Triplex-DNA stabilization by hydralazine and the presence of anti-(triplex DNA) antibodies in patients treated with hydralazine

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Hydralazine is an antihypertensive drug that elicits anti-nuclear antibodies in patients as an adverse effect. We investigated the ability of hydralazine to promote/stabilize the triplex DNA form of poly(dA)·2poly(dT). Under conditions of low ionic strength, the polynucleotide melted as a double helix with a melting temperature (T_m) of 55.3 °C. Hydralazine destabilized this duplex form by reducing its T_m to 52.5 °C. Spermidine (2.5 μM), a natural polyamine, provoked the triplex form of poly(dA)·2poly(dT) with two melting transitions, T_{m_1} of 42.8 °C corresponding to triplex → duplex + single-stranded DNA and T_{m_2} of 65.4 °C, corresponding to duplex melting. Triplex DNA thus formed in the presence of spermidine was further stabilized by hydralazine (250 μM) with a T_{m_1} of 53.6 °C. A similar stabilization effect of hydralazine was found on triplex DNA formed in the presence of 5 mM Mg^{2+}. CD spectra revealed conformational perturbations of DNA in the presence of spermidine and hydralazine. These results support the hypothesis that hydralazine is capable of stabilizing unusual forms of DNA. In contrast with the weak immunogenicity of DNA in its right-handed B-DNA conformation, these unusual forms are immunogenic and have the potential to elicit anti-DNA antibodies. To test this possibility, we analysed sera from a panel of 25 hydralazine-treated patients for anti-(triplex DNA) antibodies using an ELISA. Our results showed that 72% of sera from hydralazine-treated patients contained antibodies reacting toward the triplex DNA. In contrast, there was no significant binding of normal human sera to triplex DNA. Taken together our data indicate that hydralazine and related drugs might exert their action by interacting with DNA and stabilizing higher-order structures such as the triplex DNA.

INTRODUCTION

Hydralazine (Figure 1) is a prototype of 'lupus-inducing drugs' that elicit anti-nuclear antibodies and a syndrome resembling systemic lupus erythematosus in recipients [1–3]. This adverse effect has reduced its usage as an antihypertensive drug, at least with larger doses. Among the anti-nuclear antibodies elicited by hydralazine, anti-histone antibodies are the most prominent; however, antibodies reacting with single-stranded and Z-DNA are also found in the serum of patients taking this drug [4–6]. The origin of these antibodies in the serum of drug-treated patients is not known at present. Several studies, including those from our laboratory, indicated the possible interactions of hydralazine and other lupus-inducing drugs with DNA [7–9]. Sequence and conformational specificity has been observed in these interactions. Our results have specifically revealed that hydralazine binds with high affinity to DNA sequences capable of assuming the Z-DNA conformation [5]. Potential Z-DNA-forming alternating purine–pyrimidine sequences are widely dispersed in native DNAs and their frequency of occurrence is far higher in DNA isolated from the serum of lupus patients compared with normal controls [10,11]. As Z-DNA is immunogenic [12,13], we examined whether the serum of hydralazine-treated patients contained antibodies reacting with this conformation of DNA; we found significantly higher levels of Z-DNA-binding antibodies in 82% of patients compared with normal controls [5]. These results indicate a possible mechanism involving the induction and stabilization of immunogenic Z-DNA in hydralazine-treated patients.

Triplex DNA has attained much attention in recent years because of its potential use as an anti-gene strategy for treating cancer and immunological disorders, as well as its potential in genome mapping [14–17]. Triplex-DNA-forming poly(purine)·poly(pyrimidine) sequences are widely dispersed in the human genome [18]. Triplex DNA can form *in vivo* either by the binding of an oligonucleotide to a continuous stretch of purines or pyrimidines or as a part of H-DNA (hinge DNA) which consists of folded regions of triple- and single-stranded DNA. The presence of multivalent cations such as Mg^{2+} or spermine is essential for triplex-DNA stabilization at physiological pH. We have demonstrated a structural specificity for polyamines in triplex-DNA stabilization [19]. This result is particularly important because of the ubiquitous nature of these molecules and their up-regulation in lupus and related autoimmune diseases [20–23].

Triplex DNA is weakly immunogenic, and monoclonal and polyclonal anti-(triplex DNA) antibodies have been raised [24,25]. Burkholder et al. [26] have also found the presence of triplex DNA in chromosomal-loop regions by using fluorescence antibody. On the basis of these results, we questioned whether hydralazine is capable of inducing and/or stabilizing triplex DNA in labile sequences. We therefore examined the effect of low concentrations of hydralazine on the melting temperature T_m of duplex and triplex forms of poly(dA)·poly(dT) and found that hydralazine only has a destabilizing effect on duplex DNA. Triplex DNA was not induced by hydralazine treatment alone, but this drug stabilized Mg^{2+}- and spermidine-induced triplex DNA, as determined by T_m measurements. Analysis of 65 sera

Abbreviation used: T_m, melting temperature.

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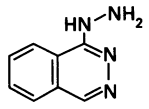

Hydralazine

Figure 1 Chemical structure of hydralazine

from 25 hypertensive patients treated with hydralazine showed the presence of antibodies binding to the triplex form in 72% of these sera.

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 was prepared in doubly-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). Spermidine trihydrochloride was also purchased from Sigma Chemical Co. and was used without further purification. HPLC analysis of this compound showed no contaminating polyamines. All other chemicals were of reagent grade purchased from Sigma.

Poly(dA) and poly(dT) were purchased from Pharmacia (Piscataway, NJ, U.S.A.). The polynucleotides were dissolved in 10 mM sodium cacodylate buffer (pH 7.2) containing 0.5 mM EDTA, and dialysed extensively from the same buffer. All experiments were conducted in this buffer. Concentrations of poly(dA) and poly(dT) were measured using molar nucleotide absorption coefficients of 8900 at 257 nm for poly(dA) and 9000 at 265 nm for poly(dT). To prepare triplex solution, poly(dA) and poly(dT) were mixed in 1:2 molar ratio in 10 mM sodium cacodylate buffer and the appropriate concentrations of spermidine and/or hydralazine were added. The solutions were heated at 90 °C for 5 min, cooled to room temperature (22 °C) and allowed to equilibrate for 16 h at this temperature before use in T_m experiments.

Calf thymus DNA was purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.), dissolved in a buffer containing 10 mM sodium cacodylate and 0.5 mM EDTA (pH 7.4), and dialysed extensively from the same buffer before use.

Immunochemicals

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 was purchased from Cappel Biochemicals (Malvern, PA, U.S.A.). Gelatin was obtained from Kodak Chemical Co. (Rochester, NY, U.S.A.).

Patient selection

Sera used in this study were collected from 25 hypertensive patients followed at the outpatient clinics of the University of Cincinnati Medical Center. Most of these patients had received antihypertensive therapy before the study, including hydrochlorothiazide, reserpine and/or methyldopa, but none had received hydralazine. Our previous studies have shown that these treatments did not produce antibodies reacting with DNA [27]. All patients were without known immunological or significant cardiac disease. Nine of the patients were male, of whom four were Caucasian and five black, and 16 were black females. The mean age was 39.9 (range 17–55 years). All patients were admitted to the General Clinical Research Center (GCRC) for pre-hydralazine study and were re-admitted for evaluation at yearly intervals. During the initial admission at the GCRC, routine laboratory studies were completed and the acetylation phenotype was determined using sulphamethazine [28,29] on those patients who had a negative history for allergic reactions to sulphonamides. After release from the GCRC, the patients were followed in the clinic. Hydralazine was used as the major antihypertensive at doses of less than 400 mg/day. Ten patients received 40 mg/day hydralazine, six 300 mg/day, two 200 mg/day and seven 150 mg/day as maintenance therapy.

We also used sera from 29 age-matched normal individuals as controls. The mean age of the group was 39.1 (range 23–55 years).

Ethical approval

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

T_m measurement

Absorbance versus temperature heating curves were obtained using a Perkin–Elmer Lambda 2 spectrophotometer [19,30,31]. The temperature of the five-cell holder was regulated by a thermoelectrically controlled programmer interfaced to an IBM PS2 computer. Melting profiles were obtained by increasing the temperature at a rate of 0.5 °C per min, with the absorbance and temperature being recorded every 30 s. T_m was taken as the temperatures corresponding to half dissociation of the complexes and the reproducibility was within ± 0.5 °C. The first derivative, dA/dT (where A is absorbance and T is temperature), of the melting curve was computer-generated and was also used to determine T_m of the triplex (T_{m_1}) and duplex (T_{m_2}) forms of the polynucleotides. T_m values obtained from the two methods did not differ by more than 0.5 °C.

Measurement of CD spectra

CD spectra of poly(dA) and poly(dT) in 1:1 and 1:2 ratio in the presence and absence of spermidine and/or hydralazine were recorded using an AVIV model 62DS CD spectrophotometer. We used a 1 cm quartz cuvette for spectral measurements.

Analysis of sera for antibodies reacting with triplex DNA

Serum samples were collected at the University of Cincinnati Medical Center from hypertensive patients treated with hydralazine over a period of several years, as indicated above. In some cases sequential samples were collected, and in others samples were obtained at single points. These samples were analysed for anti-(triplex DNA) antibodies using a published ELISA procedure [32]. A spermidine-stabilized triplex DNA form of poly(dA)·2poly(dT) was used as an authentic form of triplex DNA. A duplex form of the same polynucleotides was further used to compare the binding of the sera between the different forms. Microtitre plates were coated with poly(dA)·2poly(dT) triplex formed in the presence of 25 μM spermidine at a concentration of 10 μg/ml, washed and then treated with serum samples of hydralazine recipients or normal controls at a dilution of 1:400 (200 μg/well). The wells were

subsequently treated with peroxidase-conjugated affinity-purified goat anti-human polyclonal antibodies and the enzyme substrate, *o*-phenylenediamine. The enzyme–substrate reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4/well, and the absorbance was read at 490 nm with a microplate autoreader (Molecular Devices, Palo Alto, CA, U.S.A.).

As a control, we also measured the binding of sera from hydralazine-treated patients to the double-stranded form of calf thymus DNA, by methods described previously [5].

Analysis of anti-(single-stranded DNA) antibodies in the sera of hydralazine-treated patients

We also analysed the sera from hydralazine recipients and control subjects for the presence of anti-(single-stranded DNA) antibodies using an ELISA technique [32]. In this case, heat-denatured calf thymus DNA was used as a source of single-stranded DNA. The ELISA protocol was similar to that employed to detect anti-(triplex DNA) antibodies.

Statistical analysis

Statistical significance of the differences in absorbance values of ELISA experiments between control and hydralazine-treated groups was calculated by Student's *t* test. Comparison of absorbance values with triplex DNA, duplex and single-stranded DNA antibody titres was performed using an ANOVA program.

RESULTS

The method of continuous fractions (UV mixing curves) was used to examine the formation of duplex and triplex DNAs from poly(dA)·2poly(dT) in the presence of spermidine and/or hydralazine [19]. An inflection point at 0.5 mol fraction representing duplex DNA formation was observed both in the absence and presence of 2.5 μM spermidine and/or 50 μM hydralazine (results not shown). However, when triplex formation was examined by this method using molar fractions of the duplex DNA and a third strand of poly(dT), an inflection point representing the triplex formation was obtained only in the presence of spermidine or in the presence of spermidine and hydralazine. In order to characterize the stabilization of triplex DNA by hydralazine further, we determined the T_m of different combinations of spermidine and/or hydralazine with a Perkin–Elmer Lambda 2 UV/visible spectrophotometer.

The absorbance–temperature profile and the first derivative (dA/dT) of poly(dA) and poly(dT) mixed in 1:2 ratio in 10 mM sodium cacodylate buffer are shown in Figure 2(a). A sharp increase in absorbance centred at 55.3 °C was found, indicating the melting transition of double-helical polynucleotide. In the temperature range of 22–95 °C, there was no additional melting transition, and hence the polynucleotides existed in the duplex form even when poly(dA) and poly(dT) were mixed in a 1:2 ratio. In separate experiments, we also found that duplex DNA formed from poly(dA)·poly(dT) by mixing the polynucleotides in 1:1 ratio melted at 55.3 ± 0.2 °C.

Figure 2(b) shows the absorbance–temperature profile and derivative curve of poly(dA)·poly(dT) in the presence of 250 μM hydralazine. We used this concentration because previous experiments showed that 250 μM hydralazine exerted optimal interaction with DNA and shifted the conformation of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) to the Z-DNA form. In the presence of hydralazine, the polynucleotide melted at 52.5 °C, a temperature slightly lower than the T_m of poly(dA)·poly(dT) in the absence of hydralazine. This result is consistent with previous studies by us [7] and others showing a destabilization of duplex DNA by hydralazine [33].

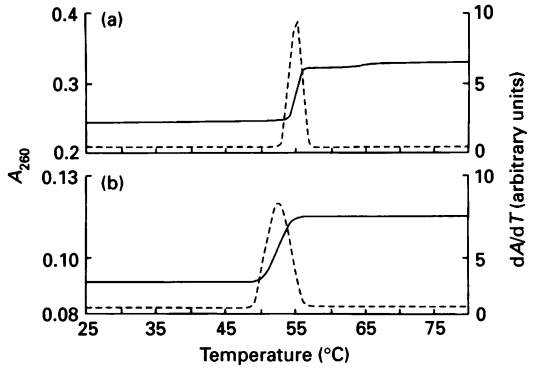

Figure 2 UV absorbance versus temperature profiles and the first-derivative plots of poly(dA)·2poly(dT) in the absence (a) and presence (b) of 250 μM hydralazine

All experiments were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.2) and 0.5 mM EDTA. ——, A_{260}; ----, dA/dT.

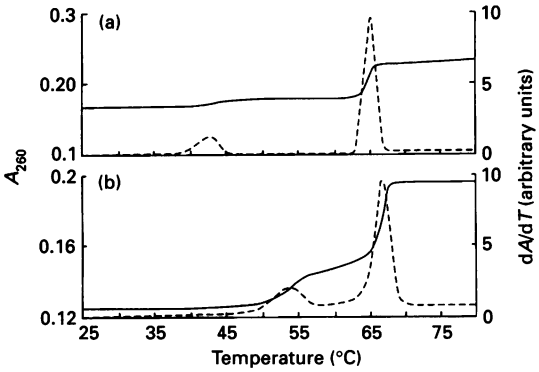

Figure 3 Effect of 25 μM spermidine (a) and 25 μM spermidine + 250 μM hydralazine (b) on the UV absorbance versus temperature profiles and the first-derivative plots of poly(dA)·2poly(dT)

The first inflection point and derivative represent the melting of the triplex (T_{m_1}), and the second inflection point and derivative represent the melting of the duplex DNA (T_{m_2}). ——, A_{260}; ----, dA/dT.

In the next set of experiments, we examined whether hydralazine is capable of interacting with triplex DNA. We used 2.5 μM spermidine to stabilize the triplex form of poly(dA)·2poly(dT). In Figure 3(a), we show the absorbance–temperature profile of poly(dA)·2poly(dT) in the presence of 10 mM sodium cacodylate buffer containing 2.5 μM spermidine. There are two melting transitions in this case, the first centred at 42.8 °C, and the second at 65.4 °C. We designated the first melting transition as T_{m_1}, corresponding to the melting of poly(dA)·2poly(dT) to duplex poly(dA)·poly(dT) and single-stranded poly(dT). The second melting transition (T_{m_2}) corresponds to the melting of duplex DNA in the presence of spermidine. It is interesting to note that spermidine increased T_{m_2} by 12.9 °C. In order to confirm that this increase was due to the stabilizing effect of spermidine on the duplex form of poly(dA)·poly(dT), we determined the effect of spermidine on the T_m of authentic poly(dA)·poly(dT). Our results are presented

Table 1 Effect of spermidine on the T_m of the duplex form of poly(dA)·poly(dT)

T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.2) and 0.5 mM EDTA.

Concentration of spermidine (μM)	T_m (°C)
0	55.4
2.5	65.4
10	68.0
25	72.5
50	75.6
100	81.9

Table 2 Effect of hydralazine on the T_m of the spermidine- or Mg^{2+}-stabilized triplex form of poly(dA)·2poly(dT)

All T_m measurements were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.2) and 0.5 mM EDTA.

Concentration of spermidine (μM)	Concentration of Mg^{2+} (mM)	Concentration of hydralazine (μM)	T_{m_1} (°C)	T_{m_2} (°C)
0	0	0	–*	55.3
2.5	0	0	42.8	65.4
2.5	0	25	44.0	65.0
2.5	0	50	46.2	64.2
2.5	0	100	49.8	65.0
2.5	0	250	53.6	66.2
0	5	0	64.6	83.9
0	5	25	66.6	83.3
0	5	50	69.7	83.0
0	5	100	72.1	82.7
0	5	250	83.3	83.3

* No T_{m_1} was observed above 22 °C.

in Table 1. Spermidine has a dose-dependent stabilizing effect on the T_m of duplex DNA.

The addition of 250 μM hydralazine to poly(dA)·2poly(dT) triplex had a significant effect on T_{m_1} and T_{m_2} as shown in Figure 3(b). The increases in T_{m_1} and T_{m_2} in the presence of hydralazine were 10.8 and 0.8 °C respectively. Thus hydralazine exerted a remarkable stabilizing effect on the triplex form of poly(dA·2poly(dT) even in the absence of a marked effect on the T_m of duplex DNA. There was also a significant increase in the amplitude of the T_{m_1} peak in the presence of hydralazine, suggesting structural alterations in triplex DNA stabilized by this drug. We further examined the dose-dependent effect of hydralazine on the T_m of triplex DNA formed in the presence of both spermidine and Mg^{2+}. There was a significant effect of hydralazine on the stability of triplex DNA formed in the presence of these two cations (Table 2).

We next recorded the CD spectra of poly(dA)·poly(dT) in 1:1 and 1:2 ratio in the presence of different concentrations of spermidine (Figures 4a and 4b). In the duplex form, poly(dA)·poly(dT) had two negative bands at 205 and 246 nm, and three positive peaks at 217, 260 and 282 nm (Figure 4a). The intensity of the 282 nm peak was slightly lower than that of the 260 nm peak for duplex DNA. At a concentration of 2.5 μM, spermidine had only a minor effect on reducing the intensity of

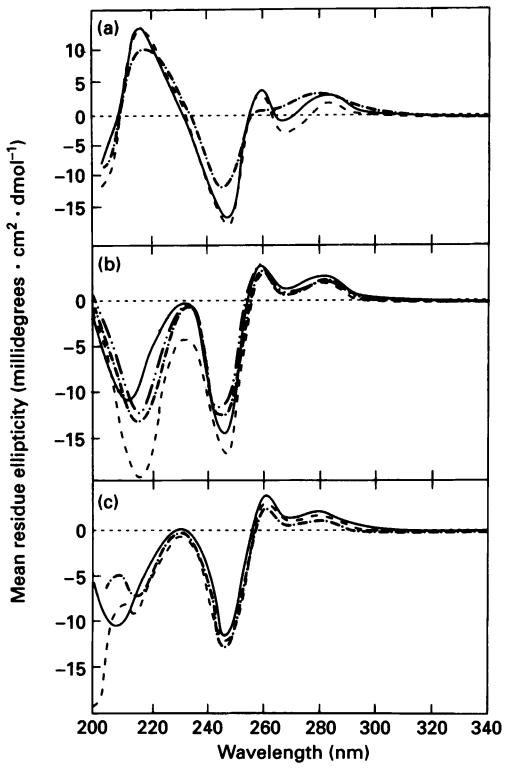

Figure 4 CD spectra of (a) poly(dA)·poly(dT) in the presence of 0 (——), 2.5 (– – –) and 25 (– · – ·) μM spermidine, (b) poly(dA)·2poly(dT) in the presence of 0 (——), 2.5 (– · – ·), 10 (– · · – · ·) and 25 (– – –) μM spermidine and (c) poly(dA)·2poly(dT) in the presence of 2.5 μM spermidine and 0 (——), 50 (– – –) and 250 (– · – ·) μM hydralazine

All CD spectra were recorded at 22 °C in a buffer containing 10 mM sodium cacodylate (pH 7.2) and 0.5 mM EDTA.

the 282 nm positive peak and in provoking a negative band at 268 nm, whereas 25 μM spermidine caused a reduction in the peak intensity of both the positive band at 217 nm and the negative band at 246 nm. In addition, the positive band at 260 nm was eliminated and a broad positive band centred at 280 nm was formed.

The distinctive feature of poly(dA) and poly(dT) mixed in 1:2 molar ratio was the complete disappearance of the 217 nm positive band and the appearance of a strong negative band centred at 212 nm (Figure 4b). With the addition of spermidine, the intensity of this band sharpened with a shift in the peak maxima to 217 nm. This negative band is considered to be a characteristic feature of triplex DNA [31]. There were no major alterations in the nature or intensity of other bands. Addition of hydralazine to poly(dA)·2poly(dT) in the presence of 2.5 μM spermidine slightly reduced the peak intensity and caused the appearance of a second negative peak at wavelengths below 200 nm (Figure 4c). The alterations in the CD spectra of poly(dA)·poly(dT) and poly(dA)·2poly(dT) in the presence of spermidine as a single agent or spermidine and hydralazine in combination indicated conformational alterations caused by these ligands in duplex and triplex DNA. UV melting experiments further indicated that these conformational transitions were associated with the stabilization of the duplex- and triplex-DNA structures.

Hydralazine is known to elicit antibodies reacting with histones, single-stranded DNA and Z-DNA in patients treated with

Table 3 ELISA results from sera from hydralazine-treated patients and controls

DNA on microtitre plate	A_{490} Normal controls	A_{490} Hydralazine-treated patients	P
Single-stranded DNA (calf thymus)	0.042 ± 0.03	0.15 ± 0.16	< 0.0001
Double-stranded DNA (calf thymus)	0.002 ± 0.001	0.005 ± 0.008	> 0.1
Triplex DNA [poly(dA)·2poly(dT)]	0.045 ± 0.02	1.095 ± 1.01*	< 0.0001
Duplex DNA [poly(dA)·poly(dT)]	0.035 ± 0.02	0.492 ± 0.37*	< 0.001

*$P < 0.002$ between binding of triplex and duplex DNA.

this drug. The molecular basis of anti-nuclear antibody production by hydralazine is not as yet clear. As triplex DNA was reported to be immunogenic, we next examined whether serum from patients treated with hydralazine contained antibodies reactive with this form of DNA. To accomplish this, we used an ELISA protocol used in our previous studies [32]. Microtitre plates were coated with the triplex form of poly(dA)·2poly(dT) in the presence of 50 μM spermidine to test for the presence of anti-(triplex DNA) antibodies. Polynucleotides in 1:1 molar ratio (duplex) were used as a control. Furthermore, the binding of sera to single-stranded DNA was also studied.

Table 3 presents the results of our ELISA experiments. Absorbance values obtained for the binding of sera from hydralazine-treated patients and controls to different forms of DNA show significant differences in the binding of the sera to these forms. Sera from normal controls exhibited only negligible binding to double- and single-stranded forms of calf thymus DNA. There was considerable binding of these sera to the duplex form of poly(dA)·poly(dT), which might be due to its ability to adopt unusual structures [33]. Sera from hydralazine-treated patients showed greater binding to the triplex form of the polynucleotides, and the mean binding of the sera to the duplex form of the same polynucleotides was significantly less ($P < 0.002$). Although these sera exhibited detectable binding to single-stranded calf thymus DNA, the mean absorbance value was 7-fold lower than that to the triplex form of poly(dA)·2poly(dT). The binding of these sera toward double-stranded calf thymus DNA was negligible. These data demonstrate that sera from hydralazine-treated patients bind triplex DNA with increased avidity compared with the duplex form of the same polynucleotides or calf thymus DNA.

DISCUSSION

Our results show for the first time that hydralazine, a phthalazine derivative with a reactive hydrazine group at the C-3 position and an antihypertensive drug with lupus-inducing side effects, is capable of stabilizing the triplex DNA form of poly(dA)·2poly(dT). This stabilization effect is found with both Mg^{2+}- and spermidine-stabilized triplex DNA. Triplex formation of these polynucleotides is associated with conformational perturbations, as detected by CD measurement. Our results further demonstrate a differential mode of interaction of hydralazine with duplex and triplex DNA. As reported for calf thymus DNA [7,34], this drug caused slight destabilization of the duplex form of poly(dA)·poly(dT), whereas a 10.8 °C increase in the T_m of the triplex form of poly(dA)·2poly(dT) was found. Both duplex and triplex forms of the polynucleotides were also stabilized by spermidine and Mg^{2+}.

The mechanism of binding of hydralazine to duplex and triplex forms of DNA is not known at present. Earlier studies by Dubroff and Reid [8] showed that hydralazine could covalently interact with thymidine and deoxycytidine, and hydralazine adducts of these bases were isolated by HPLC. An intercalative mode of binding of hydralazine to DNA was proposed by Sinha and Patterson [9] who found strong binding of this drug to DNA. In this context it is interesting to note that Scaria and Shafer [31] found that a well-known DNA intercalator, ethidium bromide, stabilized the triplex form of poly(dA)·2poly(dT) in the buffer conditions used in our experiment. Since the third strand in triplex DNA binds through the major groove of DNA, this groove may become relatively unavailable for accommodation of the drug. Thus triplex DNA may bind through the minor groove of DNA or via intercalation of the drug molecule between DNA bases. Alternatively, hydralazine binding to triplex DNA might be governed by electrostatic interaction between the positively charged amino group of hydralazine and the negative phosphate groups on DNA. As in the case of the binding of polyamines to DNA [35–37], both modes of interaction may be operative, providing sequence specificity in the binding of hydralazine to DNA.

Although triplex DNA has been described in the literature for more than three decades [38,39], its biological relevance and potential therapeutic implications have come to the forefront of DNA structural chemistry only recently [40,41]. Triplex-forming sequences consisting of purine–pyrimidine tracts are present in the regulatory regions of several genes, including c-*myc* [42], interleukin 2 receptor [43] and DNA polymerase α [44], and represent up to 1% of certain eukaryotic genome [18]. Only a limited number of ligands have been demonstrated to stabilize triplex DNA. These include polyamines [19,45–47], $MgCl_2$ [14–17], ethidium bromide [31] and benzo[*e*]pyridoindole derivatives [48]. Our recent studies [19] demonstrate the ability of polyamines to stabilize triplex DNA and the importance of ionic and structural factors necessary to provoke this form. In the present study, we demonstrate that triplex DNA induced by polyamines can be further stabilized by agents such as hydralazine, a drug that appears to affect the conformational and structural stability of DNA. As spermidine and other polyamines are natural constituents of the cells [20], and their levels are high in disease states [49], it is possible that polyamines induce triplex-DNA formation in serum where nucleosome-length DNA fragments are formed by drug action or other mechanisms. Thus the discovery of hydralazine-induced stabilization of triplex DNA is an important step in understanding the nature of agents that stabilize this form of DNA.

Our serological analysis of 25 hydralazine-treated patients shows that the majority of sera collected at different time points contain antibodies reacting with the triplex form of poly(dA)·2poly(dT). The mean absorbance indicating binding of sera to triplex DNA is 3-fold higher than the binding of the same sera to the duplex DNA form of the same polynucleotides ($A = 1.095 \pm 1.01$ compared with 0.492 ± 0.37; $n = 65$; $P = 0.002$). Binding of these sera to single-stranded calf thymus DNA is very low ($A = 0.15 \pm 0.16$). Previous studies showed that their binding to double-stranded calf thymus DNA was also negligible, whereas they exhibited high levels of binding toward the Z-DNA form of polynucleotides [5]. Increased binding of hydralazine-related sera to the duplex form of the polynucleotides might be a consequence of disproportionation of the polynucleotides toward the triplex form in the presence of the protamine sulphate

used to achieve nucleic acid binding on microtitre plates. The affinity of these antibodies for unusual forms of DNA (triplex and Z-DNA) suggests that these forms could have acted as immunogenic stimuli and elicited these antibodies.

In summary, we provide evidence for high-affinity binding and stabilization of poly(dA)·2poly(dT) by hydralazine, a drug frequently associated with anti-nuclear antibody production in treated patients. We also show that the majority of sera from a panel of hydralazine-treated patients exhibited high-affinity binding for the triplex form of poly(dA)·2poly(dT). The binding of these sera to the same polynucleotides was significantly lower in the absence of a triplex-stabilizing ligand. These data suggest that a possible mechanism for anti-nuclear antibody production in hydralazine-treated patients might involve the induction and stabilization of immunogenic forms of DNA, including higher-order structures such as triplex DNA.

This work was supported by a research grant from the New Jersey Chapter of the Arthritis Foundation, by the W. H. Conzen Endowment of the Schering-Plough Foundation, and by funds from the GEIER Foundation of Cincinnati. The technical assistance of the following individuals is gratefully acknowledged: Dr. G. D. Kulkarni (CD and T_m measurements), Mr. Yong-Ping Yan and Rajesh Balagani (T_m measurements) and Dr. Uma Gunnia, Mr. Yong-Ping Yan and Mrs. Sreedevi Adhikarakunnathu (ELISA experiments).

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Received 1 June 1995; accepted 12 June 1995