Hydralazine induces Z-DNA conformation in a polynucleotide and elicits anti(Z-DNA) antibodies in treated patients

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We studied the effect of hydralazine, an antihypertensive drug with lupus-inducing side effects, on the conformation of poly(dG-m5dC)-poly(dG-m5dC) and a plasmid with a 23 bp insert of (dG-dC)₈·(dG-dC)₈ sequences. Using an e.l.i.s.a. with a monoclonal anti-(Z-DNA) antibody Z22, we found that hydralazine provoked the Z-DNA conformation in poly(dG-m5dC)-poly(dG-m5dC) at 250–500 μM concentration. The supercoiled form of hydralazine-treated plasmid bound to Z22 in a gel-retardation assay. To examine further whether Z-DNA could act as an inciting agent in anti-nuclear antibody production in patients, we analysed 65 sera from 25 hypertensive patients taking hydralazine and found anti-(Z-DNA) antibodies in 82% of these sera. Sera from age-matched normal controls showed no binding to Z-DNA. Data on sera drawn sequentially from four hypertensive patients showed that antibodies were present after the drug treatment. These data demonstrate the presence of a high incidence of anti-(Z-DNA) antibodies in patients treated with hydralazine and suggest that a possible mechanism for the production of autoantibodies in drug-related lupus might involve the induction and stabilization of Z-DNA by drugs.

INTRODUCTION

Hydralazine (Figure 1) is a phthalazine derivative with a reactive hydrazine group at the C-3 position. It is an effective antihypertensive agent (Morrow et al., 1953; Zacest and Koch-Weser, 1972; Hager, 1982). A side effect of this drug is the occurrence of anti-nuclear antibodies in many patients, with a lupus-like clinical syndrome occurring in some of these patients (Harmon and Portanova, 1982; Reidenberg, 1983; Rubin, 1989; Hess, 1988). The molecular basis for the induction of anti-nuclear antibodies is not known (Hess, 1988). Several lines of investigation suggest that hydralazine interacts with DNA and alters its structure and/or conformation, thereby conferring on it immunogenic properties. For example, Dubroff and Reid (1980) showed that hydralazine reacted with thymidine and deoxyctydine to form covalent adducts. Sinha and Patterson (1983) found strong binding of hydralazine to DNA and postulated intercalation of the drug between DNA bases. This drug also induced initiation of DNA repair in primary cultures of rat hepatocytes and was mutagenic in the Ames test, further suggesting drug-DNA interaction (Flora et al., 1982). Melting-temperature measurements showed that hydralazine destabilized double-helical DNA (Eldredge et al., 1974).

Thomas and Messner (1986) reported that hydralazine and other related lupus-inducing drugs facilitated the salt-induced right-handed B-DNA to left-handed Z-DNA transition of poly(dG-m5dC)-poly(dG-m5dC), a synthetic polynucleotide that is very susceptible to assuming the Z-DNA conformation under a variety of environmental conditions. This work was conducted using spectroscopic methods, in which hydralazine shifted the midpoint of transition induced by NaCl. This result is important because B-DNA is a poor immunogen whereas Z-DNA is immunogenic and produces anti-(Z-DNA) antibodies in experimental animals (Lafer et al., 1981; Zarling et al., 1984; Gunnia et al., 1991). Therefore the induction and stabilization of Z-DNA by hydralazine might be an antigenic stimulus for the production of anti-nuclear antibodies in hydralazine-related lupus.

Blocks of potential Z-DNA-forming alternating purine-pyrimidine (APP) sequences, including (dG-dC)₈·(dG-dC)₈ and (dA-dC)₈·(dG-dT)₈ blocks are widely dispersed in native DNAs (Hamada et al., 1982; Hamada and Kakunaga, 1982; Schroth et al., 1992), including the immunoglobulin and the human Vβ6 T-cell antigen receptors genes (Slightom et al., 1980; Li et al., 1991). Covalent modification in this region of the immunoglobulin gene by acetylaminofluorene promoted the formation of Z-DNA, as demonstrated by c.d. spectroscopy (Kilpatrick et al., 1984). From nucleotide sequence analysis, Van Helden (1985) reported that the frequency of occurrence of potential Z-DNA-forming APP blocks is far higher in DNA isolated from the serum immune complexes of systemic lupus erythematosus (SLE) patients than in normal eukaryotic DNAs. In addition, DNA fragments isolated from DNA-anti-DNA immune complexes of sera from SLE patients have higher G+C (50–60%) content than the average 38% G+C content in total human DNA (Sano and Morimoto, 1982). Polynucleotides and plasmids containing GC blocks of >10 bp undergo a facile transition to the Z-DNA form in the presence of salts, polyamines and metal complexes (Pohl and Jovin, 1972; Behe and Felsenfeld, 1981). We (Thomas et al., 1991) found that plasmids with inserted blocks of (dG-dC)₈·(dG-dC)₈ sequences undergo a facile transition to the Z-DNA conformation in the presence of natural polyamines. Since hydralazine and polyamines share the reactive amino groups in their chemical structure, we questioned whether hydralazine–DNA interaction could provoke the Z-DNA form in a conformationally labile synthetic DNA. This investigation seeks to obtain direct evidence for hydralazine-induced Z-DNA formation using a monoclonal anti-(Z-DNA) antibody, Z22.

Antibodies that react to the Z-DNA conformation were found in the sera of patients with SLE and rheumatoid arthritis (Lafer et al., 1983; Sibley et al., 1984; Thomas et al., 1988). Anti-(Z-
DNA) antibodies were also detected in the sera of patients receiving procainamide, an antiarrhythmic drug that is implicated in drug-related lupus (Mongey et al., 1992). Bergen et al. (1987) demonstrated the production of specific monoclonal anti-(Z-DNA) antibodies from immunized autoimmune MRL-Ipr/Ipr mouse spleen cells, thereby suggesting the existence of Z-DNA-specific antibodies in murine and human lupus. Despite these intriguing findings, the origin of these antibodies is not known at present. On the basis of the hypothesis that hydralazine–DNA interaction would help to alter the conformation of blocks of DNA sequences to the immunogenic Z-DNA form and trigger the production of autoantibodies, we analysed sera from 25 patients receiving this drug and found that 82% of these sera contained statistically significant elevation of anti-(Z-DNA) antibodies compared with age-matched normal controls. In a prospective study of four of these patients over a period of 1–2 years, we also found that antibody production occurred after drug administration.

EXPERIMENTAL

Chemicals and polynucleotides

Hydralazine (reagent grade) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) in its hydrochloride form and was used without further purification. A stock solution of hydralazine was prepared in double-distilled water at a high concentration so that the amount of the drug solution added to the polynucleotide solution was kept to a minimum (approx. 10 μl/ml). Calf thymus DNA was purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.), dissolved in a buffer containing 150 mM NaCl, 1 mM sodium cacodylate and 0.15 mM EDTA (pH 7.4), and dialysed extensively from the same buffer before use. Poly-(dG-dC)·poly(dG-dC) was purchased from Pharmacia (Piscataway, NJ, U.S.A.), dissolved in a buffer containing 50 mM NaCl, 1 mM sodium cacodylate and 0.15 mM EDTA (pH 7.4) and dialysed from the same buffer. Poly(dG-dC)·poly(dG-dC) was purchased from Pharmacia, and converted into the brominated form by methods described by Lafer et al. (1981). Poly(dA-dT)·poly(dA-dT) was also obtained from Pharmacia.

Recombinant plasmids

The plasmid DNAs used in this study were a gift from Dr. David Haniford of the University of Toronto. The parental plasmid pDPL6 (2.2 kb) was constructed from pBR322 by removing the Z-DNA-forming region and the tetracycline-resistance gene (Haniford and Pulleyblank, 1983a,b). The recombinant plasmid pDHgl6 was constructed by inserting a 23 bp (dG-dC)₈·(dG-dC)₈ sequence into pDPL6. We propagated this plasmid in Escherichia coli HB101 and prepared milligram quantities of DNA by methods described by Pulleyblank et al. (1983). The purification process involved lysis of bacterial cells with Pronase, centrifugation to remove bacterial chromosomal DNA, differential poly(ethylene glycol) precipitation of plasmid DNAs, RNAase and Pronase treatments, phenol extraction and ethanol precipitation. The DNAs were characterized by agarose-gel electrophoresis using a horizontal gel apparatus in Tris/borate/EDTA buffer. The superhelical density of this plasmid was −0.03 under which its conformation was in the B-DNA form as determined by gel electrophoresis and monoclonal anti-(Z-DNA) antibody-binding assay.

Immunocahemicals

Monoclonal anti-(Z-DNA) antibody Z22 was a gift from Professor David Stollar, Tufts University School of Medicine, Boston, MA, U.S.A. Protamine sulphate (type III), alkaline phosphatase-conjugated affinity-purified goat anti-mouse polyvalent immunoglobulins and phosphatase substrate were obtained from Sigma Chemical Co. Peroxidase-conjugated rabbit anti-(human immunoglobulin) antibody was purchased from Cappel Biochemicals (Malvern, PA, U.S.A.). Gelatin was obtained from Kodak Chemical Co. (Rochester, NY, U.S.A.).

Patient selection

Twenty-five hypertensive patients were selected from the outpatient clinics of the University of Cincinnati Medical Center. Most had received antihypertensive therapy before the study, including hydrochlorothiazide, reserpine and/or methyldopa but none hydralazine. Previous studies have shown that these treatments do not induce anti-(Z-DNA) antibodies (Mongey et al., 1992). All patients were without known collagen vascular or significant cardiac disease. Nine were male, of whom four were Caucasian and five Black, and 16 were Black females. The mean age was 39.9 (17–55) years. All patients were admitted to the General Clinical Research Center for prehydralazine study and were re-admitted for evaluation at yearly intervals. During the initial admission to this centre, routine laboratory studies were completed and the acetylation phenotype was determined using sulphamethazine (Price-Evans and White, 1964; Litwin et al., 1981) on those patients who had a negative history for allergic reactions to sulphonamides. After release from the centre, the patients were followed in the clinic. Hydralazine was used as the major antihypertensive drug at doses < 400 mg/day. Ten patients received 40 mg/day, six 300 mg/day, two 200 mg/day and seven 150 mg/day as maintenance therapy.

Sera drawn from 29 age-matched normal individuals (26 Caucasians and three Blacks) were used as controls. The mean age of this group was 39.1 (23–55) years.

Ethical approval

This study was approved by the Institutional Review Boards of the University of Cincinnati Medical Center and the University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School.

Immunological studies

Fluorescent anti-nuclear antibodies (FANA) were determined on sera collected during monthly clinic visits and a titre of 1:10 or higher was considered positive (Litwin et al., 1981). Antibodies to poly(A) were measured by a standard binding assay (Spencer-Green et al., 1986). Binding levels of > 2 S.D. of mean of normal sera were considered elevated.
**E.I.i.s.a. of hydralazine–polynucleotide complex**

Poly(dG-m5dC)-poly(dG-m5dC) was diluted to 10 μM in buffer containing 50 mM NaCl, 1 mM sodium cacodylate and 0.15 mM EDTA, and treated with hydralazine solution so that the final drug concentrations were 0, 10, 50, 100, 250, 500 and 1000 μM. Polynucleotide–drug complexes were incubated at 37 °C for 2 h before use in our E.I.i.s.a. experiment designed to detect the formation of Z-DNA conformation using an anti-(Z-DNA) antibody. E.I.i.s.a. was conducted by a protocol described elsewhere (Thomas, 1991). Briefly, this method consisted of coating Costar microtitre plates with 300 μl of 0.0001% polyamine sulphate, followed by 200 μl of polynucleotide (10 μg/ml concentration) or polynucleotide–hydralazine complexes to immobilize the DNA conformation and then titrating the immobilized DNA with a monoclonal anti-(Z-DNA) antibody. The amount of Z-DNA formation was quantified by treating the plates with an alkaline phosphatase-conjugated affinity-purified anti-(mouse polyclonal immunoglobulin) antibody, followed by the enzyme substrate, p-nitrophenyl phosphate. The enzyme–substrate reaction was stopped after 1 h by adding 50 μl of 1 M NaOH/well. Absorbance was then read on a V̂max kinetic microplate autoreader (Molecular Devices, Menlo Park, CA, U.S.A.) at 405 nm. Control experiments were performed with polynucleotides incubated at 37 °C in the absence of hydralazine.

**Gel electrophoresis**

We examined the ability of hydralazine to provoke the Z-DNA conformation in pDHg16 using the gel-retardation assay (Fried and Crothers, 1981). In this set of experiments, the plasmid DNA was incubated with hydralazine for 16 h at 37 °C and then treated with Z22 for 2 h. The mixture was then loaded on to a 0.9% agarose gel using a Bio-Rad gel-electrophoretic apparatus and electrophoresis was conducted at 50 mA for 16 h at 22 °C. At the end of the electrophoresis, the gel was stained with ethidium bromide and photographed under u.v. light.

**Analysis of anti-(Z-DNA) antibody in the sera of patients treated with hydralazine**

Serum samples were collected from hypertensive patients treated with hydralazine over a period of several years. In some cases, sequential samples were collected, whereas in other cases, samples were obtained at single points. These samples were analysed for anti-(Z-DNA) antibodies using a published e.I.i.s.a. procedure (Thomas et al., 1988). Brominated poly(dG-dC)·poly(dG-dC) was used as a source of Z-DNA because this modified polynucleotide exists in the Z-DNA conformation at low and moderate NaCl concentrations (Lafer et al., 1981). All DNA solutions were made in a buffer of 150 mM NaCl, 1 mM sodium cacodylate and 0.15 mM EDTA (pH 7.4) for coating on the microtitre plates. The Z-DNA conformation was confirmed by c.d. spectroscopy before its use in the e.I.i.s.a. (Thomas and Messner, 1986). Microtitre plates were coated with brominated poly(dG-dC)·poly(dG-dC) at a concentration of 10 μg/ml, washed, and then treated with serum samples from hydralazine recipients or normal controls at a dilution of 1:400 (200 μl/well). The wells were subsequently treated with peroxidase-conjugated affinity-purified goat anti-(human immunoglobulin) polyclonal antibodies and the enzyme substrate o-phenylenediamine. The enzyme–substrate reaction was stopped by the addition of 50 μl of 2.5 M H₂SO₄/well and the absorbance was read at 490 nm with a microplate autoreader.

Poly(dG-dC)·poly(dG-dC) was used as a B-DNA control with DNA sequences compatible with that of the Z-DNA used. S1 nuclease-treated calf thymus DNA was also used to examine the binding of patient sera to native B-DNA conformation. Poly(dA-dT)·poly(dA-dT) was further used as a control because this polynucleotide was resistant to assuming the Z-DNA form (Thomas and Thomas, 1990).

**RESULTS**

Figure 2 shows typical results from our e.I.i.s.a. of poly(dG-m5dC)·poly(dG-m5dC) complexed with hydralazine at five different concentrations. Up to a hydralazine concentration of 100 μM, there was no binding of the monoclonal antibody Z22 to the polynucleotide. The absorbance values were in the background range of 0.00±0.002. At 250 and 500 μM concentrations of hydralazine, there was a positive value for absorbance, indicating the recognition of Z-DNA by Z22 on the microtitre plate. The optimum absorbance value was 0.2 at 500 μM hydralazine. At 1000 μM, however, there was a decrease in absorbance, suggesting that the polynucleotide had undergone conformational alterations that were not recognizable by Z22. These results are similar to that observed in the case of poly(dG-m5dC)·poly(dG-m5dC) treated with a Z-DNA inducer, hexamidine rhodium chloride (Thomas and Thomas, 1990). Control

![Figure 2 Detection of hydralazine-induced Z-DNA formation in poly(dG-m5dC)·poly(dG-m5dC) by e.I.i.s.a.](image-url)

The polynucleotides were incubated for 16 h at 37 °C in the presence of hydralazine, and then coated on a microtitre plate. Z-DNA formation was detected by use of a monoclonal anti-(Z-DNA) antibody, Z22.
experiments with poly(dG-m3dC) · poly(dG-m3dC) alone produced no binding to Z22 under the conditions of our experiment. Under the same ionic and drug concentrations, there was no conversion of poly(dG-dC) · poly(dG-dC) into the Z-DNA form. It is known (Behe and Felsenfeld, 1981) that the unmethylated polynucleotide requires more drastic conditions than its methylated analogue to assume the Z-DNA conformation. Previous studies have shown that the binding of Z22 is a sensitive marker of Z-DNA formation since this monoclonal antibody shows no reactivity toward ssDNA or double-stranded (ds) B-DNA under the conditions employed in our experiment (Thomas et al., 1988).

We next examined whether hydralazine could provoke the Z-DNA conformation in recombinant plasmid pDHg16 containing a 23 bp insert of potential Z-DNA-forming (dG-dC)2 · (dG-dC)2 sequences using the gel-retardation assay. In this case, the plasmid was allowed to react with different concentrations of hydralazine, and then with Z22 at a concentration of 1.5 μg/ml. The plasmid–hydralazine-Z22 complex was then loaded on 0.9 % agarose gel and electrophoresis was conducted at 50 mA for 16 h. The gel was treated with ethidium bromide and photographed under u.v. light. Figure 3 shows our results. In the absence of any additive, plasmid pDHg16 separated into three distinct regions, identified as nicked circular (top), linear (middle) and supercoiled (bottom). The supercoiled form showed several closely placed bands, which could be attributed to topoisomerons formed by thermal relaxation of the plasmid. Treatment of pDHg16 with 500 and 1000 μM hydralazine produced an additional band, m1, indicative of altered conformation/multimerization of the plasmid DNA.

Another band, m2, was slightly visible even in the untreated plasmid, but its intensity was increased by treatment with hydralazine (lane 5 of Figure 3). Neither of these bands was affected by the anti-(Z-DNA) antibody. Addition of Z22 to pDHg16 produced a retardation in the mobility of the circular and supercoiled forms on the gel, but there was no reactivity with the linear form. Z22 showed strong reactivity toward pDHg16 treated with 500 μM hydralazine. At 1000 μM hydralazine, there was a reduction in the binding of Z22 to pDHg16, suggesting the transition of the Z conformation to a form not recognizable by the monoclonal anti-(Z-DNA) antibody. Another possibility is that hydralazine and Z22 were competing for the same binding sites on DNA. Gel-retardation experiments thus show that hydralazine stabilizes unusual conformations of pDHg16 that are recognizable by Z22 over a narrow range of the drug concentration.

It should be pointed out that the (dG-dC)n sequences of plasmid pDHg16 adopt a Z-DNA conformation under conditions of superhelical stress and react with Z22 even in the absence of hydralazine or any other Z-DNA inducer (results not shown). However, in this case the retardation on the gel was less dramatic than that observed in lane 4 of Figure 3.

Hydralazine has been extensively used as an antihypertensive drug, and we therefore tested for anti-(Z-DNA) antibodies in the sera of 25 patients on this drug by using an e.l.i.s.a. As the ability of hydralazine to elicit anti-DNA antibodies had been previously shown to depend on the acetylator phenotype of the individual, we determined the acetylation status of patients in this study. Thirteen patients were classified as slow and eight as rapid acetylators. Acetylation phenotyping was not determined in four patients because of a history of sulphonamide allergy. Positive FANA test results were found in the slow acetylators. The staining patterns were homogeneous and titres varied from 1:20 to 1:320. Elevated titres to poly(A) were found in ten of 13 slow and three of eight rapid acetylators (Litwin et al., 1981). Clinical evaluation of and detailed laboratory findings on these patients have been reported elsewhere (Litwin et al., 1981). A panel of age-matched normal controls were also studied in this experiment. All sera were used at a dilution of 1:400 for the e.l.i.s.a. The results of e.l.i.s.a. experiments on sera from hydralazine-treated patients and normal controls are presented in Table 1. The mean absorbance of sera from hydralazine-treated patients was 0.42 ± 0.32 compared with 0.04 ± 0.01 for age-matched normal controls. Some 82 % of sera from hydralazine-treated patients had absorbance values 2 S.D. higher than that of the normal controls. The binding of the sera to poly(dG-dC) · poly(dG-dC), a polynucleotide that existed in the B-DNA form under conditions in which Br-poly(dG-dC) · poly(dG-dC) assumed the Z-DNA form, was negligible (A00 = 0.061 ± 0.067). There was no reactivity of these antibodies with microtiter plates

![Figure 3: Gel-retardation assay of pDHg16–hydralazine complex by monoclonal anti-(Z-DNA) antibody, Z22](attachment:image-url)

Table 1: E.L.I.s.a. results for sera from hydralazine-treated hypertensive patients and age-matched normal controls

<table>
<thead>
<tr>
<th>DNA on microtitre plate</th>
<th>Normal controls</th>
<th>Hydralazine recipients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-denatured calf thymus</td>
<td>0.042 ± 0.03</td>
<td>0.15 ± 0.16</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Br-poly(dG-dC) · poly(dG-dC) (Z-DNA)</td>
<td>0.04 ± 0.01</td>
<td>0.42 ± 0.32</td>
<td>&lt; 0.00001</td>
</tr>
<tr>
<td>Poly(dG-dC) · poly(dG-dC)</td>
<td>0.035 ± 0.02</td>
<td>0.061 ± 0.067</td>
<td>&gt; 0.1</td>
</tr>
</tbody>
</table>

**Figure 3** Gel-retardation assay of pDHg16–hydralazine complex by monoclonal anti-(Z-DNA) antibody, Z22

Lanes are as follows: 1, molecular-size marker (HindIII digest of λ-DNA); 2, pDHg16; 3, pDHg16+500 μM hydralazine; 4, as lane 3+Z22 (1.5 μg/ml); 5, pDHg16+1000 μM hydralazine; 6, as lane 5+Z22 (1.5 μg/ml). Bands denoted m1 and m2 represent an altered conformation and multimerization respectively of pDHg16. Nicked circular, linear and supercoiled forms of the plasmid are indicated.
Whereas higher considerably hydrazaline-treated patients. Procedure. To ssDNA and Z-DNA. To reacted with hydralazine-treated patients.

Figure 4 A prospective study of anti-DNA antibody elicitation in two hydralazine-treated patients (a and b)

Serological analysis was by e.i.i.s.a. with Br-poly(dG-dC) · poly(dG-dC) (Z-DNA) (●), ssDNA (■), poly(dG-dC) · poly(dG-dC) (▲) and calf thymus dsDNA (○).

dC) · poly(dG-dC) was negligible before the administration of the drug. Antibody production occurred with drug intake, peaked at approx. 3 months and remained elevated over a period of 1–2 years. There was a fall in anti-(Z-DNA) antibody levels toward the end of the study.

Discussion

Our results show that hydralazine, an antihypertensive drug with a frequent association with autoantibody production and a lupus-like syndrome in about 10% of the recipients, provokes conformational alternations in a synthetic DNA that can easily switch to the left-handed Z-DNA conformation. Although long repeats (dG-m³dC) sequences, as used in our e.i.i.s.a. experiments, are not found in human DNA, blocks of potential Z-DNA-forming (dG-dC)ₙ sequences are widely dispersed in the regulatory elements of a large number of genes studied so far (Schroth et al., 1992). Methylation at the C-5 position of these sequences occurs as a control mechanism of gene expression. The concentration of hydralazine that provoked the Z-DNA conformation in poly(dG-m³dC) · poly(dG-m³dC) is about 250 µM, a concentration approx. 25-fold higher than the steady-state level of circulating hydralazine in treated patients (Karlsson and Molin, 1975; Ludden et al., 1981). However, the DNA phosphate/drug ratio in our experiment is about 0.04 and compares well with that calculated on the basis of circulating DNA [ng/ml (Steinman, 1984)] and drug concentrations [10 µM (Ludden et al., 1981)] in patients. In addition, the effective concentration of cationic drugs and other ions near DNA is calculated to be much higher than that of the bulk concentration because of electrostatic interactions (Manning, 1978). Thus the concentration of hydralazine that stabilized the Z-DNA form in polynucleotide and plasmid pDHg16 is in the physiologically compatible range.

Our data further demonstrate the presence of antibodies that react with the Z-DNA conformation in the sera of the majority of patients receiving hydralazine. Anti-(Z-DNA) antibody formation appears to be unrelated to anti-ssDNA antibody formation, because of a low correlation between the two types of antibodies (r = 0.56). These data thus suggest that a potential mechanism for autoantibody formation in hydralazine-related lupus may involve the binding of the drug to DNA and consequent formation of an immunogenic Z-DNA conformation. The Z-DNA thus formed may stimulate the production of anti-DNA autoantibodies in hydralazine-treated patients. In experimental animals, Z-DNA is a known immunogen and produces anti-(Z-DNA) antibodies (Lafer et al., 1981; Zarling et al., 1984; Gunnia et al., 1991). Earlier studies (Lafer et al., 1981) indicated that covalent modification of DNA to a stable Z-DNA conformation, such as bromination of poly(dG-dC) · poly(dG-dC), was necessary to induce an anti-(Z-DNA) antibody response. However, recent results with polyamine-polynucleotide complexes demonstrate that ligand-induced Z-DNA formation is sufficient to produce anti-(Z-DNA) antibodies in experimental animals (Gunnia et al., 1991). In this context, it is important to note that bacterial DNAs are shown to elicit anti-DNA antibodies in mice and to stimulate in vitro proliferation of mouse splenocytes (Glakeson et al., 1989; Messina et al., 1991; Terada et al., 1992).

In a previous study, Thomas et al. (1988) evaluated the binding of monoclonal anti-(Z-DNA) antibody Z22 to calf thymus ss and ds DNA and found no binding using e.i.i.s.a. Br-poly(dG-dC) · poly(dG-dC) bound to an extent (A₄₉₀ = 0.69 ± 0.01) comparable with that of calf thymus ds DNA (A₄₉₀ = 0.41 ± 0.05) to a monospecific anti-dsDNA antibody.

coated with poly(dA-dT) · poly(dA-dT) or calf thymus dsDNA. This set of experiments clearly showed the presence of antibodies that reacted with Z-DNA in the sera of the vast majority of hydralazine-treated patients.

We also analysed these sera for antibodies bound to ssDNA. In this case, calf thymus DNA was boiled in a water bath for 45 min, and quickly cooled on ice to freeze the single-stranded form. E.i.i.s.a. for ssDNA was conducted in the same way as for the anti-(Z-DNA) antibody except for the use of denatured calf thymus DNA on the microtitre plates. The mean A₄₉₀ for sera from hydralazine recipients was 0.15 ± 0.16 compared with 0.042 ± 0.03 for normal controls (Table 1).

DNA bases are exposed to the solvent surface in ssDNA, whereas these bases are buried in the central core of double-helical DNA. Z-DNA, by virtue of its zig-zag arrangement of phosphate groups along the backbone structure, exposes bases to considerably higher degree than does B-DNA. There was thus the possibility of cross-reactivity of anti-(DNA antibodies) between ssDNA and Z-DNA. To test this possibility, we analysed absorbance values of ssDNA and Z-DNA using a linear regression procedure. The correlation coefficient obtained from this analysis was 0.56.

We also analysed sequential serum sample drawn from hydralazine-treated patients. The results of this prospective study on two patients are shown in Figure 4. Comparable results were obtained in two other cases (result not shown). In all cases, absorbance due to the binding of sera to Br-poly(dG-dC) · poly(dG-dC) was negligible before the administration of the drug. Antibody production occurred with drug intake, peaked at approx. 3 months and remained elevated over a period of 1–2 years. There was a fall in anti-(Z-DNA) antibody levels toward the end of the study.
reference serum from the Centers for Disease Control (Atlanta, GA, U.S.A.). These data suggest that the binding of Z22 is to the dsZ-DNA form of hydralazine-treated poly(dG-m5dC)·poly(dG-m5dC). The results of our gel-shift experiments indicate an increased affinity of Z22 for the supercoiled form of pHgl6 and negligible binding to its linear form. These results suggest that Z-DNA-facilitating factors such as superhelical density are important in hydralazine-induced changes in short segments of DNA. In vivo, hydralazine could act as one of the factors that stabilize Z-DNA, although hydralazine itself may be a weak Z-DNA-inducing agent unless a long stretch of Z-DNA-forming sequences, such as found in poly(dG-m5dC)·poly(dG-m5dC), is readily encountered in the serum or on the cell surface.

Thomas and Messner (1986) demonstrated the ability of procainamide and hydralazine to facilitate the conversion to Z-DNA of poly(dG-m5dC)·poly(dG-m5dC) in the presence of 1 M NaCl. The present series of experiments were conducted at a lower concentration (50 mM) of NaCl and hence salt-induced transition is not necessary for the induction and stabilization of Z-DNA by hydralazine. Zacharias and Koopman (1990) also analysed the effect of hydralazine on circular supercoiled plasmids as models of chromosomal loop domains. They found that the binding of a Z-DNA-specific autoimmune antibody to a Z-DNA-containing plasmid was greatly perturbed by hydralazine. Direct interaction of the drug with DNA was proposed as the basis for hydralazine-induced changes in the superhelicity and antibody binding of plasmid DNA. On the basis of the interaction of hydralazine with plasmid DNA, Zacharias and Koopman (1990) proposed that the in vivo capacity of this drug to elicit anti-nuclear antibodies might be related to its ability to alter the structure and conformation of chromosomal DNA domains or nucleosomes, thus liberating antigenic structural epitopes in DNA and/or DNA-associated proteins. The results presented in the present paper confirm and extend these previous observations and provide data for the first time that demonstrate the ability of hydralazine to provoke the Z-DNA conformation in susceptible DNA sequences to a left-handed Z-DNA form in conditions of low ionic strength. In addition, our results indicate other unusual conformations and/or multimerization of the plasmid in the presence of hydralazine.

The serological evaluation of a group of hydralazine-treated patients is consistent with our hypothesis that drug-induced Z-DNA might be the antigenic stimulus for the production of autoantibodies. In the majority of the patients studied (82%), antibodies reacting with the Z-DNA form of Br-poly(dG-dC)·poly(dG-dC) were found. Negligible binding of these sera to poly(dG-dC)·poly(dG-dC) was demonstrated and this B-DNA form indicates that the binding specificity of the immunoglobulins is toward the Z conformation rather than the (dG-dC) sequences. Furthermore, the non-reactivity of these sera toward calf thymus dsDNA and poly(dA-dT)·poly(dA-dT) shows that the binding of sera to the microtitre plate is negligible. Anti-sDNA antibodies were also present in many patients and there was a low correlation between anti-Z(DNA) and anti-sDNA antibodies. There were no anti-(Z-DNA) or anti-sDNA antibodies in age-matched normal controls. In addition, in our prospective study of four patients, it was clear that anti-(Z-DNA) antibody formation occurred after hydralazine treatment of these patients.

The role of slow acetylation in hydralazine-related lupus has been reported (Perry et al., 1970; Litwin et al., 1981). Anti-(Z-DNA) antibodies were more prevalent in these patients than other anti-nuclear antibodies, including anti-sDNA and anti-dsDNA antibodies.

Elicitation of anti-nuclear antibodies by hydralazine and other 'lupus-inducing' drugs has been documented in the literature over the past few years (Harmon and Portanova, 1982; Reidenberg, 1983; Hess, 1988; Rubin, 1989). Although anti-dsDNA antibodies are believed to be unique markers of SLE (Tan et al., 1988), anti-histone antibodies are predominantly found in drug-related lupus (Rubin, 1989). Recent studies (Mongey et al., 1992) show that these antibodies are present in asymptomatic patients as well, suggesting that their presence may be a prelude to expression of clinical disease. Burlingame and Rubin (1991), as well as Rubin et al. (1992), reported that sera from patients suffering from drug-related lupus have increased affinity for a complex formed from histone H2A–H2B and DNA compared with their binding to individual histones or DNA. This complex can be considered as a model of nucleosomal substructures formed by enzymic degradation of chromatin. Conformational alterations of DNA produced by histones in these complexes have not been documented, but several reports indicate the possibility of unusual DNA structures formed by the interaction of DNA with proteins, polyamines and amino acids (Haniford and Pulleyblank, 1983a,b; Rich et al., 1984; Wells, 1988; Zacharias et al., 1988). These include B-DNA to Z-DNA transition as well as B-DNA to C- and A-DNA transitions. Our finding of the presence of anti-(Z-DNA) antibodies in 82% of patients taking hydralazine suggests that sera from patients taking certain drugs bind with increased affinity to non-B-DNA conformations. The ability of hydralazine to provoke the Z-DNA conformation in poly(dG-m5dC)·poly(dG-m5dC) further supports the hypothesis that lupus-inducing drugs might interact with labile sequences of serum or cell-surface DNA and present it in an immunogenic form. Purification and sequencing of these extracellular DNAs may shed further light on the role of DNA conformation dynamics in anti-nuclear antibody production in drug-related and idiopathic forms of lupus.

In summary, our data provide a possible biochemical pathway for the induction of anti-nuclear autoantibodies in hydralazine-related lupus. Studies on these patients who really are ‘experiments of nature’ are of great importance for our understanding of autoantibody formation and its possible role in disease expression, and of metabolic and possibly genetic responses of the host.

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