Unique poly(dA)·poly(dT) B'-conformation in cellular and synthetic DNAs

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ABSTRACT

Poly(dA)·poly(dT), but not B-form DNA, is specifically recognized by experimentally induced anti-kinetoplast or anti-poly(dA)·poly(dT) immunoglobulins. Antibody binding is completely competed by poly(dA)·poly(dT) and poly(dA)·poly(dU) but not by other single- or double-stranded DNA sequences in a right-handed B-form. Antibody interaction with poly(dA)·poly(dT) depends on immunoglobulin concentration, incubation time and temperature, and is sensitive to elevated ionic strengths. Similar conformations, for example, (dA)₄₋₆·(dT)₄₋₆, in the kinetoplast DNA of the parasite *Leishmania tarentolae* are also immunogenic and induce specific anti-poly(dA)·poly(dT) antibodies. These antibody probes specifically recognize nuclear and kinetoplast DNA in fixed flagellated kinetoplastid cells as evidenced by immunofluorescence microscopy. Anti-poly(dA)·poly(dT) immunofluorescence is DNase-sensitive and competed by poly(dA)·poly(dT), but not other classical double-stranded B-DNAs. Thus, these unique cellular B'-DNA helices are immunogenic and structurally similar to synthetic poly(dA)·poly(dT) helices in solution.

INTRODUCTION

In aqueous solutions poly(dA)·poly(dT) has a right-handed double-stranded B'-type DNA conformation with 10.1 base pairs per helical turn [bp/t; (1,2)]. Core histones do not bind to relatively lengthy linear poly(dA)·poly(dT) polymers (3,4) or to long (dA)_n·(dT)_n tracts inserted into recombinant plasmids (5,6). Furthermore, *Fok I* restriction endonuclease cleaves (dA)_n·(dT)_n only minimally, while it efficiently cleaves other classical B-DNA sequences (S. Diekmann and M. Robert-Nicoud, unpublished observations).

In contrast poly(dA-dT)·poly(dA-dT), like B-type natural DNAs, assembles onto nucleosomes (7). The differences between these structures are summarized in Table 1 and Figure 1 shows computer-generated conformations. Poly(dA)·poly(dT) differs from normal B-form DNA in X-ray diffraction (8,9), Raman (10), UV circular (11), and linear dichroic spectra (12). A likely interpretation is that poly(dA)·poly(dT) may adopt a unique structure, referred to as B'-DNA. We report here that experimentally induced immuno-

Table 1. COMPARISON OF POLY(dA)·POLY(dT) AND POLY(dA-dT)·POLY(dA-dT)

PROPERTY	poly(dA)·poly(dT)	poly(dA-dT)·poly(dA-dT)
conformation	B'	B
isomerization	very difficult	B→A B→Z
base-pairs per helix turn (1,2)	10.1 ± 0.1,	10.6 ± 0.1
<u>in vivo</u> and <u>in vitro</u> assembly as nucleosomes (3-7)	not assembled on synthetic polymer or recombinant plasmid	assembled as nucleosome
anti-poly(dA)·poly(dT) and anti-kinetoplast IgG binding	specifically bound	not bound

globulins (Igs) specifically recognize unique features of the (dA)_n·(dT)_n conformation in synthetic DNA in solution and in natural DNA sequences in fixed cells.

MATERIALS AND METHODS

1. Synthetic Polymers and Immunizations. Synthetic DNAs and RNAs were from Pharmacia, P-L Biochemicals. Polymers were dissolved in buffer containing 5 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA. Four- to five-month-old female New Zealand White rabbits were each inoculated at multiple intradermal sites with 150 µg poly(dA)·poly(dT) or with 50 µg pKS414 (13) plasmid DNA cut with Hind III (New England Biolabs). pKS414 is a pBR322 derivative containing a deletion from base-pair 235 to 2352 and has a kinetoplast DNA fragment of 414 bp (13,14) inserted in the Hind III site. The DNAs were complexed 1:1.5 (wt/wt) with methylated bovine serum albumin (BSA, Sigma) and emulsified in complete Freund's adjuvant (Behring). Subsequent booster inoculations were emulsified in incomplete adjuvant. Serum was incubated at 56°C for 15 min and stored frozen at -70°C. Anti-nucleic acid Ig binding activity was measured by radioimmunoassay. IgGs were purified as described by

Zarling et al. (15). For titrations, rabbit IgG concentrations were adjusted to an initial concentration of approximately 1.27 mg/ml, assuming that 1 A₂₈₀ Unit is 1.4 mg/ml IgG. Circular dichroism spectra were measured in a Jobin-Yvon Dichrograph V.

2. Radioimmunoassays (RIA) and Competition Experiments. Reactions (60 μ l) were performed with antibodies in standard RIA buffer containing final concentrations of 40 mM Tris-HCl, pH 7.5, 4 mM EDTA, and 50 mM NaCl. Polynucleotides were [³²P]5'-end-labeled for RIA experiments with polynucleotide kinase (16). In standard or competitive RIAs, approximately 0.05 μ g ³²P-poly(dA).poly(dT) substrate (about 6000 cpm, specific activity 120 cpm/ng DNA) was pre-equilibrated at 37°C for 5 min with various concentrations of unlabeled competitor DNA or RNA. Next, 50 μ l of serum or purified IgG was added. Primary antibody-antigen reactions were incubated for 1 hour at 37°C. Subsequently, the IgG fraction of goat anti-rabbit IgG (Behring or Miles) was added and incubated for an additional hour at 30°C. Immune complexes were centrifuged at 10K $\times$ g at room temperature and immunoprecipitates were washed three times by resuspension and centrifugation in buffer containing 200 mM NaCl, 40 mM Tris-HCl, pH 7.6, 4 mM EDTA, and then dissolved in 0.2 M NaOH. Finally, 5.0 ml of Aquasol or Aqualuma (NEN-Dupont or Baker) was added and the radioactivity measured in a liquid scintillation counter (Beckman). The experimental background ranged from 2-5% in different experiments and was subtracted from the data shown.

3. Cells, Immunofluorescent Staining, Specificity Tests, and Nuclease Digestions. Cells of the Dipteran fly parasite, *Crithidia luciliae*, were grown in culture and fixed on toxoplasmosis slides (Bellco) in ice-cold ethanol and subsequently in acetone for 10 min each. Slides were stored at -20°C. Sera or purified IgG were diluted in phosphate buffered saline [(PBS); 0.14 M NaCl, 3 mM KCl, 4 mM Na₂HPO₄, 2 mM KH₂PO₄ containing 1 mg/ml nuclease free bovine serum albumin (BSA) (BRL)] and approximately 15 to 25 μ l of antibody dilution was incubated with prewarmed fixed cells for 30 minutes at 37°C. Cells were washed twice in PBS for 5 minutes each. Next, 15 to 25 μ l of the IgG fraction of affinity purified goat anti-rabbit IgG conjugated with FITC (GARIG-F) was applied at a concentration of 50 μ g/ml in PBS containing 1 mg/ml BSA. Slides were then washed, blotted, and mounted in Mowiol 4.88 (Calbiochem). Phase contrast and fluorescent microscopy was performed with Zeiss or Olympus epi-illumination microscopes fitted with the manufacturer's filter combinations. Fluorescent images were photographed with Kodak Tri-X Pan film.

In competition experiments, 5 μ l of pretitered antibody was incubated with 20 μ l of competitor polynucleotide at the given final concentrations. Following a 60-min preincubation at 37°C, the mixtures were centrifuged for 15 min at 10K $\times$ g and the supernatant applied to fixed cells as described. For nuclease digestions, cells on slides were incubated with DNase I (Boehringer) or RNase A (Sigma) at a concentration of 0.5 mg/ml in PBS buffer for 1 hr at 37°C. Reactions with DNase were supplemented with 5 mM MgCl₂ and this concentration of MgCl₂ had no effect on immunofluorescence. Cells were washed with PBS and incubated with the primary antibody and then GARIG-F, as described above.

RESULTS AND DISCUSSION

1. Antibodies Recognizing Unique poly(dA)·poly(dT) Conformations

a. Synthetic or Natural (dA)_n·(dT)_n Sequences Are Immunogenic. In two separate experiments, we injected three rabbits, lacking any measurable endogenous nucleic acid antibodies, with poly(dA)·poly(dT) and followed the time-course of the immune response (Figure 2). All animals produced an immunoglobulin (Ig) response which peaked within the first three months after inoculation, as measured by radioimmunoassay with ³²P-labeled homologous DNA (Figure 2 and similar data not shown). In different animals endpoint serum titers of up to approximately a 1/2000 dilution of serum were observed. However, the response could not be boosted to higher levels by repeated immunization. Increasing the amount of DNA in the booster inoculation to either 200 or 300 μ g also had no effect.

Similarly, two rabbits immunized with a 414 base-pair parasite kinetoplast DNA fragment (13,14) produced anti-DNA Ig as measured with a ³²P-poly(dA)·poly(dT) substrate (Figure 2). Both rabbits immunized with pKS414 produced anti-DNA Ig. However, similar to anti-poly(dA)·poly(dT) responses, the titers could not be increased by subsequent booster inocu-

Figure 1. Comparison of B'-form poly(dA)·poly(dT) (left side) and B-form poly(dA-dT)·poly(dA-dT) (right side) conformations. The structure of poly(dA)·poly(dT) proposed by Arnott et al. (8) from fiber diffraction data has been used as starting coordinates in the AMBER program of Weiner and Kollmann (28). The result of the minimization for poly(dA)·poly(dT) is compared with poly(dA-dT)·poly(dA-dT) constructed from the atomic positions indicated by the data of Arnott and Hukins (29). The figures were constructed using the SHAKAL plot program kindly provided by Dr. E. Keller. The N-6 position of adenine is labeled (N, nitrogen; H, hydrogen) as well as a phosphate (P) of the dA-strand.

Figure 2. Timecourse of the immune response to poly(dA)·poly(dT) (●, ○) and kinetoplast DNA (*). Booster inoculations are indicated with arrows. Serum titers were measured by RIA with ^{32}P -poly(dA)·poly(dT) substrates and normalized to 1 with respect to the highest value obtained. Approximately 35% of the ^{32}P -poly(dA)·poly(dT) probe was precipitated with a 10-fold dilution of immune serum.

lations. In each animal the antibody titers against poly(dA)·poly(dT) fell significantly after the third to fourth month post-injection.

In other similar immunization regimes, left-handed Z-DNA is immunogenic and the Ig response can be boosted, while many synthetic B-DNAs of similar lengths are relatively weakly or nonimmunogenic (15,17,18). In general, inoculation of normal animals with synthetic or natural DNA does not elicit immunoglobulins specifically directed against double-stranded DNA with a canonical right-handed B-type structure (17,19,20). However, single-stranded DNAs can be immunogenic (for reviews see 20-23). Thus, poly(dA)·poly(dT) and certain kinetoplast DNA sequences are immunogenic *in vivo*, in contrast to B-DNA, which is relatively nonimmunogenic.

b. Specificity of Anti-DNA Immunoglobulins and Effect of NaCl. The specificity of anti-poly(dA)·poly(dT) Ig binding was measured by competition between ^{32}P -labeled poly(dA)·poly(dT) and varying amounts of unlabeled polynucleotides. Poly(dA)·poly(dT) was specifically and completely competed only by poly(dA)·poly(dT) and poly(dA)·poly(dU) (Figure 3). Poly(rA)·poly(dT) only partially competed, suggesting certain similarities in the two structures including common sugar pucker configurations (8) (Figure 3). There was no

Figure 3. Specificity of the immune response to poly(dA)·poly(dT) (left) and kinetoplast DNA (right). Competition between ^{32}P -poly(dA)·poly(dT) and various unlabeled DNAs for binding (A) anti-poly(dA)·poly(dT) serum antibodies and (B) anti-kinetoplast serum antibodies. ^{32}P -poly(dA)·poly(dT) (0.09 μg for reactions shown in (A) and 0.04 μg for those in (B), with the exception of the poly(dA) reactions, which contained 0.007 μg substrate) was competed with unlabeled: (●), poly(dA)·poly(dT); (□), poly(dA)·poly(dU); (○), poly(dA-dT)·poly(dA-dT); (△) poly(rA)·poly(rU); (✱), poly(rA)·poly(dT); and (+), single-stranded poly(dA). The competition reactions shown in (A) with (●), (○), and (+) were in RIA buffer without NaCl.

significant competition with double-stranded poly(dA-dT)·poly(dA-dT), poly(dG)·poly(dC), poly(rA)·poly(rU), or single-stranded poly(dT), poly(dG), or poly(dC) (Figure 3 and similar data not shown). Similar specificities were observed for the other antibodies and with purified IgGs (see below).

Serum antibodies were concentrated by ammonium sulfate precipitation, dialyzed at 4°C against 10 mM sodium acetate, pH 5.5, and IgG extracted in bulk with DEAE-Sephadex (A-50) (15). In this fraction both IgM and IgG specific anti-poly(dA)·poly(dT) responses were detected. The serum from rabbit 5, injected with poly(dA)·poly(dT), contained about 30% of the total activity in IgM enriched fractions and 70% in IgG. IgGs were purified by chromatography on protein A-Sepharose (15).

Anti-poly(dA)·poly(dT) IgG or anti-kinetoplast IgG binding to ^{32}P -poly(dA)·poly(dT) substrates were both specifically and completely competed by poly(dA)·poly(dT). Furthermore, there was no reactivity of these polymers with IgG purified from normal rabbit serum. The extent of the RIA monitored binding reaction directly depended on both the amount of antibody added and

Figure 4. The effect of NaCl on the binding of anti-poly(dA)·poly(dT) antibodies to ^{32}P -poly(dA)·poly(dT). The NaCl concentration was adjusted to the indicated final concentration in standard RIA buffer. The data represents the mean value of two independent measurements.

the inclusion of second antibody to efficiently form sedimentable cross-linked networks (data not shown).

One anti-kinetoplast and one anti-poly(dA)·poly(dT) Ig showed a small but significant reactivity with single-stranded poly(dA) (Figure 3 and data not shown). At neutral pH, poly(dA) has strong stacking interactions that yield a helical structure even as a single-strand (24). Interwound into a double-helix, with poly(dT) as the opposite strand, poly(dA) may retain some features of its own helical form (8,9), consistent with the observed partial reactivity of the anti-poly(dA)·poly(dT) antibodies with single-stranded poly(dA). However, this partial reactivity could also be due to trace amounts of single-stranded DNA in the immunogens.

Antibody binding decreased linearly with the logarithm of the ionic strength and was completely inhibited in reactions supplemented with 400 mM NaCl (Figure 4). Binding of anti-poly(dA)·poly(dT) or anti-kinetoplast DNA antibodies was optimal at 37°C in the presence of 50 mM NaCl at 37°C.

The effect of NaCl could be due to effects on the antibody, the antibody-DNA interaction, or the DNA itself. The effect of NaCl on the UV circular dichroic (CD) spectra of poly(dA)·poly(dT) showed no significant structural changes in poly(dA)·poly(dT) at NaCl concentrations ranging from 50 to 500 mM (data not shown). Thus, the effect may not be on the DNA alone.

Figure 5. Anti-poly(dA)·poly(dT) recognition of cellular DNA. Fixed *Crithidia luciliae* cells were preincubated with 0 (A) or 0.5 mg/ml DNase I (B) before application of anti-poly(dA)·poly(dT) antibody and GARIG-F. Note that nuclear and kinetoplast staining was eliminated in cells treated with DNase I. Nonspecific staining of the basal body by normal rabbit IgG was also eliminated by DNase I. In A the immunofluorescence of cellular DNA is indicated by: n, nucleus; k, kinetoplast; and b, basal body. The bar denotes 5 μ m. The photographic exposure and development times in A and B were identical.

c. Protein Recognition of B'-DNA Conformation. Poly(dA)·poly(dT) displays a double helical structure which is different from normal B-DNA. The dA·dT base pairs fold into the narrower minor groove (9). The phosphates of the A-strand point into the major groove coming close to the N-6 of the adenines and, therefore, the lower part of the major groove is less notched compared with normal B-DNA (see Figure 1). These structural differences may be recognized by proteins and enzymes. The restriction endonuclease Fok I cuts B-form DNAs approximately 10 times faster than the B'-conformation of poly(dA)·poly(dT) when cloned next to the Fok I recognition site in recombinant plasmids (Diekmann and Robert-Nicoud, unpublished experiments). In addition, the long (dA)·(dT) stretches are only partially cut. As shown here, experimental anti-poly(dA)·poly(dT) immunoglobulins specifically bind this helix (Figure 3).

Minicircles of kinetoplast DNA from *Leishmania tarentolae* (and some other parasites) contain repeats of the DNA sequence dC(A)₅₋₆T which are believed to cause the slower electrophoretic migration of dC(A)₅₋₆T containing DNA fragments in gels (25,26). These effects are interpreted as a sequence-directed curving of the fragment. Helical repeat measurements of these stretches yield 10.4 ± 0.1 (12) base pairs per helical turn, a value between

those of classical B-form DNA (10.6 ± 0.1) and of B'-form poly(dA)·poly(dT) (10.1 ± 0.1). This result suggests that the stretches of (dA)₅₋₆ (about half of a helix turn) have B'-like conformation, while other parts of the sequence may have a B-conformation. This alteration is assumed to cause curving (for a review, see 27). Rabbits injected with kinetoplast DNA, containing these repeat sequences, generate IgGs which can specifically recognize synthetic poly(dA)·poly(dT) (Figure 3) indicating that the (dA)₅₋₆ stretches within the repeats are the immunogens. In addition, the data suggest that the short dA₅₋₆ stretches have a B'-form structure similar to that of long poly(dA)·poly(dT) sequences.

The permanent curvature in certain kinetoplast DNA repeats or small regions around nucleosomes may be affected by conformation- and/or sequence-specific cellular proteins with binding specificities similar to the antibodies described here. In some cases, bivalent cellular proteins could be involved in DNA curving and/or nucleosome positioning.

2. Recognition of Cellular DNA Helices

a. (dA)_n·(dT)_n Conformations in Nuclei and Kinetoplasts. Figure 5 shows fixed kinetoplastid flagellates incubated with rabbit anti-poly(dA)·poly(dT) antibody and subsequently stained with GARIG-F. Both nuclear and kinetoplast DNAs were specifically stained (Figure 5A). This immunofluorescence was dependent on the specific antibody, its concentration, and the time and temperature of incubation. The staining of the basal body (observed by phase contrast microscopy) was nonspecific as evidenced by reactivity with normal rabbit antibodies. However, no nuclear or kinetoplast immunofluorescence was observed with second antibody alone or with preimmune or other normal rabbit antibodies.

b. Tests for Specificity. Preincubation of anti-poly(dA)·poly(dT) or anti-kinetoplast DNA antibodies with excess (50-250 µg/ml) poly(dA)·poly(dT) completely eliminated the immunofluorescent staining in both the nucleus and the kinetoplast (data not shown). However, preincubation of these antibodies with the same amount of poly[d(A-T)] had no effect.

When the fixed parasites were digested with 0.5 mg/ml DNase I, fluorescence was eliminated (Figure 5B). Digestion with RNase A had no effect. Thus, anti-poly(dA)·poly(dT) antibodies specifically recognize cellular kinetoplast and nuclear DNAs. These cellular DNA conformations are, therefore, immunologically similar to the B'-form of poly(dA)·poly(dT) in solution.

SUMMARY

In summary, the B' structure of poly(dA)·poly(dT) and similar kinetoplast DNA sequences are sufficiently different from classical B-DNA to give rise to a humoral immune response in rabbits. The data suggest that the short tracts of (dA)₄₋₆·(dT)₄₋₆ in the kinetoplast DNA fragment are similar in structure to poly(dA)·(dT).

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