anti-DNA antibody in the urine may be a valuable monitor of the efficacy of the DNase in dissolving immune deposits in the kidney.

Histopathology. Severe glomerular changes were seen only in the diluent-treated mice. Moderate changes were seen in the DNase-treated animals. Immunofluorescence demonstrated that

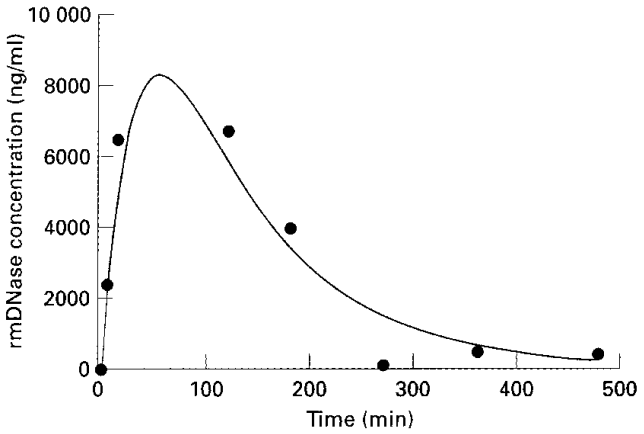

Fig. 2. Pharmacokinetics of rmDNase in male NZW mice following i.p. administration at 5 mg/kg. rmDNase levels in excess of 1 μg/ml were maintained for approximately 5 h.

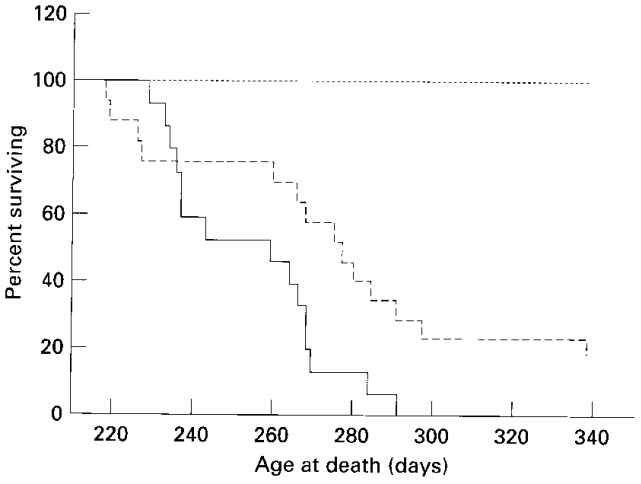

Fig. 3. Mortality rates of NZB/W mice. ——, Excipient; – – ––, DNase; . . . . . , dexamethasone.

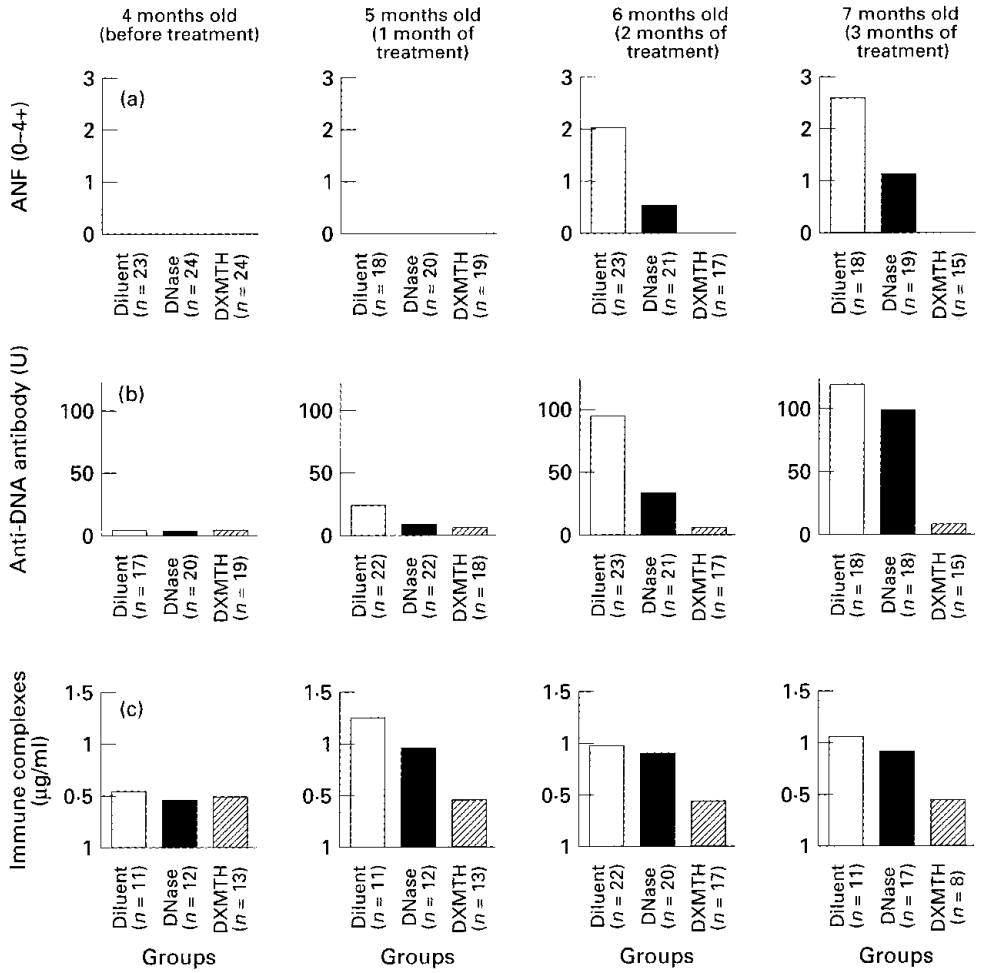

Fig. 4. Median values for serum antinuclear antibodies (a), anti-DNA antibodies (b) and immune complexes (c) measured every month in NZB/W hybrids. ANF, Antinuclear factor.

the DNase-treated animals showed substantially less staining for IgG than diluent-treated animals. In contrast to what is seen in human lupus nephritis, deposits of C3 were less intense than deposits of IgG in all groups.

Treatment of murine lupus in NZB/W F_1 hybrid mice with DNase from the age of 7 months for 3 weeks

A fall in anti-DNA antibody accompanied by a fall in serum IgG was seen in the control animals, whereas in the DNase-treated

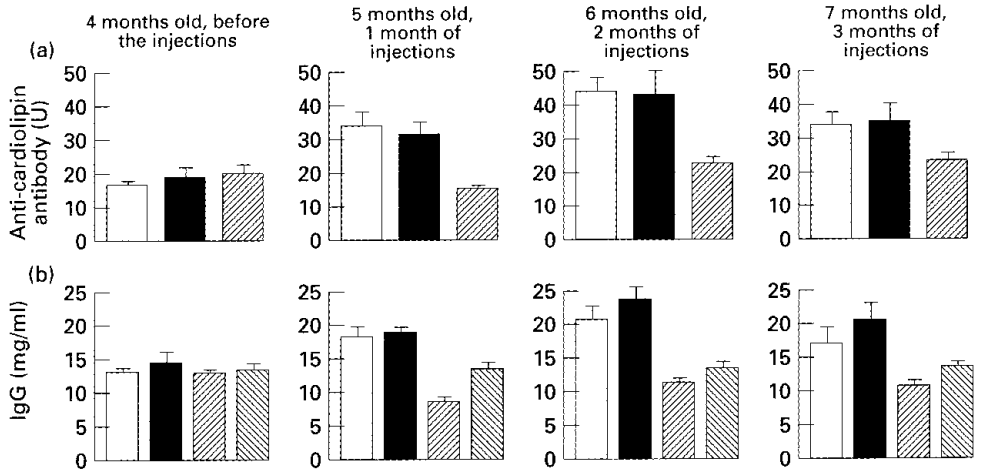

Fig. 5. Serum concentration of IgG (a) and anti-cardiolipin antibodies (b) in NZB/W mice (mean + s.e.m.) measured by ELISA. □, Diluent; ■, DNase; ▨, dexamethasone; ▧, normal mice.

Table 1. Serum anti-DNase antibodies in three groups of NZB/W hybrid mice at various time intervals

	Antibody titre to rmDNase (means) Age (months)			
Treatment group	4	5	6	7
Diluent	0	0·1	0·1	0
DNAse	0	2·9	3·0	2·8
Dexamethasone	0	0	0	0

Antibody titre is the log of the serum dilution that produced an absorbance equal to twice the absorbance obtained from a pool of normal mouse sera.

animals less change was seen in both parameters. Moreover, the variation between animals at this stage of the disease was substantial. However, the DNase-treated animals had less proteinuria (Fig. 7a) and substantially lower serum creatinine (Fig. 7b) than untreated controls. Serum creatinine levels were significantly decreased in the DNase group compared with the diluent group after 3 weeks of treatment.

There was an impressive reduction in the severity of the histopathological changes in the kidney (Fig. 8a) in the DNase-treated group. Two animals in the diluent-treated group died during treatment, while no deaths occurred in the DNase-treated group. Immunofluorescence demonstrated that the DNase-treated animals showed substantially less staining for IgG (Fig. 8b) and C3 (Fig. 8c) than the diluent-treated animals. The difference between the DNase and diluent groups was statistically significant for the intensity of histological lesions and the deposits of IgG and C3. *Z*-test statistics were calculated to test for the difference between groups in the histopathology and immunofluorescence data after 3 weeks of treatment. The difference between DNase and diluent group was highly statistically significant ($P = 0{\cdot}0006$).

DISCUSSION

In the first experiment three groups of mice were treated, respectively, with saline alone, with dexamethasone (1 mg/kg) and with DNase. A striking finding was the ability of this dose of dexamethasone totally to inhibit the development of the disease, both the clinical manifestations and the development of antinuclear antibodies—although the ability of high dose hydrocortisone (10 mg/kg per day) to prevent renal disease and prolong life in these mice has previously been reported [32]. The ability of steroids to inhibit SLE in mice is remarkable if not surprising. Mice are much more readily immunosuppressed by corticosteroids than man [33]. They seem to tolerate relatively high doses well. It is also known that corticosteroids have a marked effect on other autoimmune diseases in rodents such as autoallergic encephalitis [34]. Unfortunately, extensive experience has shown that such findings do not translate to humans, where corticosteroids are both more toxic and less immunosuppressive than in mice.

The DNase-treated animals developed both the anti-DNA antibodies and the nephritis leading to death about 1 month after control animals. This represents a prolongation of the survival by about 30%. It is not immediately clear why the effect of the DNase does not last much longer. It is possible that antibody formation to the DNase is limiting its effect. Antibodies to the enzyme were found in the treated group and not in either of the control groups, but the levels were not high, they did not rise and their role in inhibiting enzyme function *in vivo* is so far not clear. The strain of mouse from which the DNase was cloned is unknown, and it is possible that there are allotypic sequence differences which provide immunogenicity. In any event, the formation of anti-DNase antibody in the treated group did not prevent them from doing better than the diluent-treated controls. The absence of any anti-DNase antibody in control mice at times when they had high titres of anti-DNA antibody conflicts with the finding of Puccetti *et al*. [35] that many anti-DNA antibodies bind to DNase. Their suggestion that DNase may be the antigen giving rise to anti-DNA certainly does not seem to be the case in NZB//W F_1 mice.

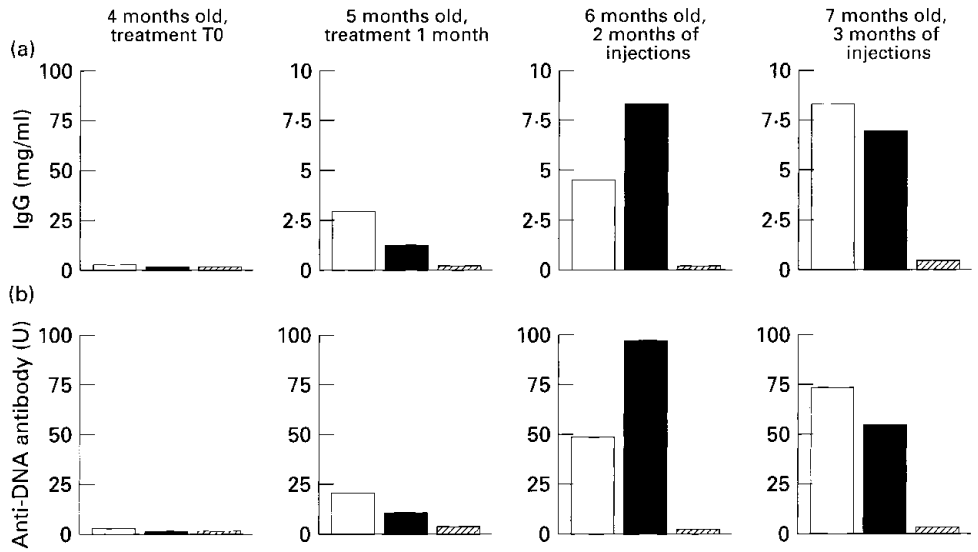

Fig. 6. Urinary IgG (a) and anti-DNA antibodies (b) in NZB/W hybrids measured at monthly intervals. □, Diluent; ■, DNase; ▨, dexamethasone.

Possibilities other than the formation of anti-DNase should also be considered. Treatment was given at a single dose level determined, at least in part, by the availability of the enzyme for the study. This may not have permanently maintained active DNase at inflammatory sites, and indeed when inflammation progresses due to the involvement of other antigen/antibody systems more DNase may be needed to overcome the quantity of actin released. A third possibility is that after this time, disease manifestations are due largely to antigen/antibody complex systems that do not involve DNA nucleoprotein. However, this is unlikely in the light of the findings of the second experiment.

In the first experiment the reduction of antibody titres is seen for anti-DNA antibodies and not for cardiolipin antibodies or for total IgG levels. The concomitant rise of anti-DNA antibodies in the urine suggests that this may reflect the destruction of DNA-containing IC in the kidney. The severity of the glomerulonephritis in the New Zealand mice was much less in the DNase-treated animals than in controls, whereas the kidneys of the dexamethasone-treated animals were entirely normal.

In the second experiment, a different protocol was used and animals at 28 weeks, already at the height of their anti-DNA antibody response and approaching the terminal part of the disease, were treated with DNase or with saline daily for 3 weeks and then killed for histology. At this stage of the disease the measurement of anti-DNA antibody levels is no longer particularly informative and more substantial falls in antibody and IgG levels were seen in control animals than in the DNase-treated animals. The variation between individual animals at this stage of the disease was also

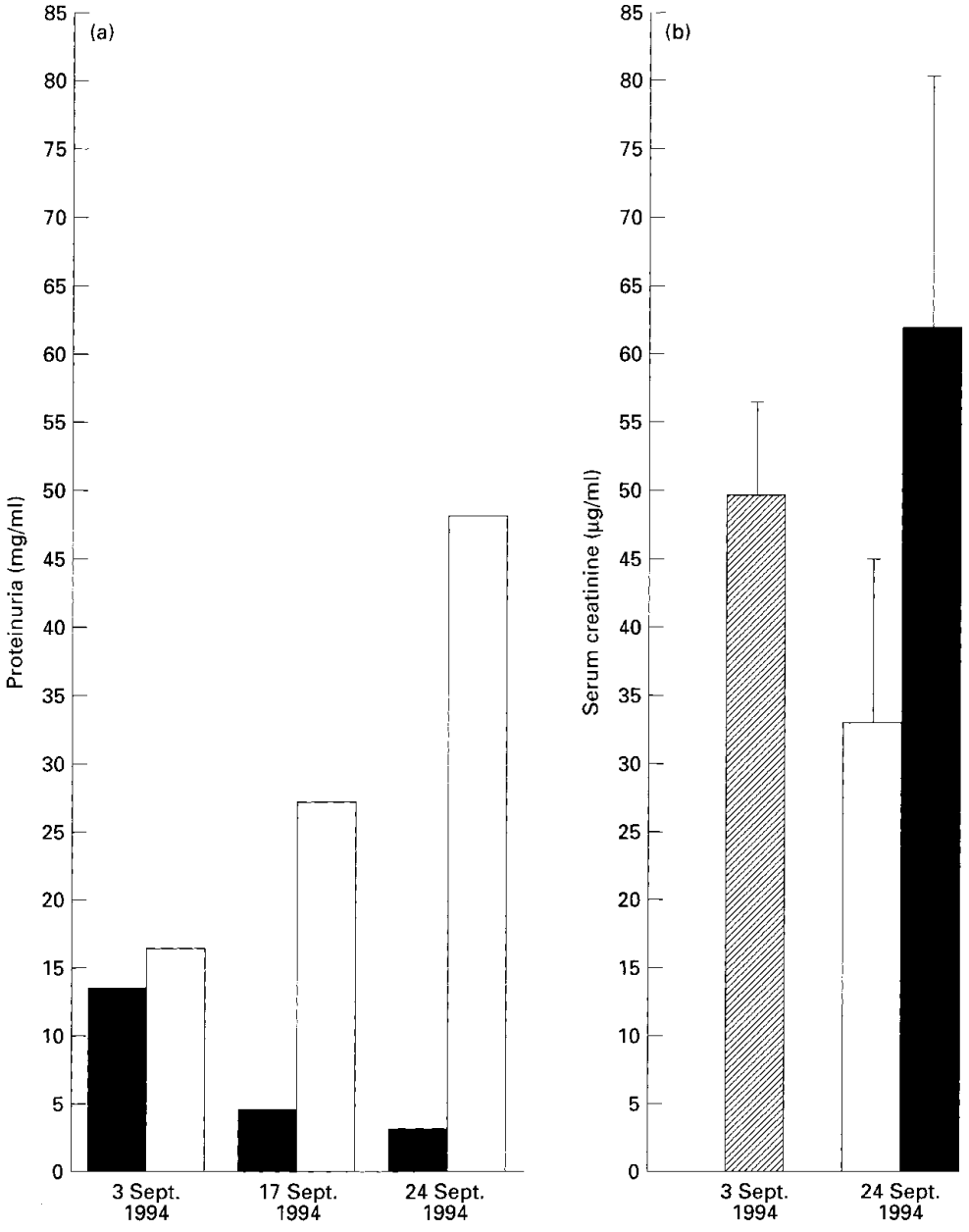

Fig. 7. Proteinuria (a) and serum creatinine (b) in 7-month-old NZB/W F_1 hybrid mice before treatment (3 Sept. 1994), in the course of treatment with DNase or diluent and after 3 weeks of treatment (24 Sept. 1994). ▨, Before treatment; ■, DNase; □, diluent.

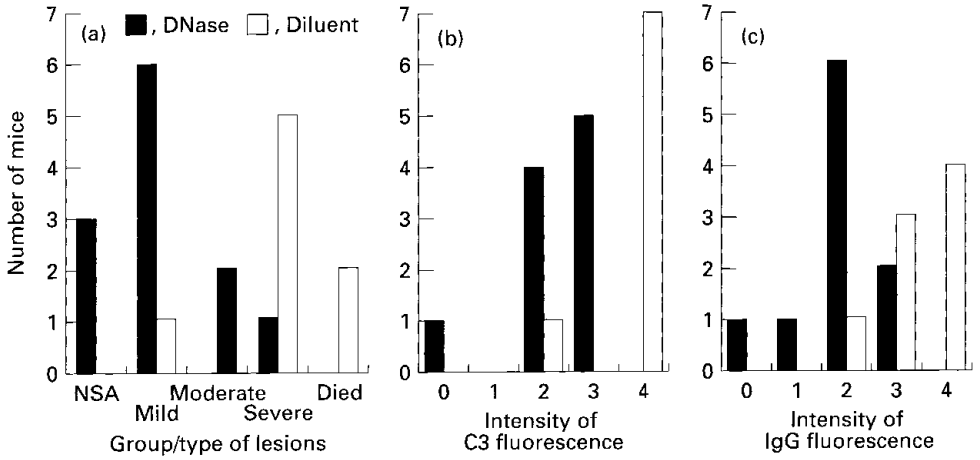

Fig. 8. Type of histopathology lesions (a), direct immunofluorescence of C3 (b) and IgG (c) deposits in the kidneys of 7-month-old NZB/W F_1 hybrids after 3 weeks of treatment with DNase or diluent.

marked. However, DNase-treated animals had substantially lower serum creatinine levels and less proteinuria than untreated controls, and there was an impressive reduction in the severity of histopathological changes in the kidney. This experiment demonstrated that even at a late stage of the disease there is still involvement of antigen (and presumably of antigen/antibody complexes) deposited in the kidney which can be broken down by the enzyme. The severe renal changes seen in control animals seem to develop late in the disease. The rate of development of nephritis in the NZB/NZW mice is clearly much more rapid than that seen in human lupus.

The results also confirm that DNA-containing antigens play an important role in SLE and contribute more substantially to the progress of the disease than the other antigen/antibody systems giving rise to IC in SLE. One reason for this may be the observations of Izui *et al*. [36] that DNA can enter the kidneys directly through the basement membrane and that IC can then form *in situ* at sites where it may be particularly difficult to clear. Another explanation arises from our own studies of immune complexes [37,38] demonstrating that for IC to fix complement and to be capable of interacting with polymorphs, a high epitope density on the antigen is of considerable importance. DNA, of course, has an extremely high epitope density since the antibodies to dsDNA react with epitopes on the sugar phosphate background which repeat continuously along the large molecule. Similarly, the antibodies to native DNA nucleoprotein react with frequently repeating epitopes on the surface of the nucleosome.

Since the DNase-treated mice do develop high titres of anti-DNA antibodies, albeit later in the disease, it is clear that the regimen of treatment we have used is not sufficient to abolish the antigenicity of DNA and DNA-nucleoproteins entirely. Whether a more complete abrogation of anti-DNA responses can be achieved by using larger doses of DNase can be investigated when further supplies of the recombinant murine enzyme are available. The delay in death is paralleled by the delay in formation of anti-DNA antibodies, which might suggest that if a more powerful inhibition of this response can be obtained, the clinical outcome could be improved further.

The results reported are enough to encourage a trial of human DNase in human SLE, where an unmet need certainly exists for therapeutic agents less toxic than corticosteroids or non-specific immunosuppressants. DNase has a good safety profile [31] and the recombinant human enzyme is available in amply sufficient amounts to treat patients. The bovine enzyme was given parenterally, and even intrathecally, without any ill effects and the recombinant human enzyme has already been given to substantial numbers of patients with cystic fibrosis. Here it is given by the respiratory tract, which is probably a more severe test of its immunogenicity than giving it subcutaneously.

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