ANTIBODIES TO DNA

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I. INTRODUCTION

The study of anti-DNA antibodies has several motivations. One derives from the role of anti-DNA antibody formation as part of an autoimmune response that is characteristic of the disease systemic lupus erythematosus (SLE). It is hoped that an understanding of the nature and origin of these antibodies and of their target sites can lead to more specific control of the disease than is presently available. A second motivation is the consideration of anti-DNA antibodies as model systems for protein recognition of nucleic acid components and conformations — systems in which adequate amounts of proteins with varying recognition sites can be used to analyze the interaction. A further aim is the development of specific antibodies as biochemical reagents for detection of unique conformations that may constitute a small fraction of the total nucleic acid of complex biological systems.

Work in this field has been under way since about 1957, when DNA was identified as a reactant for SLE autoantibodies.[1-4] The studies were accelerated when, in the early 1960s, methods were developed for experimental induction of antibodies to nucleic acids,[5-9] which until then had been considered widely to be nonimmunogenic. A further acceleration has come from the ability to prepare homogeneous monoclonal representatives of both auto-antibodies and experimentally induced antibodies. Contributions to the understanding of the autoimmune phenomena and clinical correlations are reviewed elsewhere.[10-13] This article will consider the questions of specificity of several kinds of anti-DNA antibodies and their applications as biochemical reagents. Stages in the development of these aspects have been examined in earlier reviews also.[14-23]

When models of helical DNA are displayed, they usually portray the structure as a straight elongated rod built of base-paired nucleotides that are essentially uniform in most bond angles. Recently, there has been growing interest in the potential for variability in the DNA helix. The interest has arisen from X-ray diffraction studies of polynucleotide fibers[24] and from the descriptions at atomic resolution of the crystal structure of helical oligonucleotides.[25-27] In the structure of a B-helical crystal dodecamer for example, there occurs variability in the angle between the base and the sugar, in the sugar puckering, and in the propeller twisting and roll orientation in the base pairs, and there is a bend along the helix axis.[25] More dramatic differences have been seen in crystal structures taking up an A-helical form[26] or the recently described left-handed Z-helical structure.[27]

A second basis for interest in helical diversity has been the repeated demonstration that certain base sequences are essential for specific functions. The direction of RNA polymerases to specific promoter sites, the localization of specific sites for origin of replication, the interaction of repressors with regulatory gene sites, and the recognition of unique structures by restriction nucleases all indicate that proteins can recognize local information content that depends on the primary sequence of nucleotides. It is possible, therefore, that this

recognition depends in part on local helical conformation. As reviewed by Wells et al.,[28] it is also possible that structural variability such as the formation of locally melted regions, supercoiling,[29] cruciform formation,[30] or the presence of a multistrand structure[31] may determine the specificity of interactions with proteins.

The analysis of this potential variability within complex structures such as chromosomes or interphase chromatin presents considerable difficulty for physical techniques. Many such techniques require purified materials and optically clear solutions (UV, IR, and CD) or relatively large quantities of material (NMR) or even more specific conditions (crystallization). Many physical measurements are averaging, so that a very small amount of an unusual form may not be detected readily against large signals from the quantitatively dominant structure(s). Certain enzymes, such as S1 nuclease, provide probes that can be used with more complex materials, but may not be specific for one unique structure; S1 nuclease could cleave at locally denatured regions, the looped ends of cruciform structure,[30] or at B-Z-DNA junctions.[32] The generation of antibodies specific for unique conformations can provide sensitive and selective reagents that can measure the presence of specific conformations present as minor components in complex materials of biological origin.

Several kinds of antibodies to nucleic acids have been described, and some have been discussed in previous reviews.[14-23] Antibodies may be directed against normal purine or pyrimidine bases of DNA or short sequences of bases (reviewed in Reference 18). Such antibodies can be induced by chemically synthesized protein conjugates of these structures[6,7,9] and can react with single-stranded nucleic acids in which the corresponding bases are exposed to solvent. They can detect small amounts of unpaired bases that may be present in a DNA preparation that is largely in a native helical conformation. Similarly, antibodies directed against chemically modified bases serve as highly selective reagents for detecting such bases when they are accessible within the DNA (reviewed in References 21 to 23). Quite different antibodies have been induced by immunization with helical nucleic acids, usually injected as complexes formed between helical synthetic polynucleotides and methylated bovine serum albumin (reviewed in References 18 to 20). Although native DNA itself has not been immunogenic in this form, many helical structures that differ from DNA have induced immune responses. Nearly all of the antibodies that are formed recognize only the differences between the immunogen and native DNA as they do not react with naturally occurring native B-DNA. For example, antibodies to A-helical forms such as double-stranded RNA or RNA-DNA hybrids have shown high selectivity for either of these forms of helix, and have served as reagents for the identification of the corresponding dsRNA helixes in virus-infected cells[33,34] or of annealed RNA-DNA hybrid in polytene chromosomes of *Drosophila*.[35-37] As will be described in this review in some detail, antibodies of high selectivity have been induced by left-handed Z-DNA, and they provided the first identification of Z-DNA in chromosomes. Weaker immune responses have been induced by right-handed helical examples of polydeoxyribonucleotides; the resulting antibodies were also highly selective. Triple-helical nucleic acids have also induced specific antibodies, allowing distinctions among various classes of triple-helical structure.[19,20] An additional source of anti-DNA antibody, including populations that will react with native DNA, is the serum of patients with autoimmune disease SLE.[10-13] The immunizing agent for these antibodies is not known, and their specificity is not as highly selective as those of many of the above-induced antibodies, but they do provide valuable reagents for the measurement of naturally occurring native B-DNA.

II. ANTIBODIES TO Z-DNA

A. Structural Features of Z-DNA

The first description of a DNA helix based on X-ray crystallography at atomic resolution contained a surprise.[27] The helix was left-handed and a zigzag line rather than a smooth

spiral connected the phosphorous atoms of the backbone; hence the name Z-DNA. The crystal was that of a hexamer, dCdGdCdGdCdG. Hexamers were aligned end-to-end in the crystal to form long chains with a continuous helix geometry. The discovery of this structure reawakened interest in the observation made several years earlier by Pohl and Jovin[38] that the polymer poly(dC-dG) underwent a sharp structural transition as the ionic strength of its solution was increased between 2 and 4 *M* NaCl. Pohl and Jovin observed a change of the CD spectrum as the ionic strength increased, from one characteristic of native DNA to one that was inverted, with a trough at 290 nm and a peak at 260 nm. They predicted that this CD inversion could represent as marked a change as a conversion from a right-handed to a left-handed helix. The crystallization of an oligomer in the left-handed form bore out this prediction, but the crystal was formed under conditions of low ionic strength, suggesting that an equilibrium exists in which some of the oligonucleotide is in left-handed form even under conditions in which most is a more conventional right-handed helix. NMR analysis of solutions,[39] infrared spectroscopy of films and solutions,[40] and laser Raman spectroscopy of both dissolved polymer and crystallized oligomer[41] confirmed that the crystal and the structure in solution of high ionic strength are identical Z-helixes. Rich et al.[42] have provided a detailed review of the chemistry and biology of Z-DNA.

The finding of a new helix geometry of such distinctive structure raised speculation as to whether it could exist in naturally occurring DNA in which its interconversion with the B form could perhaps serve as a recognition signal of functional importance. Klysik et al.[43] showed that both the Z form of poly(dC-dG) and B forms of mixed base sequence DNA could exist in a single chain in 5 *M* NaCl when the Z-forming (dG-dC) segment was about one third of the total length of the DNA or in supercoiled plasmid DNA when the segment was only about 1.3% of the total. Later studies showed that when the poly(dC-dG) sequence was present in circular plasmid DNA, the Z-helix formation was induced by negative supercoiling and was stable even in physiological ionic strength.[32,44,45] Other conditions can also favor the formation of Z-DNA. Poly(dC-dG) can be converted to the Z form by lower concentrations of Mg^{++} or Mn^{++} than of NaCl;[46,47] other cations, most notably cobalt hexamine, even at very low concentrations, converted the polymer to the Z helix.[46] The presence of certain bases, such as 5-bromocytosine[48] or 5-methylcytosine[46] and chemical bromination of poly(dG-dC) in 4 *M* NaCl[49] also stabilize Z-DNA for study at low or moderate ionic strength.

The Z-DNA helix differs from B-DNA in several structural features that render it highly immunogenic and easily distinguished from B-DNA by the antibodies it induces. The purine bases of Z-DNA are rotated into a *syn* rather than the *anti*-conformation of B-DNA.[27] Matching this, the pyrimidines flip over as well, but their turning is accompanied by rotation of the deoxyribose, so the *anti*-configuration is maintained. With this alternating different geometry of adjacent bases, there are associated changes in the backbone geometry, the base stacking, and the orientation of the base pair axis to the fiber axis.[27] With the alternating pyrimidine-purine sequence, the repeating unit of the Z-DNA is a dinucleotide pair rather than a mononucleotide pair. Of importance, from an immunochemical point of view, the deoxyribose-phosphate backbone follows a zigzag left-handed course rather than a smoothly spiralling right-handed path. The interphosphate distances differ from those in B-DNA, and the helix is slimmer with more base pairs per helical turn than in B-DNA. Even more important, the major groove of B-DNA is replaced in Z-DNA by a convex surface on which the purine N7 and C8 and the pyrimidine C5 positions are exposed and can be accessible to antibody (Figure 1).[27,42] It could be predicted that this structure would be immunologically quite distinct from B-DNA.

B. Preparation and Properties of Polyclonal Anti-Z-DNA Antibodies

1. Brominated Poly(dG-dC)

It was recognized early that if antibodies of high specificity for Z-DNA could be obtained

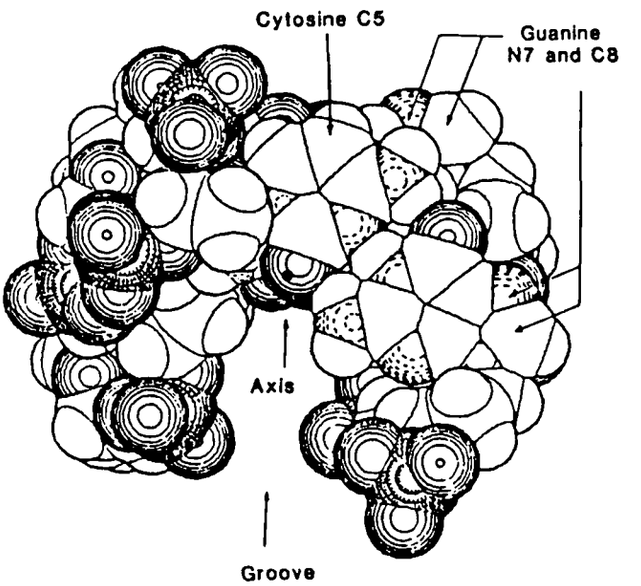

FIGURE 1. End view of Z-DNA in which three base pairs are drawn in a van der Waals diagram. In contrast to B-DNA, which has a broad major groove, the Z-DNA has a surface on which the N7 and C8 atoms of guanine and the C5 of cytosine are exposed. The axis of the base pairs is greatly displaced from the center of the fiber axis. These features, and the differences in the shape and direction of the deoxyribose-phosphate backbone, form a basis for the sharp immunological distinction between B-DNA and Z-DNA. (From Wang et al., *Science*, 211, 172, 1981. Copyright 1981 by the AAAS. With permission.)

they would be particularly useful for the search for Z-DNA as a minor component of complex biological material such as chromatin. To this end it was essential to have a means of stabilizing the Z-DNA helix so that it would not revert quickly to the B form in a solution of ionic strength suitable for injection into an animal for immunization. Suitable stabilization was provided by bromination of poly(dG-dC) which had been converted to the Z-DNA in high ionic strength.[49,50] When brominated to the extent that about 30% of the guanine residues were substituted on the 8 position and 10% of the cytosine was substituted on the 5 position, the Z-DNA structure persisted upon dialysis into a solution of physiological ionic strength, and it was suitable for immunization. The immunogen was formed as a physical complex of the brominated polymer with methylated bovine serum albumin and was injected into rabbits. It proved to be a potent immunogen, inducing about 1.2 mg of antibody per milliliter of serum.[50] The antibody reacted with brominated poly(dG-dC), but not with unmodified polymer (Figure 2). It did not react with native DNA, single-stranded DNA, single- or double-stranded RNA, RNA-DNA hybrid, or synthetic polynucleotides such as poly(dG), poly(dC), poly(dA-dT), or poly(dA)·poly(dT). The major question remaining after these findings was whether the antibody was simply recognizing brominated bases exposed on the surface of the helix that replaces the major groove. Arguing against this possibility were the findings that even high concentrations of brominated poly(G), brominated poly(dC), and brominated poly(dG)·poly(dC) did not react with the antibody.[50]

Additional observations conclusively ruled out the brominated base as a determinant of specificity. They depended on the finding that the antibody could react with its antigen under conditions of high ionic strength.[51] The reaction with brominated poly(dG-dC) in 4 *M* NaCl was as strong as in 0.2 *M* NaCl, and the reaction was also strong, in 4 *M* NaCl, with

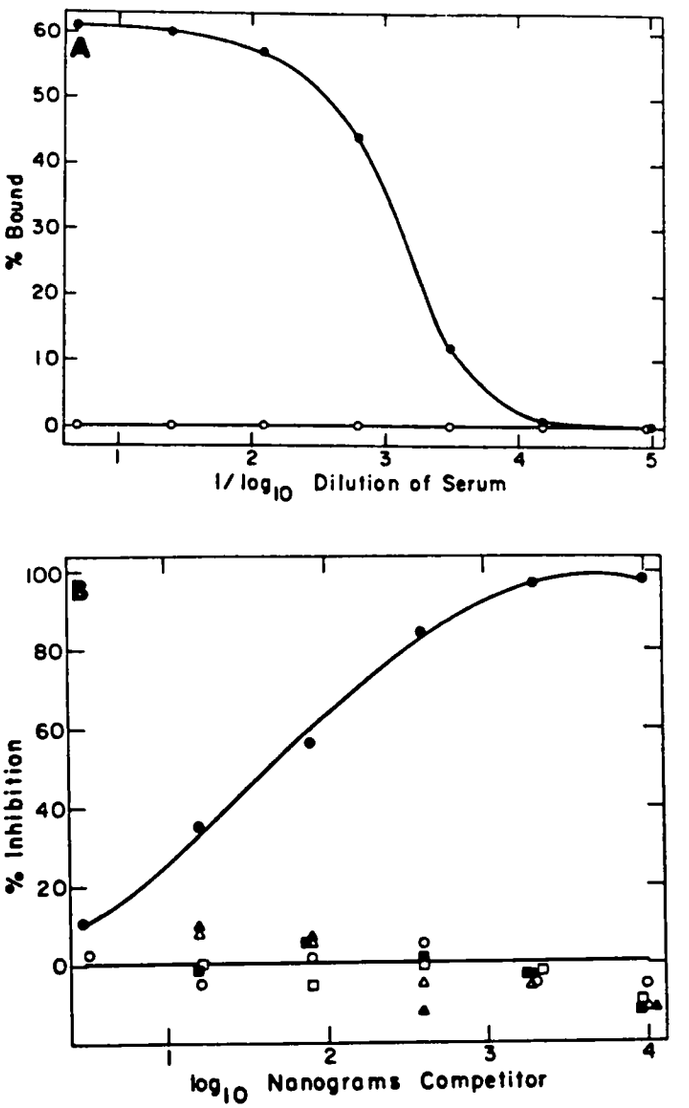

FIGURE 2. Specific binding of Z-DNA by rabbit antibodies induced on immunization with brominated poly(dG-dC). (A) Binding of rabbit serum to ^{3}H-br-poly(dG-dC) in the Z configuration (●) and lack of binding to ^{3}H-poly(dG-dC) in the B form (○); binding was measured in 0.2 *M* NaCl. (B) Competitive binding assay in 0.2 *M* NaCl in which binding of ^{3}H-br-poly(dG-dC) by rabbit anti-Z-DNA serum was measured in the presence of varying amounts of unlabeled br-poly(dG-dC) (●); poly(dG-dC) (○); br-poly(dG) (▲); poly(dG) (△); br-poly(dG)·poly(dC) (■); and poly(dG)·poly(dC) (□). The serum was used at a dilution of 1.625; it bound 28% of the labeled polymer in the absence of competing polynucleotide. (From Lafer, E. M., Valle, R. P. C., Nordheim, A., Rich, A., and Stollar, B. D., *Cold Spring Harbor Symp. Quant. Biol.*, 47, 156, 1983. With permission.)

unmodified poly(dG-dC) which completely lacked bromine.[51] Furthermore, in 1.5 *M* NaCl, the antibody reacted with Z-DNA made with poly(dG-dm5C), which forms a Z-helix at this ionic strength. The antibody therefore reacted with differing forms of polymer, but only when they were in the Z-helical form and whether they contained some brominated bases

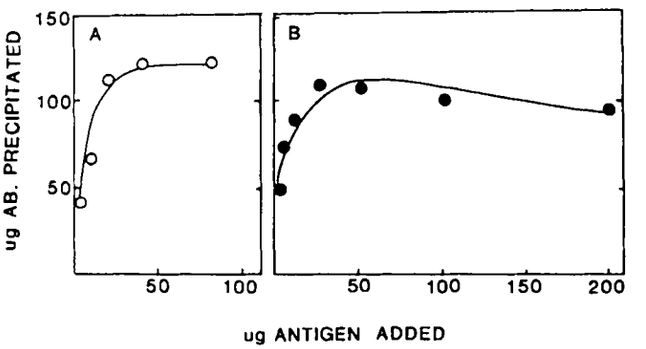

FIGURE 3. Quantitative precipitation of rabbit anti-Z-DNA antisera induced on immunization with br-poly(dG-dC). (A) Precipitation of serum 441a with br-poly(dG-dC) in 0.2 *M* NaCl, with varying amounts of polynucleotide added to 0.2 mℓ of serum. (B) Precipitation of rabbit serum 442e with unmodified poly(dG-dC) in 4 *M* NaCl, with varying amounts of polynucleotide added to 0.2 mℓ of serum. (From Lafer, E. M., Ph.D. thesis, Tufts University, Medford, Mass., 1982.)

or methylcytosine or no chemical modification at all. The reaction in high ionic strength led to precipitate formation (Figure 3), allowing immunospecific purification of the antibody by its dissociation from the washed precipitate.[51] The amount of antibody precipitated by unmodified polymer at high ionic strength accounted for nearly all the antibody precipitated by brominated polymer. The purified antibody had the same specificity as the whole serum and has served as a reagent for studying the conditions under which the DNA can exist in biologically interesting circumstances.

2. Other Immunogens for Anti-Z-DNA Antibodies

Other modified polymers have also served for the induction of anti-Z-DNA antibodies. Malfoy et al.[48] and Malfoy and Leng[52] obtained the antibodies by immunization with poly(dG-dC) modified by chlorodiethylenetriamino platinum (II) chloride (dien-Pt). This modification also stabilized Z-DNA at physiological ionic strength and provided a suitable immunogen. It induced antibodies with properties very similar to those of anti-br-poly(dG-dC). They also reacted with unmodified polymer in high ionic strength. The midpoint of the transition of the unmodified polymer to increased antibody reactivity occurred at 1.4 *M* $NaClO_4$, whereas the midpoint for the B-Z transition measured by circular dichroism occurred at 1.8 *M* $NaClO_4$, so that the antibody may have affected the equilibrium and favored the transition.[52] These antibodies also recognized Z conformation in poly(dG-dC) modified by *N*-acetoxy-*N*-acetyl-2-amino-fluorene (AAF). The antibody reactions also indicated that the polynucleotide poly(dG-dbr5C) adopts the Z form even in 0.1 *M* NaCl.[48] Analysis of precipitates formed in antibody excess revealed a ratio of nucleotide residues to IgG of eight, suggesting that each binding site of the Fab fragment covers a region smaller than about four base pairs.[48] In other studies, antibodies to Z-DNA have been induced by immunization with a variety of polymers stabilized in the Z-form (Table 1). Antibodies induced by all of these immunogens shared the general property of reacting specifically with Z-DNA, including the unmodified poly(dG-dC). Interestingly, immunization with *unmodified* poly(dG-dC) complexed to methylated BSA also induced a small amount of antibody, most of which was specific for the Z form.[50,53] This suggested that complex formation with the positively charged protein was sufficient to form or stabilize a small amount of Z-DNA in the unmodified polymer.

3. Variations in Specificity of Anti-Z-DNA Antibodies

Some variations in specificity have been observed in subpopulations of polyclonal rabbit and goat antisera to Z-DNA induced by br-poly(dG-dC). One of the rabbit sera obtained

Table 1
SOURCES OF ANTI-Z-DNA ANTIBODIES

A. Polyclonal Serum Antibodies Induced By

		Ref.
Brominated poly(dG-dC)		50, 53
Poly(dG-dC)		50
Poly(dG-dm5C)		53, 103
Poly(dG-dbr5C)		53, 54
Poly(dG^{5}dC)	(phosphorothioate in backbone)	53
Poly(dG-dIo5C)		53
Pt-dien-poly(dG-dC)	(chlorodiethylene triaminoplatinum II)	48, 52
AAF-poly(dG-dC)	(acetylaminofluorene)	55

B. Monoclonal Antibodies Induced By

Brominated poly(dG-dC)		53, 56, 57, 89
Poly(dG-dbr5C)		53
Poly(dG-dm5C)		57

C. Autoantibodies

Human SLE sera	*(small fraction of total anti-DNA population plus cross-reactive antibody)*	106

after several months of immunization did show some cross-reactivity at high serum concentration with the B form of poly(dG-dC).[50] Even when this antiserum bound this material, however, the brominated polymer was a very effective competitor for the binding. Furthermore, even when antibodies from this serum were purified from precipitates made with the B form of polymer in 0.2 *M* NaCl, they reacted as well with Z-DNA as with the B form. These results indicated that one antibody population could react with both forms, perhaps by recognizing some feature which is similar, though not identical, in the two helixes. An alternative would be that the antibody could alter the equilibrium between B- and Z-DNA, favoring the conversion to the Z form. Analysis of this possibility will be discussed below.

Varying specificities have also been observed in polyclonal antisera induced by different forms of Z-DNA. Zarling et al.[53,54] found that some rabbit sera induced by br(dG-dC) reacted about equally with several forms of Z-DNA, whereas others reacted much more strongly with the brominated polynucleotide than with the Z-form of poly(dG-dm5C). Sharper selectivity was observed with some sera that were induced by poly(dG-dbr5C); one such serum reacted preferentially with polymers containing five-substituted cytosine, whether the substitution was a bromine or a methyl group.[53,54] When anti-Z-DNA antibodies were induced by immunization with AAF-poly(dG-dC), about 70% of the antibodies were precipitable by brominated poly(dG-dC) at low ionic strength or by unmodified poly(dG-dC) in a concentrated salt solution.[55] After removal of this population of antibodies, there remained some antibodies in the serum that reacted only with AAF-poly(dG-dC). These antibodies were not directed against the AAF structure itself, as they did not react with AAF-modified poly(dG) poly(dC).[55]

Still another structural variation tested for its effect on Z-DNA formation was modification of the helix backbone by replacement of one of the ester oxygen atoms with sulfur.[53] This phosphorothioate-containing analog of poly(dG-dC), i.e., poly(dG^{5}dC), assumed a left-handed conformation and reacted with anti-Z-DNA antibodies induced by br-poly(dG-dC).

It did not, however, react with antibodies induced by poly(dG-dm5C) or poly(dG-dbr5C) and the antibodies induced by it did not react with the methylcytosine-containing polymer or with the Z form of poly(dT-dG) poly(dbr5C-dA).[53,54] Anti-poly(dG^{S}dC) did react with br-poly(dG-dC). It appears that the phosphorothioate analog does share common structural features of Z-DNA, but that the combination of the convex surface and the backbone of this polymer provide unique structural features as well.

C. Monoclonal Anti-Z-DNA Antibodies

From the above results, it may be concluded that a variety of subpopulations of anti-Z-DNA antibodies occur in polyclonal sera, recognizing different regions of the Z-DNA helix or slightly varying conformations of that helix. The potential for recognition of varying sites on the Z-DNA helix was confirmed in studies of monoclonal antibodies.[53,54,56,57] The first such examples were derived from animals immunized with br-poly(dG-dC).[56] Among eight monoclonal antibodies, two distinct patterns of reactivity were observed. One antibody, for example, reacted with poly(dG-dC) or brominated poly(dG-dC), but did not react with polymers containing substituents on the 5 position of cytosine even under conditions in which such polymers were clearly in the Z-helical configuration. With brominated poly(dG-dC), this antibody reacted nearly as well in 4 *M* sodium chloride as it did in 0.2 *M* sodium chloride. These properties suggested that this antibody recognized the convex surface of Z-DNA that replaces the major groove of B-DNA (Figure 4A) and is thus very sensitive to structural changes on this surface, on which the 5 position of cytosine is exposed.[58] The lack of interference by high ionic strength further suggests that the interaction does not involve ionic bonds as would be expected if the antibodies were combining with the phosphate group of the backbone.

A second monoclonal antibody was much more accommodating of substitutions on that Z-DNA surface and reacted equally well with unmodified polymer in high ionic strength and with poly(dG-dbr5C) or poly(dG-dm5C).[56] In addition, this antibody reacted with mixed based sequences that occur in Z-DNA-forming regions of supercoiled plasmid DNA and with sequences of poly(dT-dG)·poly(dC-dA) in supercoiled plasmids.[59] The reactions of this antibody were more sensitive to ionic strength, and more antibody was required for a reaction with br-poly(dG-dC) in 4 *M* sodium chloride than in 0.2 *M* sodium chloride.[56] These properties suggested that this antibody reacts with a site that includes the phosphate group in part and a portion of the convex surface that is not perturbed by addition of substituents to the five-position of cytosine (Figure 4B). Similar variations in specificity of monoclonal antibodies induced by brominated poly(dG-dC) were observed by Zarling et al.[53,54] and Lee et al.[57] In addition, a monoclonal antibody derived from an animal immunized with poly(dG-dbr5C) reacted with that polymer or with poly(dT-dG)·poly(dbr5C-dA), but not with unmodified poly(dG-dC) in high ionic strength.[53] Monoclonal antibodies requiring a substituent on the five-position of cytosine were also obtained from an animal immunized with poly(dG-dm5C).[57] These two types of antibodies appear to react with the convex surface of the Z-DNA, as did some of the anti-br-poly(dG-dC) antibodies. Certain of the monoclonal antibodies, therefore, may serve as more selective reagents for particular forms of Z-DNA, whereas others may serve as more general anti-Z-DNA reagents.

D. Application of Anti-Z-DNA Antibodies to the Search for Z-DNA in Biological Systems

1. Polytene Chromosomes

A major reason for preparing antibodies specific for Z-DNA was the potential for using them to test whether Z-DNA actually occurs in biological systems. The first test used polytene chromosomes of *Drosophila melanogaster*.[51] In acid-squashed fixed chromosomes examined by immunofluorescence, antibody was seen to bind reproducibly to many regions throughout the chromosome (Figure 5). The specificity of the binding was supported by the use of immunospecifically purified antibody and by the fact that the immunofluorescent staining

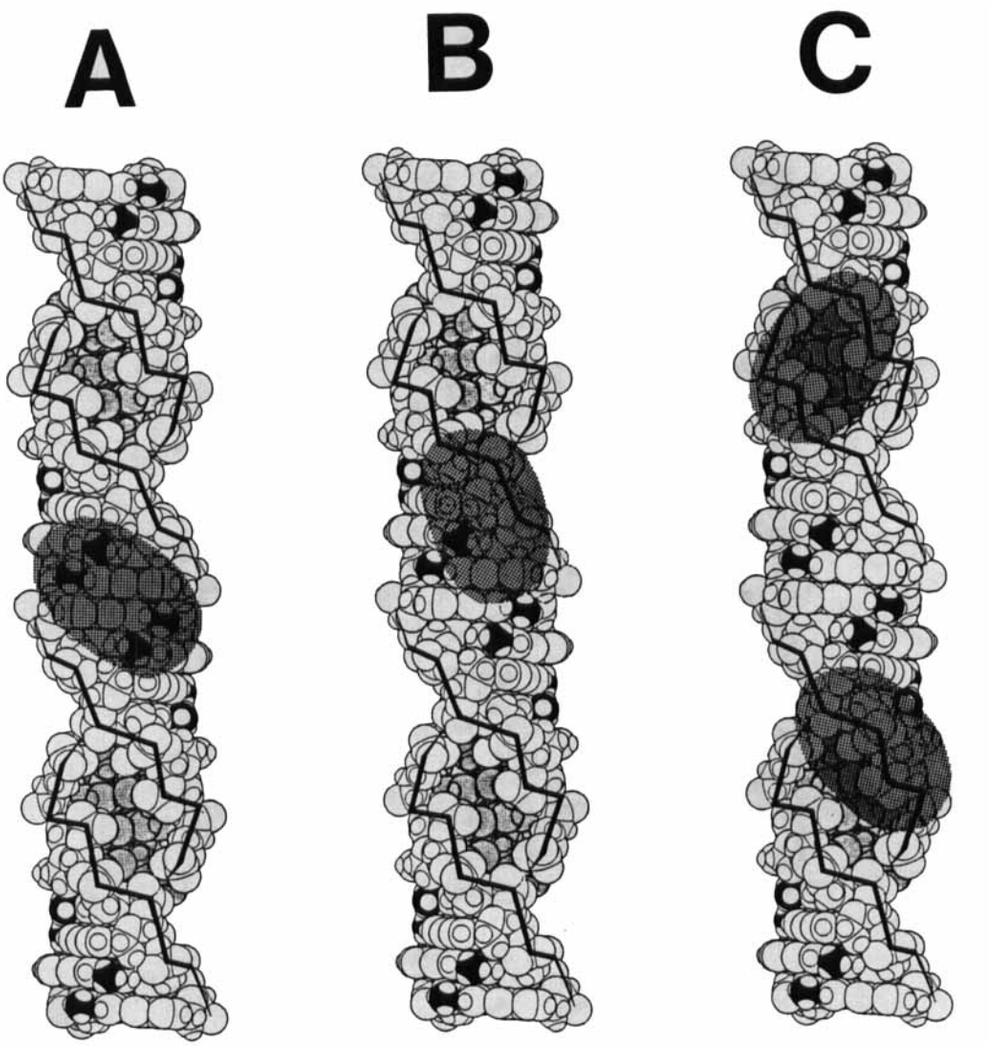

FIGURE 4. Proposed binding sites of anti-Z-DNA antibodies. Computer drawing of the van der Waals surface of the Z-DNA form of poly(dG-dm5C). Black atoms are methyl carbons. The black zigzag line connects phosphorus atoms along the polynucleotide chain. The shaded areas represent possible antibody recognition sites corresponding to a 15 × 20 Å area that would correspond to the binding surface of an Fab portion of the IgG. (A) and (B) are sites for monoclonal antibodies with varying specificity for Z-DNA of given base sequences and with varying sensitivity to ionic strength. (C) represents possible sites of binding of highly salt-sensitive SLE autoantibodies with Z-DNA selectivity. (From Lafer, E. M., Möller, A., Valle, R. P. C., Nordheim, A., Rich, A., and Stollar, B. D., *Cold Spring Harbor Symp. Biol.*, 47, 161, 1983. With permission.)

was specifically blocked by pre-incubation of the antibodies with soluble Z-DNA forms of synthetic polymers.[51] Staining was less bright if the chromosomes were prefixed with formaldehyde, but in these studies, as in subsequent ones,[60-62] varying types of fixation including ethanol alone, acetic acid alone, or acetic acid and ethanol have yielded strikingly positive results.

a. Localization of Z-DNA in Polytene Chromosomes and the Role of Tissue Fixation

There have been two issues which have aroused considerable discussion in relation to the immunofluorescent staining of polytene chromosome. One concerns the localization of the

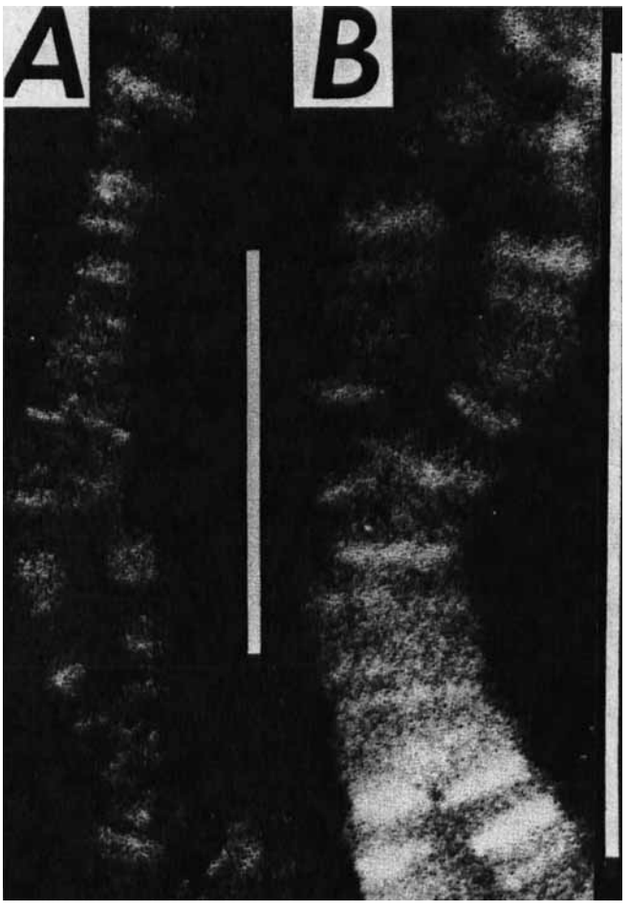

FIGURE 5. Reproducible pattern of immunofluorescent staining of polytene chromosome of *Drosophila melanogaster* by anti-Z-DNA antibody. Chromosomes were fixed and squashed in 50% acetic acid. Two examples of asynapsis show similar fluorescence in homologous regions of unpaired homologues of chromosomal arm. (From Nordheim, A., Pardue, M. L., Lafer, E. M., Möller, A., Stollar, B. D., and Rich, A., *Nature (London)*, 294, 420, 1981. Copyright 1981, Macmillan Journals Limited. With permission.)

antibody binding. In the studies of Nordheim and colleagues,[51,63] alignment of phase-contrast micrographs and immunofluorescent micrographs led to the conclusion that much of the staining was in interband regions, although certain bands and certain parts of puff regions were also stained. Lemeunier et al.[60] observed similar patterns of staining with *Drosophila melanogaster* preparations, but in chromosomes of *Chironomus thummi* the fluorescence was much more restricted, particularly to two bands on chromosome four. A quite different result was reported by Arndt-Jovin and co-workers,[53,54,62,64] who studied both *Chironomus* and *Drosophila*. Comparing the DNA distribution as revealed by a red-fluorescing DNA-binding dye and the immunofluorescent pattern, they concluded that the antibody binding consistently paralleled the DNA concentration, as the immunofluorescent staining was present almost entirely in bands. Thus, all three laboratories agreed that specific immunofluorescence occurs in polytene chromosomes with anti-Z-DNA antibodies, but drew different conclusions about the location of the corresponding Z-DNA. In attempts to clarify the differences, antibody reagents were exchanged between the Nordheim-Pardue group and Arndt-Jovin-

Zarling group, but each laboratory still confirmed its own findings with the reagents of the other laboratory. This suggested that some aspect of the fixation of chromosome preparation was different. Because these two laboratories have had consistent results that are different, an important recent development has been the finding of Alonso and co-workers[189] that either pattern of staining can be obtained in one laboratory depending on fixation conditions used. A combined exposure to acetic acid and ethanol for a significant time yielded a predominant staining of bands, whereas fixation with acetic acid alone or separate exposure to acetic acid and ethanol for short periods yielded staining in interbands and some puffs, as well as a few bands, as seen by Nordheim and co-workers.[51,63] It appears, therefore, that certain regions in interbands and some puffs have a greater tendency toward Z-DNA formation than do more numerous Z-DNA-forming sequences in the bands throughout the genome and are either in that conformation *in situ* or are readily converted to it during mild fixation procedures.

The important contribution of fixation was emphasized further when chromosomes that were dissected and placed simply in buffer with spermidine and a very low concentration of formaldehyde were examined.[61,65] These chromosomes, considered to be close to the native state and not subjected to acid or ethanol fixation, showed no immunofluorescent staining with anti-Z-DNA antibody. Chromosomes that were exposed for 5 sec to acetic acid showed staining particularly in interband regions, and chromosomes exposed to acetic acid for 25 sec showed general staining with prominent band fluorescence. Related findings were made by Robert-Nicoud et al.[62] These workers found very slight staining of native chromosomes with high concentrations of antibody, and a marked increase of staining when chromosomes were subjected to acid or high temperature. The critical pH range over which a marked increase of reactivity occurred was between 2.5 and 2. They also noted that Z-DNA was detected after chromosomes were heated to 70 to 90°C in low ionic strength at neutral pH, but not by exposure to 0.35 *M* NaCl, which was sufficient to cause some decondensation of chromatin and exposure of histone H3.

Both the variable pattern of staining and the requirement for fixation have given rise to the second issue of controversy, the question of whether the Z-DNA really exists in the chromosomes or is produced entirely during the fixation. One mechanism proposed for the effect of fixation involves the protonation of bases. Poly(dG-dC) undergoes a B to Z transition at low pH at a rate that is faster than the transition in 4 *M* sodium chloride.[62] Again, the critical pH range for this enhanced Z-DNA formation is between 2.5 and 2.[62] On the other hand, the appearance of reactive Z-DNA in chromosomes can occur at high temperature or with ethanol fixation without acid.[61,62] An important driving force under all these conditions appears to be torsional strain which may result from removal of chromatin proteins and disruption of nucleosome structure. The evidence for a mechanism involving torsional strain comes from the result of treatment of fixed chromosomes with topoisomerase I or very low nicking concentrations of DNAse 1.[61,62,65] Treatment of fixed chromosomes with these enzymes leads to a complete loss of reactivity with anti-Z-DNA antibody, whereas exposure to these enzymes before fixation did not prevent the appearance of Z-DNA.

These results, however, do not give a complete answer to the question raised by the weak or negative result with unfixed chromosomes of whether Z-DNA exists in vivo. It is possible that Z-DNA does occur and is masked by Z-DNA-binding proteins which may be required for its stabilization; for example, proteins with selective binding to Z-DNA have been identified in preparations from *Drosophila*.[66] It is possible that such proteins are among the ones that are removed by fixation and that the fixation conditions can stabilize Z-DNA in their place. Thus, the lack of immunofluorescent staining cannot give a definitive negative answer to the question of whether Z-DNA exists in the native chromosomes. A plausible interpretation of data available is that the fixation conditions reveal most of the DNA regions

that have the potential for forming Z-helical structure and this amount, estimated at 0.02 to 0.1% of the total DNA,[62,64] may in fact be much more than what exists in vivo. Given the findings that sequences with Z-DNA-forming potential do exist, and that the conditions for driving the formation of Z-DNA (an increase in negative supercoiling) can occur under physiological conditions,[32,44,45] it is likely that some fraction of these sequences is in fact in the Z conformation at any given time. An alteration in the nucleosome packing density, which could occur as chromatin structure is altered for transcription or replication,[67] could yield the necessary increase in negative supercoiling density. With reversible changes in chromatin configuration, the B to Z transition could also be reversible and thus serve as an important signal subject to modulation by changes in nucleosomal organization.

b. Analysis with Sequence-Specific Anti-Z-DNA Antibodies

Some antisera, such as those induced by br-poly(dG-dC), appear to recognize Z-DNA forms with a wide variety of base compositions and do not indicate what sequences may be involved at an immunoreactive chromosomal site.[50,53] The availability of monoclonal antibodies has provided reagents with more selective specificity. One monoclonal antibody for example (Z44), binds br-poly-(dG-dC) very strongly, but does not recognize Z-DNA of other sequences or even poly(dC-dG) derivatives in which there are substituents on the five-position of cytosine.[56] This antibody bound to sequences of alternating dG-dC that were present in a supercoiled circular DNA, but did not bind to inserted sequences of poly(dT-dG)·poly(dC-dA).[59] This antibody also failed to stain polytene chromosomes under conditions in which a polyclonal anti-Z-DNA serum or another more generally reactive monoclonal antibody gave bright immunofluorescence, indicating that a purely alternating (dC-dG) sequence was not responsible for Z-DNA formation at that site.[59] In comparisons of reactions of different monoclonal antibodies, there was a correlation between the ability to react with plasmid DNA that contained poly(dT-dG)·poly(dC-dA) inserts and the ability to react with polytene chromosomes, but the latter reaction may also have involved other naturally occurring sequences of mixed base composition. Zarling et al.[53,54] have also found differences in the immunofluorescence caused by monoclonal antibodies and polyclonal antisera. Most striking was the difference of staining of the terminal telomeric regions of *Chironomus* chromosomes, which were stained brightly with the polyclonal rabbit antiserum, but weakly with a monoclonal antibody (D11).[53,68] The monoclonal antibody reacted with derivatives of the poly(dG-dC) family of Z-DNA, but not with members of the poly(dT-dG)·poly(dC-dA) family.[68] They concluded that telomeric regions may be rich in the latter sequences and that they may be responsible for Z-DNA formation in such regions. It remains to be determined whether other monoclonal antibodies with strict specificity for base sequence within Z-DNA can be generated.

2. Immunohistochemical Identification of Z-DNA in Other Cells

a. Stylonychia

Another interesting identification of Z-DNA by immunofluorescence was in ethanol- or acetic acid-fixed nuclei of the ciliated protozoon *Stylonychia* sp.[69] These organisms contain two nuclei.[70] One, a micronucleus, consists of chromatin comparable to that of normal interphase nuclei. The second, a macronucleus, contains gene-size fragments of DNA with many copies of individual genes that were generated during an earlier polytene stage of development. The macronucleus is the site of gene transcription and mRNA formation; the micronucleus undergoes usual changes in mitosis but is not a site of major transcriptional activity. Anti-Z-DNA antibodies caused bright immunofluorescence of the macronucleus, but not of the micronucleus.[69] When the macronucleus underwent division by fission, a

"fission band" across the nucleus was not stained well by anti-Z-DNA antibodies, but was stained by a monoclonal autoantibody that reacted with B-DNA. These results provided a hint that the Z-DNA formation could be related to a function, namely transcription, but did not establish a mechanism.

b. Mammalian Nuclei and Mammalian and Plant Chromosomes

By immunofluorescence or immunoperoxidase techniques, Z-DNA has been identified in other nuclei and chromosomes. Morgenegg et al.[71] found reactivity in many, but not all, nuclei in tissue sections of rat brain, kidney, liver, and testes. In seminiferous tubules, for example, nuclei of spermatogonia at the periphery were regularly reactive, whereas few of the more highly differentiated spermatocytes and spermatids located toward the center of the tubule were stained. In cerebellar cortex, some cell nuclei were reactive, but not others. These results hint at developmental changes in chromatin that may require or facilitate Z-DNA formation. Again, these were fixed tissues, but several different fixation procedures, not all acidic, revealed the Z-DNA. Prefixation with formaldehyde hindered its appearance. In metaphase mitotic chromosomes of animal or human cells, Z-DNA was also present in a nonrandom distribution.[72,73] In both human and new world monkey (*Cebus*) chromosomes, Z-DNA immunoreactivity was prominent in T-bands and R-bands and weak in G-bands, and it was suggested that homologous Z-forming regions were conserved.[73] Anti-Z-DNA antibodies also caused immunofluorescence of plant nuclei and showed particularly bright staining in the telomeric regions of isolated metaphase chromosomes of rye plants.[74]

Flow cytometry has allowed additional quantitation and measurement of changes in large numbers of lymphocyte nuclei during cell cycle changes.[185] In ethanol-fixed resting lymphocytes, Z-DNA was measurable in about 85% of the cell nuclei. After stimulation by phytohemagglutinin, all nuclei became reactive with the antibody during progressive passage through G1, S1, and G2 phases of the cell cycles. The fluorescent signal with anti-Z-DNA antibody did not double with a doubling of the DNA content, but the same was true for fluorescence with an autoantibody that reacted with B-DNA, so that no specific cell cycle stage correlation of Z-DNA appearance could be made. There was no burst of Z-DNA formation during G1, in which RNA synthesis increased greatly.

3. Supercoiled Circular DNA

Linear nucleic acids of biological origin have not been found to contain Z-DNA conformation under conditions of physiological ionic strength and pH, or at high ionic strength, although inserted (dG-dC) sequences comprising a significant portion of a restriction fragment of plasmid DNA can do so at high ionic strength.[43] Thus, even when regions with the potential for forming Z-DNA occur as a small portion of a large linear DNA molecule, their B-DNA conformation is quite stable. Linear DNA samples that were "G-C-rich" did not compete for binding by anti-Z-DNA antibody even in high ionic strength.[186] Z-DNA formation, however, can occur under physiological conditions when the appropriate sequences occur within circular DNA and the DNA is supercoiled. This has been demonstrated by two-dimensional gel electrophoresis (with the presence of intercalating agents in one dimension,[44,45] and by the appearance of B-Z junctional regions that are sensitive to cleavage by S1 nuclease).[32] It is also readily demonstrated by reactions with specific antibodies.[53,75,76] When poly(dG-dC) sequences of 32 base pairs or longer were inserted into a bacterial plasmid, and the plasmid was grown in a bacterial host and reisolated, it was found to react with anti-Z-DNA antibody as long as it was in the supercoiled state, but when it was converted to a relaxed circular molecule, it no longer reacted.[76] The amount of supercoiling present in naturally grown plasmid was sufficient to induce the Z-DNA formation in these plasmids (Figure 6). With inserts of poly(dG-dC) of shorter lengths, the natural degree of supercoiling was not sufficient to cause Z-DNA formation, but when a higher degree of negative supercoiling was induced by incubation of the plasmid DNA with topoisomerase and ethidium

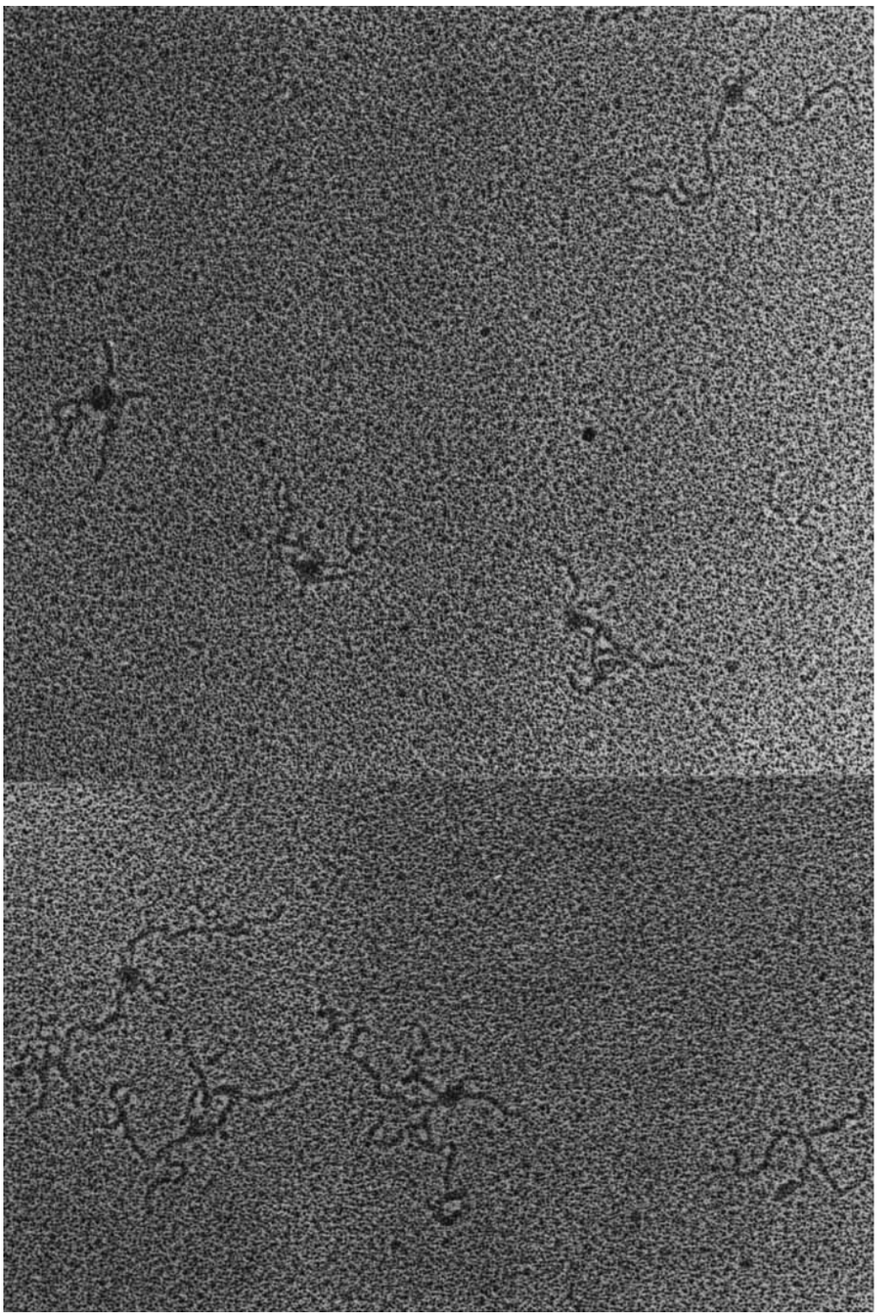

FIGURE 6. Electron microscopy of antibody bound to single sites of supercoiled pBR322 plasmids containing inserted (G-C) sequences, (pLP32).

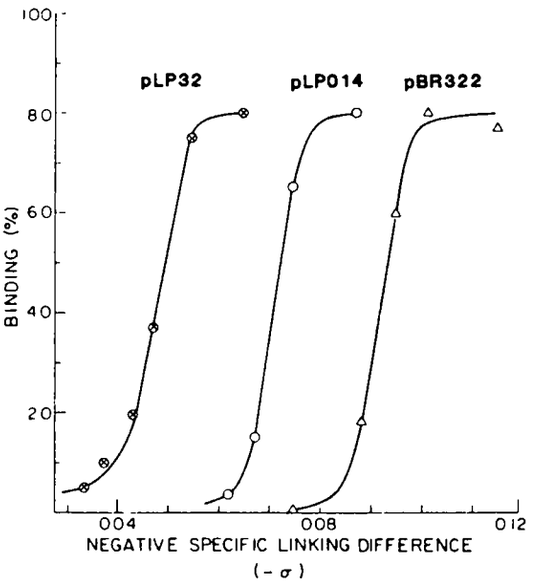

FIGURE 7. Immunochemical titration of Z-DNA formation on supercoiling of plasmid DNA. Anti-Z-DNA antibody (860 n*M*) was incubated with DNA of pBR322 plasmids or pBR322 plasmids into which a segment of 32 base pairs of alternating (C-G) sequence (pLP32) or a segment of 14 base pairs of (C-G) sequence (pLP014) was inserted. Plasmid DNA was supercoiled by incubation with varying concentrations of ethidium bromide and toposomerase I followed by removal of the ethidium bromide and enzyme. The specific linking differences were determined by agarose gel electrophoresis. After incubation, the mixtures were filtered through nitrocellulose filters, which bound antibody-plasmid complexes, but not free DNA. (From Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C., Stollar, B. D., and Rich, A., *Cell*, 31, 312, 1983. Copyright 1983, Massachusetts Institute of Technology. With permission.)

bromide, Z-DNA appeared in these plasmids also. In fact, with a high degree of supercoiling there was an appearance of Z-DNA even without an inserted sequence in the pBR322, indicating that one or more naturally occurring sequences with Z-DNA-forming potential do occur in the plasmid (Figure 7). With antibody cross-linked to the DNA by glutaraldehyde and with the use of restriction nucleases, it was possible to identify the site of this Z-DNA formation as a region in which alternating pyrimidine-purine sequences occurred over a 14-base pair region with 1 base pair out of this alteration pattern.[76]

Z-DNA was subsequently found in the coiled circular DNA of SV40 virus at a naturally occurring degree of negative supercoiling and was found to occur at interesting sites in the DNA.[77] Three sequences were prominent for their Z-DNA formation. Two occurred in the 72-base pair repeat enhancer sequences of the early promoter, and the third was just outside one of these enhancer regions. In all three sites, which are in a nucleosome-free region of the SV40 mini-chromosome, there were sequences of eight base pairs showing pyrimidine-purine alteration. A search of the literature found several other such sequences, ranging from 8 to 13 base pairs with no more than 1 base pair out of alteration, and with a similar 60- to 80-base pair spacing between them.[77] They occurred in regions of interest for regulation of gene expression. Many of them occurred in the long terminal repeat regions of retrovirus

cDNA, in control regions of DNA tumor virus genomes, and in intron regions of immunoglobulin or globin genes. These were not strictly (dC-dG) or (dT-dG) sequences, but it is of interest that significant stretches of the latter do appear in many copies in the eukaryotic genome,[78,79] and when such sequences occur in circular DNA, they do take up the Z-DNA conformation.[80-82] The reaction of Z-DNA-containing plasmids with antibody has been demonstrated by antibody-mediated retention of plasmid on nitrocellulose filters,[76,77,80] slowing of the migration of plasmid DNA in polyacrylamide electrophoresis,[53,83] and electron microscopy.[76,83] In PM2 viral DNA, electron microscopy revealed a clustering of Z-DNA regions in one portion of the DNA.[83] The simple antibody-binding assay allows a sensitive and precise measurement of the transition from B- to Z-DNA with increasing supercoiling.[76] In addition, study of populations with varying degrees of supercoiling in vivo can be facilitated by the use of immobilized antibody as affinity columns for isolation of Z-DNA-containing plasmids.[84]

From these many studies with supercoiled DNA, one may conclude that naturally occurring sequences with the potential for Z-DNA formation occur relatively frequently, that supercoiling is a major force that can drive the B to Z transition, and that this can occur under physiological conditions of ionic strength and pH. Indeed, the transition is favored at lower ionic strengths.[85] This is an important consideration in evaluation of the reactions of chromatin and chromosomes with anti-Z-DNA antibodies. There is evidence that DNA is organized within domains in such chromosomes with the domains looping out from a fixed scaffolding structure of chromatin protein.[86] This would have the effect of constraining a looped domain in a circular DNA form, meaning that negative supercoiling within the domain could influence the amount of Z-DNA formation. The amount of negative supercoiling will in turn depend on the nucleosomal organization within the domain, as the winding of DNA around the nucleosomal protein core offsets the negative supercoiling to the extent of about one super helical turn per nucleosome.[87] This means that modulation of nucleosomal packing within the domain could vary the amount of Z-DNA present and this may in turn determine the binding of specific regulatory proteins to the domain.[88]

4. Does the Reaction with Antibody Cause the Formation of Z-DNA?

A major question that has arisen during the use of antibody to measure DNA in tissues and chromosomes has been whether the antibody itself drives the formation of Z-DNA by stabilizing it and therefore affecting the equilibrium between B and Z forms. A model for such an influence has been presented by Jovin et al.[68] In a test of the model, those investigators found that when poly(dG-dC) was incubated with anti-Z-DNA antibody at 53°C in 1.5 *M* NaCl, there was a progressive increase in antibody binding over a period of several hours, indicating that a transition occurred from the B form to the immunoreactive Z form; under this condition, the polymer in the absence of antibody did not undergo conversion to Z-DNA as measured by physical chemical means. It was concluded that the antibody could drive the formation of Z-DNA, as expected, probably by stabilizing one equilibrium form.[68] Malfoy and Leng[52] also observed that, with their antibody, binding of poly(dG-dC) became apparent at concentrations of sodium perchlorate that were lower than those required for the transition of polymer alone, again suggesting that the antibody favored the transition.

With some antibodies prepared in the author's laboratory, the transition from B-DNA occurred with a midpoint of 2.25 *M* NaCl in the presence or absence of antibody at room temperature or 53°C as measured by antibody binding and changes in the UV-absorption spectrum. Other anti-Z-DNA antibodies did lower the transition midpoint to 2.0 *M* NaCl.[187] These antibodies had a higher affinity for the Z-form than did the antibodies that did not influence this transition. We also noted that antibody was able to bind poly(dG-dC) in very low ionic strength (10 m*M* Tris® with no added NaCl); this was so even with the antibody that caused no change in the 2.25 *M* NaCl transition. In the absence of antibody, the polymer

had a B-DNA spectrum in low ionic strength, whereas in the presence of antibody it had a spectrum that was characteristic of a mixture of B- and Z-DNA. Thus, under this condition we also measured the influence of antibody in stabilizing an equilibrium form and favoring Z-DNA formation. A further indication that the antibody can stabilize Z-DNA was the observation that when immune precipitates were formed in high ionic strength, centrifuged, and resuspended in a solution of 0.14 *M* NaCl, the precipitates dissolved slowly, gradually releasing the polymer antigen and free antibody. The multivalent attachment of antibody to the polynucleotide in the precipitate thus stabilized the Z-DNA conformation under conditions in which the Z to B transition of polymer alone is very rapid. In other experiments, large amounts of antibody measured Z-DNA in plasmids that were not supercoiled enough to be measurable as containing Z-DNA when low concentrations of antibody were used. In this case also, the antibody may have favored the transition in plasmids under conditions that were very near to those required for the B to Z conversion. Antibody may detectably affect the equilibrium and favor the accumulation of the Z-DNA form. Even relatively large amounts of antibody, however, do not substantially drive Z-DNA formation in conditions distant from the transition as with linear DNA in 0.15 *M* NaCl. Low concentrations of antibodies of moderate affinity should be used for detection of Z-DNA in tissues and cells and isolated plasmids to make it unlikely that the observed Z-DNA is a product of the activity of the antibody.

5. Z-DNA in Form V DNA

Form V DNA is a complex structure that results when two circular single-stranded chains that are complementary to each other are allowed to anneal.[89] They cannot form a completely B-helical circle, and regions of right-handed B-helix that are formed must be compensated by such features as negative supercoiling and left-handed duplex turns. Form V DNA has a circular dichroism spectrum with some Z-like character, notably a negative ellipticity at 290 nm. The presence of Z-DNA structure has been confirmed by reactions with specific anti-Z-DNA antibodies. Form V DNA inhibited the binding by the antibody of labeled poly(dG-dC) in 4 *M* NaCl and of dien-Pt-poly(dG-dC) in low ionic strength.[90] The antibody also specifically slowed the electrophoretic migration of form V DNA,[89] and its binding to many sites along the form V DNA molecule was visualized directly by electron microscopy.[90] In these studies, antibodies again provided a direct and relatively simple measurement of a specific structural feature and helped to establish the role of topological stress in the induction of Z-DNA structure.

6. The Search for Biological Function

The presence of Z-DNA in regions flanking coding portions of genes or between elements of a split promoter inhibited transcription of supercoiled DNA injected into *Xenopus* oocytes,[91] suggesting a potential negatively regulatory role for Z-DNA sequences, but such sequences farther upstream from coding initiation sites may have a positive effect on transcription. Anti-Z-DNA antibodies have identified Z-DNA forming sequences in enhancer regions of SV40 DNA,[77] but a specific function was not established for the Z form. The potential formation of Z-DNA in controlling regions has also been suggested by the presence of S1 nuclease-sensitive sites in the 5′-flanking regions of expressed genes and in supercoiled plasmids into which the genes were inserted.[93] There are, therefore, suggestive correlations for Z-DNA function. It is possible that microinjection of antibodies into cells could help to test these possibilities, but antibodies have not yet identified Z-DNA in the midst of such actions in vivo.

Another context in which a potential role for Z-DNA has been considered is that of DNA recombination. Haniford and Pulleyblank[94] have provided a conceptual model for this possibility, suggesting that repeated (dT-dG) sequences can serve as interacting sites for aligning homologous chromosomes. Z-DNA formation at these sites could then lead to helix un-

winding that would precede the strand association required for recombination. Indirect evidence comes from occurrence of a high frequency of recombination in negatively supercoiled plasmids with Z-DNA-forming sequence inserts.[95] A more direct observation has been the identification by Kmiec and Holloman[96] of Z-DNA in the paranemic joint formed by a circular single-stranded molecule of DNA interacting with a partly homologous linear double-stranded molecule under the influence of the rec1 protein of *Ustilago*. Contributing to the evidence for the presence of this structure was its reaction with anti-Z-DNA antibodies. The formation of Z-DNA may be necessary for the step of recombination in which homologous strands of different DNA molecules are paired, if they become aligned under conditions in which the strands cannot become intertwined.[96]

III. RIGHT-HANDED HELICAL POLYNUCLEOTIDES

In the experience of several laboratories, native B-DNA has not been immunogenic in normal animals, whether injected alone or in complexes with methylated BSA.[97] On the other hand, antibodies reactive with native DNA and other polynucleotides have been detected in sera of animals after injection of lipopolysaccharide[98,99] or peptidoglycan.[99] This presumably results from polyclonal B-cell stimulation, which may activate certain autoantibody-producing cells more than other populations.[99] Polyclonal activation may also occur when nucleic acid antigens are injected as an emulsion in complete Freund's adjuvant. A certain level of antibody reactive with DNA and other polynucleotides was observed in sera of control animals receiving adjuvant alone,[97] and no stimulation of antinative DNA or antidenatured DNA was observed above this background when native DNA-methylated BSA complexes were incorporated into the Freund's adjuvant for the injection (Figure 8). The background antibody populations resembled autoantibodies to DNA in their cross-reactivity with several polynucleotides. In view of this, special care is required to establish as clearly as possible a distinctive specificity of antibodies to a given polynucleotide if they are to be distinguished from the polyreactive background.

Even with this qualification, it is clear that helical polynucleotides that differ from B-DNA can be effective specific immunogens (Figure 8). The anti-Z-DNA antibodies described earlier do not show the cross-reactivity characteristic of polyclonally stimulated autoantibodies, and they reach concentrations far above the background levels.[48,50,53] Other distinct helical forms, such as the A-helical double-stranded RNA, DNA-RNA hybrids, and triple-helical polynucleotides all have generated high levels of antibody specific for the helical conformation of the immunogen. They have served as sensitive and specific reagents for detection of these structures as minor populations of complex biological materials; their properties have been reviewed in some detail earlier.[19,20]

Certain right-handed double-helical deoxyribonucleotide polymers have also induced antibody specific for the immunogen. Their immunogenicity itself is a first indication that they differ in structure from the average B-DNA. Further, nearly all of the antibodies generated recognize those differences almost uniquely; they react with the immunogen, but very weakly or not at all with DNA. This again indicates the existence of structural differences in the helixes. Because the background of polyclonal anti-B-DNA is low, the presence of even modest amounts of specific antibody to unique features of other polymers can usually be detected and their specificity established by competitive immunoassays.

A. Antibodies to Poly(dG)·Poly(dC) and Poly(dG)·Poly(dm5C)

Poly(dG)·poly(dC) is an example of an immunogenic right-handed helical polydeoxyribonucleotide. It has induced specific antibody in normal rabbits[100] and mice,[97] and a monoclonal antibody specific for it has been described.[101] In the earliest studies, complement fixation assays at modest dilution indicated that rabbit antipoly(dG)·poly(dC) sera reacted

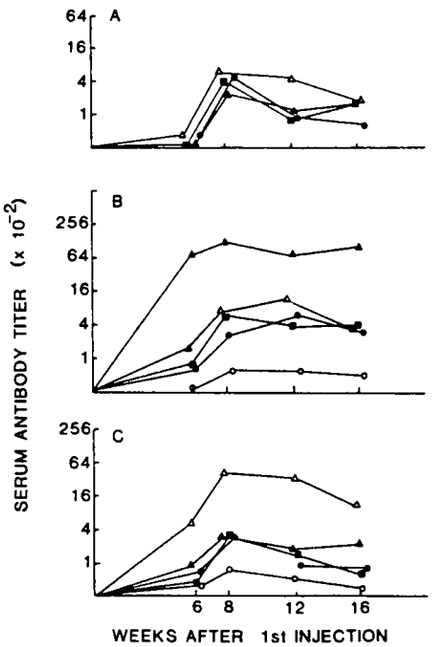

FIGURE 8. Immune responses of C57BL/6 mice to immunization with nucleic acids in adjuvant or adjuvant alone. Serum antibodies were measured in a solid-phase enzyme-linked immunoassay with: (A) native DNA; (B) Z-DNA; and (C) denatured DNA after injection of: saline only (○); methylated BSA-adjuvant (■); native DNA-MBSA-adjuvant (●); denatured DNA-MBSA-adjuvant (△); or br-poly-poly(dG-dC) (Z-DNA)-MBSA-adjuvant (▲). Points represent the mean titer for each group of mice. (From Madaio, M. P., Hodder, S., Schwartz, R. S., and Stollar, B. D., *J. Immunol.*, 132, 874, 1984. With permission.)

with that polymer, but not with native or denatured DNA, poly(dA-dT), double-stranded RNA, or DNA-RNA hybrid.[100] A recent extension of these experiments, with a competitive radioimmunoassay, indicated the presence of some antibody that cross-reacted with poly(G)·poly(dC) or poly(G)·poly(C) and some that reacted with the homopolymers poly(dG) and poly(dC), but there was still antibody specific for the poly(dG)·poly(dC).[102] The recently described monoclonal antibody is highly selective and does not react with the homopolymers, the RNA-DNA hybrid form of poly(G)·poly(dC), or even poly(dG)·poly(dm5C).[101] This report, by Lee et al.,[101] also included a description of a second monoclonal antibody from an animal immunized with poly(dG)·poly(dm5C), which showed strong reactivity with poly(dG)·poly(dC) as well as with the immunogen and weak reactivity with poly(G)·poly(dC) or poly(I)·poly(dm5C). High concentrations of phage XP-12 DNA, which contains 67% (dG + dm5C) base content, competed for binding of the latter, even though the homopolymers poly(dG) and poly(dm5C) did not. It was concluded that, in this case, the antibody was recognizing a specific conformation that depended on the presence of dm5C, and that occurred in a small fraction of the total XP-12 DNA structure.

B. Poly(dG-dC) and Poly(dG-dA)·Poly(dT-dC)

Other right-handed helical DNA analogs have induced weak responses. On immunization

of rabbits with methylated BSA complexes of right-handed poly(dG-dC), Lafer and Stollar[102] and Zarling et al.[53] both noted formation of some antibody with a specificity for the Z-DNA form of this polymer. Lafer and Stollar[102] noted, in addition, the presence of some antibody specific for the right-handed form of the polymer. It did not react with native *E. coli* DNA, native or denatured calf thymus DNA, poly(dG)·poly(dC), or poly(dA-dT). It was a small population of antibody and was not studied in more detail.

A slightly stronger response was obtained on immunization of mice with poly(dG-dA)·poly(dT-dC).[97] A distinct response has also been found in rabbits, and corresponding antibodies are currently under study in the author's laboratory.

C. A Monoclonal Anti-Native DNA Antibody from Immunized Animals

In experiments designed to prepare monoclonal antibodies specific for native DNA, Huang et al.[104] immunized mice with several different polymers over several weeks; included were poly(dA-dT), poly(dG-dC), and poly(dG)·poly(dC). After fusion of spleen cells of these animals with myeloma partner cells, 51 hybridomas producing anti-DNA antibodies were identified; 17 were specific for single-stranded DNA and 34 reacted with both single- and double-stranded DNA. An interesting question is whether these hybridomas were derived from cells that were specifically induced by the immunizing polynucleotides or whether they resulted from stimulation by the adjuvant, as discussed above. One hybridoma produced an IgM that was specifically reactive with double-stranded DNA. Its binding was not competed by single-stranded M13 or ØX174 DNA, but it did react with the double-stranded derivatives of these molecules. It also reacted with fragments that had been annealed to specific DNA probes to form duplex structures, but these probes had to be relatively long, over 500 base pairs, for optimal reactivity. An antibody with this specificity and a high affinity would be a valuable reagent for measurement of annealing of specific probes with corresponding short sequences in DNA.

D. Poly(dA-dT)

Helical poly(dA-dT) and poly(dA)·poly(dT) have not induced specific antidouble-stranded DNA antibody in most laboratories, but Lubit and Erlanger[105] did induce a response with poly(dA-dT) that was boiled, rapidly chilled, and mixed with methylated BSA for injection. As the separated strands would reanneal rapidly, at least in part, it is likely that the immunogen contained both single-stranded and double-stranded regions. The antibodies bound labeled denatured poly(dA-dT), and this binding was inhibited by deoxyadenosine, deoxythymidine, and, at very low concentrations, by poly(A). It was also inhibited by denatured DNA of calf thymus, *Pseudomonas aeruginosa*, *B. subtilis*, and *V. faba*, but the G + C-rich *Pseudomonas* DNA did not achieve complete inhibition. After absorption of the antinucleoside antibody with adenosine- and thymidine-protein conjugates, the residual antibody was specific for polymeric forms, but still reacted with the homopolymer poly(dT) and denatured DNA. Some reactivity was observed with high concentrations of native mouse DNA and poly(dA)·poly(dT), but it appears that the antibodies induced in this study were specific mainly for the single-stranded regions of the immunogen.

IV. SLE AUTOANTIBODIES TO DNA

As noted above, native DNA has generally not served as an effective immunogen, but there is a source of antibody that reacts with it. Such antibodies are present in the sera of patients with SLE and in sera of several strains of mice with a similar disorder. These sera contain some antibodies specific for denatured DNA,[18] some that react with synthetic polynucleotides, some specific for Z-DNA, and some that cross-react with denatured and Z-DNA,[106] as well as those that react with native DNA. The association of these antibodies

with clinical phenomena have been summarized elsewhere.[10-13] The antigen stimulus or the regulatory breakdown that is responsible for this production of antiself antibodies is not known and could in fact be something other than immunization by the nucleic acids.

A. Specificity of SLE Serum Antibodies

As a result of examination of many SLE sera in several laboratories, it became clear by the mid-1960s that some lupus antibodies reacted exclusively with denatured DNA, whereas others recognized both native and denatured forms, and a smaller, but important, population preferred native over denatured DNA. These findings raised questions as to whether the antibodies were recognizing primarily the purine and pyrimidine bases or the sugar-phosphate backbone. Subsequent experiments over many years have indicated that both types of recognition can occur.

An early step was the use of oligonucleotides of chemically or enzymatically degraded DNA as competitors of SLE antibody reactions with DNA in order to probe the specificity at a fine level.[107,108] This was achieved with a small nunmber of sera that were specific for denatured DNA. One reacted with high selectivity for oligonucleotides containing thymine; the most effective inhibitor obtained was d(T5).[107] When tri- or tetranucleotides were compared, the effectiveness of competition was directly related to their thymine content; with tetranucleotides, the order of effectiveness was d(T4) > d(T3C) > d(T2C2) > d(TC3), and no inhibition was caused by d(C4). All of the tetranucleotides would share the deoxyribose-phosphate backbone, so it was concluded that the specificity depended on recognition of the thymine base or stacked bases, which could also explain the requirement for DNA denaturation to expose reactive sites. One lingering question is whether the backbone geometry is in fact identical in these tetranucleotides. If the base composition or sequence could affect that geometry and alter the interphosphate spacing or orientation, for example, it could be that the real recognition was of variations in the backbone that depended on the base sequence. One serum for which this was not a consideration was an example that was reactive with purine bases alone, recognizing adenine or adenosine and, even better, methylxanthines.[108] Furthermore, Estrada-Parra and colleagues[109] found that some of the SLE antibodies would precipitate with nucleoside protein conjugates, again indicating that a significant part of their binding energy could come from recognition of the bases rather than the phosphates. Recently, Munns et al.[110] have quantified this reactivity with a solid phase assay and with absorption by nucleoside-bearing columns. They found that a large fraction of the antidenatured DNA antibody could be accounted for by antinucleoside reactivity; 80% of the antidenatured DNA was removed from several sera by a column containing a mixture of adenosine, guanosine, cytidine, and thymidine. This column removed 30% of the antinative DNA population as well, although even high molecular weight denatured DNA immobilized on a column did not absorb all of the antinative DNA activity. In examination of individual nucleosides, guanosine was particularly prominent in reacting with several sera. These findings are of interest in view of the potential use of nucleoside-bearing nonimmunogenic carriers to induce specific suppression of this antibody formation,[111] and in view of the relative strength of guanosine as an immunizing and tolerance-inducing hapten as compared to other nucleosides.[112]

The use of mononucleotides as competitive binders has also indicated a contribution of the bases to specificity. Casperson and Voss[113,114] examined the inhibition of the SLE antibody-DNA interaction by mononucleotides and oligonucleotides and polymers, and identified a population of antidenatured DNA that was very susceptible to inhibition by mono- and oligonucleotides. A second population that reacted with both native and denatured DNA required very much higher concentrations of these small DNA fragments for any interaction, and these antibodies were sensitive to changes in ionic strength or polymer configuration, indicating that a significant part of their binding depended on interaction with the backbone;

however, it was concluded that the bases and base sequences can contribute at least in small part to the binding of these antibodies as well. Contribution of the bases to specificity, either directly or by determining overall polymer structure, was also evident in detailed studies with polynucleotides. Koffler et al.[115] found that varying populations of the SLE antibodies were absorbed by and could be purified by selective precipitation with poly(dT) or poly(dC). One population was selectively reactive with poly(dT), another reacted with poly(dT) and denatured DNA, and a third with poly(dT), poly(dC), and denatured DNA.

As noted above, some antibodies that react with native DNA are not readily inhibited by mono- and oligonucleotides. If these antibodies do see the bases, the latter probably provide only a small portion of the determinant. A great deal of effort in several laboratories went into the demonstration that SLE antibodies could indeed react with native DNA. Some skepticism about this was based on the lack of immunogenicity of DNA in experimental animals and on the lingering possibility that some denatured regions could occur in isolated nucleic acids. Arana and Seligmann[116] measured the loss of reactivity of native DNA with some SLE sera as the DNA was melted, indicating that the native structure was important for these antibodies. Immunofluorescent studies with the trypanosone *Crithidia* revealed that SLE sera reacted with the kinetochore, which contains closed circular helical DNA;[117,118] in this case, there would be no question of denaturation during DNA isolation. Furthermore, purified antibody has been shown to react with isolated circular helical DNA[119] and has been visualized in this reaction by electron microscopy.[120]

A puzzling feature of the antinative DNA reaction has been that it is nearly always inhibited by denatured DNA (whereas native DNA does not inhibit the antidenatured DNA population). In competitive assays, denatured DNA is sometimes less potent than native DNA,[121] but often it can cause inhibition even at concentrations that are just as low as those required for native DNA.[122] Some of the corresponding antibodies may recognize structures, probably in the backbone, that are present on both native and denatured DNA, such as a determinant involving a portion of a single-chain backbone. The single deoxyribosephosphate chain would be available in both native and denatured DNA. Another possibility is that denatured DNA contains a significant amount of secondary structure that presents helical regions and that the antibodies are truly specific for such double-stranded regions within the denatured DNA. This appeared to be the case, at least for some sera,[122] because the inhibitory potency of denatured DNA was reduced when assays were carried out at higher temperatures (45°C as compared with 4°C), at which some base-paired regions of denatured DNA would be melted; purely native DNA would not melt at this temperature and its inhibitory effectiveness was not temperature dependent in this range.[122] Retention of reactivity after digestion with an enzyme selective for unpaired single-stranded regions, such as S1 nuclease, also supported this conclusion.[122]

In viewing the many studies of specificity of anti-DNA antibodies of SLE sera, it appears that there are indeed several populations. The distinctions among them may be somewhat artificial, as determinants may involve contributions from more than one part of the molecule. Nevertheless, some of the antibodies appear to react primarily with bases or stacked bases and react only with denatured DNA. Others may see backbone features still unique to denatured DNA. Still others may recognize backbone features common to both native and denatured DNA with minor contribution from the bases and, finally, a small number show a high degree of preference for the backbone of double-stranded DNA.

An additional feature of interest concerning the SLE anti-DNA antibodies is their range of affinities.[123-126] This has been investigated by measurements of the binding of labeled DNA, or by dissociation of preformed immune complexes by excess DNA. In some cases, affinities have been calculated on the basis of antigen concentration expressed as moles of phosphorus (nucleotide). These affinities are in the range of 10^5 to 10^6. Other reports give values of 10^{10} to 10^{11}, presumably because antigen concentration was calculated as moles

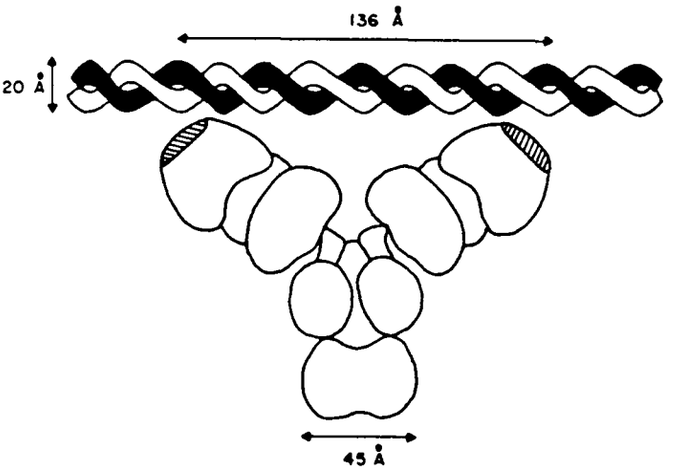

FIGURE 9. Model describing requirements for bivalent binding of one IgG molecule to a DNA fragment. The binding surface of the Fab arm of IgG is approximately 15 × 20 Å and would correspond to about three nucleotide pairs along the DNA molecule. The angle at the hinge of the IgG was taken as 115°; it is flexible, but to a minimal angle that is greater than 80°. This requires a DNA segment of at least 45 to 50 base pairs to allow both Fab regions to bind simultaneously to one molecule. (From Papalian, M., Lafer, E., Wong, R., and Stollar, B. D., *J. Clin. Invest.*, 65, 475, 1980. Copyright by American Society for Clinical Investigation. With permission.)

of high molecular weight polynucleotide. This reflects the difficulty in rationally choosing the number of nucleotides or nucleotide pairs to be used as a basis for calculating moles of antigen sites and in estimating the concentration of free antigen. The size of one site is not known with precision, and even when there is a reasonable estimate of the size of a single site, it cannot be known whether the sites are exclusive or overlapping and how often they occur in a given DNA. This problem of indeterminate site size and number has been treated for oligo(L-lysine)-DNA binding in a study by McGhee and von Hippel.[127]

The dissociation of antibody from complexes with denatured DNA was rapid, especially at 37°C, but in some cases the dissociation of antibody from native DNA was so slow as to be not measurable.[123] The latter condition probably reflects the very high affinity of multivalent binding by an antibody to one DNA molecule,[119] which complicates the evaluation of affinity, but may be very important in stabilization of immune complexes in vivo. This type of binding may also be reflected in the fact that with certain SLE sera there was a great increase in binding affinity with helical DNA fragments of 40 to 50 base pairs as compared to fragments of 20 to 30 base pairs.[128] Both fragments would be large enough to have an antigenic site (which would cover only 3 to 4 base pairs), but the 40 to 50 base-pair fragment would be just large enough to accommodate both binding sites of an IgG (Figure 9).[128] For some SLE sera, even larger DNA fragments, up to several hundred base pairs in length, were required before high binding affinity was observed. In this case, it may be necessary for the DNA, which may be flexible enough to bend over this chain length, to form a looped structure that would provide two binding sites for an IgG. Interestingly, electron micrographs suggest that antibody binding does occur at the base of looped structures.[120] These findings are also of interest in relation to the finding that the DNA present within circulating immune complexes in SLE plasma have been found to be about 50 base pairs in length in some cases and about 200 base pairs in length in others.[129] The DNA in those complexes was somewhat enriched in guanine and cytosine as compared with

the bulk of human DNA.[130] This could reflect either the prominent specificity of antibodies for guanine- and cytosine-containing regions of DNA or it could mean that such regions in immune complexes are relatively resistant to digestion by circulating nucleases.

B. Monoclonal Anti-DNA Autoantibodies

In recent years, several laboratories have attempted to clarify the nature of autoantibodies to DNA by the study of monoclonal examples.[131-144] Without immunization, spleen cells or peripheral blood cells from mice or humans with autoimmune disease have been fused with corresponding mouse myeloma or human lymphoblastoid cells to obtain hybridomas. The hybridomas were screened for production of anti-DNA antibodies. In this way, many monoclonal autoantibodies have been isolated and their specificities tested with a variety of synthetic and natural nucleic acids.

Each of the monoclonal antibodies has been tested for specificity by competitive assays with various poly-, oligo-, and mononucleotides as inhibitors of DNA binding. A general finding has been that antibodies selected for DNA binding have not bound to single- or double-stranded RNA. Separately derived anti-RNA monoclonal antibodies have been obtained by Eilat et al.[146] from NZB/NZW spleen cell hybridomas. Those anti-RNA antibodies were directed against a (G,C)-containing sequence in the RNA conformation and did not react with DNA.[146] The contribution of guanine to binding specificity was, however, reminiscent of properties of both serum and monoclonal anti-DNA specificities.

The monoclonal autoantibodies displayed nearly individualized patterns of reactivity when their reactions with a panel of poly- and oligonucleotides were examined (Figure 10).[147] There are certain recurring features, however; for example, a significant number have reacted well with poly(dT), reminiscent of significant serum antibody populations. A second recurring finding is that several react very well with poly(G) and poly(I) even though these are polyribonucleotides. They are complex polymers that can exist as single- or multistranded structures[148] and could provide a variety of conformations in which base and/or backbone may be available to antibody. It may also be important that the sugar conformation in these polymers, and therefore the phosphate group spacing, can resemble the B-form conformation of DNA rather than the A-form conformation of RNA.[149] The antipoly(dT) and antipoly(I) reactivities are not mutually exclusive and the classification of antibodies by specificity leads to overlapping groups with no completely distinct boundaries. Another criterion for grouping is reactivity with mono- or oligonucleotides. Only a few are strongly reactive with these small fragments, and guanine and thymine again were prominent in these specificities. Although the size of a determinant is not larger than about six nucleotides, often it has required considerably larger poly(dT) chains to express an effective determinant.[150] The reason for this is not clear.

Most of the monoclonal autoantibodies have shown a marked preference for denatured DNA over native DNA, but there have been several examples of antibodies that do react well with the native form.[132,141] As in the case of serum antibodies, the binding of native DNA can be inhibited by both native and denatured DNA. Again, this may mean that the antibodies recognize some feature that is available on both a single DNA chain and the helical double-stranded DNA, or that the antibodies do recognize a feature of the secondary structure of the helix and that such structures occur in collapsed or base-paired regions of denatured DNA. The latter may not apply to all of the monoclonal autoantibodies, however, because some of them react with synthetic homopolymers that do not contain base-paired regions. As noted above, the groupings are not completely distinct, and one antibody molecule may, for example, react with native DNA and poly(I).

A frequent finding has been that the autoantibodies show a greater degree of cross-reaction than do induced antibodies to nucleic acids. Experimentally induced antibodies to Z-DNA, for example, are highly selective for this structure,[50] and antibodies induced by poly(I) are

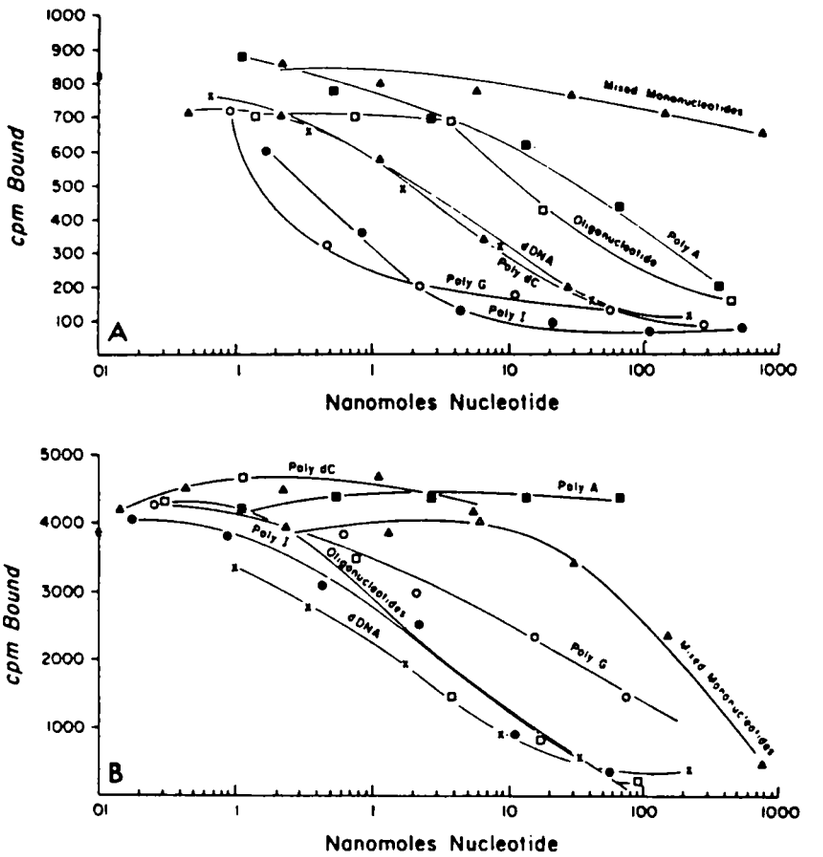

FIGURE 10. Polyreactivity of murine monoclonal anti-DNA autoantibodies. Competitive radioimmunoassays measured the ability of varying polynucleotides and oligonucleotides to compete for the binding of antibodies to DNA immobilized on polystyrene tubes. Antibody binding was detected by ^{125}I-labeled goat antimouse IgG. Binding to denatured DNA was performed in the presence of soluble competitors. (From Andrzejewski, C., Jr., Rauch, J., Lafer, E., Stollar, B. D., and Schwartz, R. S., *J. Immunol.*, 126, 228, 1981. With permission.)

selectively reactive with this immunogen.[151] Neither these nor other induced antipolynucleotide sera react with native DNA. A single monoclonal autoantibody, however, may react with poly(I), poly(dT), native and denatured DNA, and Z-DNA.[143] In such cases, it seems unlikely that the specificity is determined mainly by the purine or pyrimidine base. It seems more likely that these antibodies recognize an accessible feature of the sugar phosphate, and that there is a requirement for a specific spacing of the phosphate groups for a given antibody-combining site. One antibody may find this appropriate spacing in the double-helical structure of native DNA. Because denatured DNA and some synthetic homopolymers are much more flexible than native DNA, it is possible that they could contain the appropriate spacing for the antinative DNA antibody as well as many other local conformations, so that the antibody that reacts with native DNA can react with them as well. On the other hand, a different antibody may require one of the spacings that is present in the flexible denatured form, but not in the rigid native helix, and such an antibody would be one of the more frequent type with a strong preference for denatured DNA. With either type of antibody, the requirement for a specific three-dimensional spacing of the phosphates characteristic of DNA is still important, as nearly all of them fail to react with a single- or double-stranded RNA. In no

case has a precise definition of the antigenic determinant been made, and that may await the ability to crystallize a fragment of monoclonal antibody with bound nucleic acid.

In view of the cross-reactivity with many polynucleotides and because the origin of the autoantibodies is unknown, experiments have been conducted to determine whether the antibodies that react with DNA could recognize a repeating phosphate group in other kinds of structures, particularly phospholipids. Such a possibility was suggested also by the finding of Guarnieri and Eisner[152] that rabbit antibodies induced by cardiolipin immunization were able to bind to DNA incorporated into liposomes. Some of the monoclonal antibodies selected for their anti-DNA activity have, in fact, been found to react with cardiolipin and phosphatidic acid[153] as well as the phospholipids of certain bacterial cell walls.[188] These findings are of particular interest in relation to the laboratory findings that have been recorded in studies of SLE serum. For many years it has been known that such sera may give a false/positive test for syphilis in an assay that depends on reaction with cardiolipin, and the sera may also contain an anticoagulant activity measured in an assay that depends on the presence of phospholipid.[154] An example has been found of a monoclonal antibody that reacts with both DNA and cardiolipin and has the lupus-like anticoagulant activity.[147] This must reflect the specificity of the antibody-combining site and its recognition of appropriately spaced phosphate groups on the backbone of nucleic acid or on the surface of phospholipid containing vesicles (Figure 11). Anti-DNA antibodies of SLE sera can also be inhibited by cardiolipin.[155] Additional cardiolipin-reactivity antibodies that do not recognize DNA are also present in SLE serum.[155,156] This would suggest that the antibodies that react with DNA could be part of a larger family of antibodies directed primarily against nonnucleic acid structures, and some caution must be exercised in their use as biochemical probes. They may be used, however, as useful controls in comparisons of the more unique reagents. For example, native polytene chromosomes that show little or no reactivity with anti-Z-DNA antibodies were able to react with monoclonal antinative DNA, indicating that the nucleic acid was accessible in the chromosome.[65]

As more examples of the autoantibodies are examined, more specific reagents may be obtained. Antibodies obtained by Tron et al.[141] were reactive with poly(dA-dT), but not with poly(dG-dC). One example recently studied in the author's laboratory showed similar selectivity while another reacted with native DNA and with synthetic poly(dG-dC), but not with poly(dA-dT). These findings raise the question whether the antibodies can recognize local variations in helix structure that would depend on local base composition. The antibody bound to many sites on a closed circular plasmid DNA, however, at low concentrations the (dG-dC)-selective example did show preference for a few particular regions of the plasmid. The ability of these antibodies to discriminate between two right-handed helixes of the B-DNA family raises two possible interpretations, both remarkable, as the basis for their selectivity. One possibility is that the helix geometry of poly(dA-dT) and poly(dG-dC) (i.e., the shape and orientation of the easily accessible sugar-phosphate backbones) are so distinct as to be easily distinguished by the proteins. The second interpretation is that the antibodies do make some contact with the bases and thus distinguish the base sequences. This would require that a portion of the antibody actually reach into the major groove to a depth of several angstroms. Antigen-binding sites of crystallized Fab fragments have, so far, fit with the long-standing notion that they are cavities or grooves at the Fab tip.[157] Could it be that an edge of the antigen-binding cavity can enter the major groove to contact the bases while the inner part of the antibody cavity makes contact with the deoxyribose-phosphate? Again, crystallography of a monoclonal Fab fragment-ligand complex with an antibody of this specificity may be required to resolve these alternatives.

V. ANTIBODIES TO BASES, NUCLEOSIDES, AND NUCLEOTIDES

One of the earliest methods for production of anti-DNA antibodies was the use of small

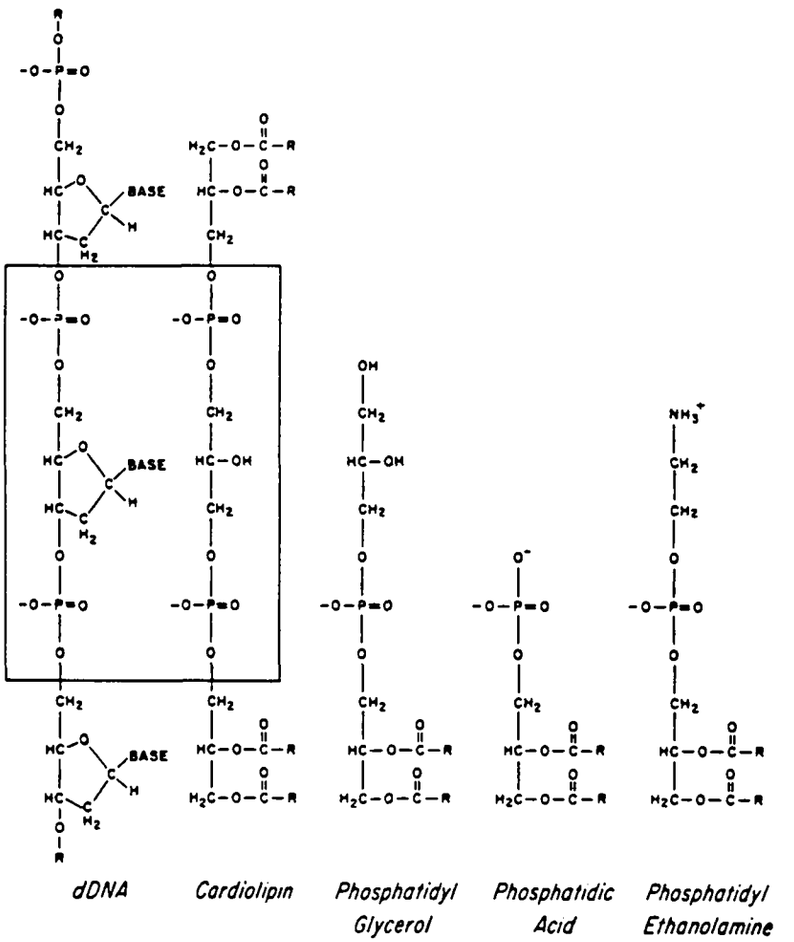

FIGURE 11. Comparison of chemical structures of phospholipids and DNA showing the separation of phosphodiester groups by three carbon atoms in cardiolipin and DNA, as pointed out by Guarnieri and Eisner.[152] Repeating phosphate groups of phosphatidic acid and phosphatidyl glycerol would also be available on liposomal surfaces. Positively charged phospholipids were less reactive. (From Lafer, E., Rauch, J., Andrzejewski, C., Jr., Mudd, D., Furie, B., Furie, B., Schwartz, R. S., and Stollar, B. D., *J. Exp. Med.*, 153, 907, 1981. Copyright permission of the Rockefeller University Press. With permission.)

DNA components as haptens, conjugated covalently to protein carriers for immunization. Purine[158] or pyrimidine bases,[159] nucleosides,[169] or oligonucleotides[160,161] have all served for this purpose. The resulting antibodies have shown considerable specificity for the purine or pyrimidine bases and even for regions within the base structure. Munns et al.[162] recently have reexamined the range of cross-reactions of antibodies induced by nucleosides of guanine, 7-methylguanine, and cytosine. They concluded, for example, that the major recognition site of guanine involved the N1, C6, O6, and N7 atoms, whereas the C4 and N4 of cytosine were dominant for anticytidine antibodies. Beyond specificity for the bases, antibodies induced by nucleosides and nucleotides gain additional binding energy and selectivity by interaction with the pentose and phosphate[6,9] or, in oligonucleotides, with base sequences as well.[163] In the latter case, conformational features of certain sequences may be recognized, as in reactions of antibodies to (A-A-U) and to (A-A-U-U).[163]

Antibodies to bases, nucleosides, or nucleotides react with single-stranded nucleic acids in which the purine and pyrimidine bases are exposed to solvents. Antibodies to the normal DNA bases have served as reagents for identifying single-stranded regions in cells during the S phase of the cell cycle,[164] or in sites near lesions caused by UV irradiation[165] or photo-oxidative damage.[166] Antithymidine antibodies have also been used to quantify circulating serum thymidine, as a measure of cell death.[167]

In recent years, increased emphasis has been placed on studies of modified bases. Specificities of antibodies for methylated bases were described by Levine and colleagues.[168] These antibodies have provided sensitive and selective probes for naturally modified bases, such as 7-methylguanine (in RNA), 6-methyladenine, and 5-methylcytosine. Storl et al.[169] measured 6-methyladenine in denatured DNA specifically in prokaryotic samples known to contain this modified base. A quantitative complement fixation assay did not, however, yield a reliable linear relationship between the degree of immunochemical reactivity and the amount of 6-methyladenine present. The discrepancies could result from variable accessibility of the base within the secondary structure that does form in denatured DNA. The selectivity of this type of antibody was demonstrated in an immunoelectron microscopy study of the DNA of phage fd, in which the base occurs just once. Ferritin-labeled antibody was visualized at one site in the DNA.[22]

5-Methylcytosine is a naturally occurring modified base that has been of great interest because of its frequent (though not perfect) correlation with regulation of gene expression.[170,171] There are many examples in which unmethylated CpG sequences are associated with genes in the actively transcribed state, whereas the methylation of C in the same site of the same gene occurs when the gene is not being transcribed. Antibodies specific for 5-methylcytosine have been applied to the localization of the base both in chromosomes and in isolated DNA and restriction fragments of DNA. By immunofluorescence or immunoperoxidase techniques, 5-methylcytosine has been shown to be concentrated in heterochromatin regions of mammalian metaphase chromosomes, particularly in certain centromeric regions.[172-176] This concentration occurs particularly in repeated sequences of satellite DNAs, both in CG sequences within AT-rich satellites and within GC-rich satellites. The staining patterns with antibodies indicated a variability from species to species in which chromosomes were stained,[173-177] and a polymorphism of staining patterns among unrelated humans.[178] These techniques also identified concentrations of 5-methylcytosine in amplified rRNA genes in both human[179] and rat hepatoma cells;[180] the amplified genes rich in the methylated base were not actively transcribed.

These antibodies also detected regions of 5-methylcytosine concentration in the condensed bands of polytene chromosomes of *Sciara* and *Drosophila* after the chromosomal DNA was denatured by photo-oxidation or UV light.[181] This result was surprising because only very low levels of this methylated base have been detected in insects, and adult *Drosophila melanogaster* was reported to have none. The immunohistochemical analysis was carried out with purified antibodies, and their reaction was blocked specifically by 5-methylcytidine, but not by other bases or nucleosides. In contrast to these results with polytene chromosomes, no 5-methylcytosine was detected by immunohistochemical analysis of mitotic metaphase chromosome.[181] The methylation restricted to the polytene stage may be related to maintenance of certain gene regions in the inactive state in condensed chromatin of bands in spite of the amplification of polytenization.

The presence of a minor modified base can be identified in isolated DNA that has been denatured and blotted onto nitrocellulose. Sano et al.[182] measured 5-methylcytosine in nuclease restriction fragments of *Chlamydomonas* DNA obtained at different stages of the life cycle, showing little methylation in the DNA of chloroplasts of vegetative cells or male gametes, but more extensive methylation in fragments from female gametes and zygotes. They also measured the base in satellite I DNA from calf thymus DNA, showing that the

method could detect 0.5 pmol of base in a sample of 100 ng of DNA.[182] In the case of single-stranded ØX174 DNA, which contains but one methylated base in the molecule, the antibodies could detect 0.02 pmol in a 40-ng sample.[182] Garrard and colleagues[183] used an electrophoresis and blotting technique to determine the distribution of 5-methylcytosine in various nucleosome populations; they found that 80% of the methylated base was associated with nucleosomes that contained H1 histone, possibly reflecting the presence of the base in condensed chromatin that is not actively transcribed.

As well as the naturally occurring modified bases, chemically and physically modified bases have been studied immunochemically. Corresponding antibodies have been valuable reagents for identifying and quantifying the formation of lesions and their repair. These include antibodies to UV-irradiated DNA, photo-oxidized DNA, and to several DNA adducts formed by alkylating agents and platinum derivatives. The immunoassay of carcinogen-modified DNA components has been the subject of a recent extensive review.[23]

VI. SUMMARY

Less than 30 years ago, nucleic acids were generally considered to be nonantigenic. There were only a few reports on the induction of antibodies with DNA-containing, but very heterogeneous, nucleoprotein preparations. Beginning with the identification in the late 1950s of SLE autoantibodies that react with DNA, and progressing with the demonstration in the early 1960s that anti-denatured DNA antibodies can be induced experimentally, there has been a great growth in the number and kinds of immunochemical probes available for specific nucleic acid structures. These include normal or modified bases, oligonucleotides, and single-stranded and helical polymers. The more recent development of monoclonal antibodies to nucleic acids and components enhances the precision of their applications as biochemical probes.

A potential limitation is often raised in discussions of the use of the antibodies as specific probes. Although a given serum or even monoclonal antibody can be demonstrably specific for a given conformation when many isolated nucleic acids and synthetic polymers are tested, could it be possible that some other structure, not tested in that survey, might be what is detected when the antibodies are used to probe complex biological materials? Could something other than Z-DNA, perhaps displaying some partial feature of it, be responsible for the immunofluorescence seen with chromosomes? Such a possibility is difficult to rule out totally with any structural probe, therefore, many studies seek additional confirmatory indications of the nature of the target. So far, the antibodies and additional tests have been concordant. In one instance, antibodies provided a discriminating negative result; although the circular dischroism spectrum of a mitomycin-poly(dG-dC) complex suggested that the polymer was assuming a Z-DNA conformation, anti-Z-DNA antibody did not detect it, and this negative finding was supported by other independent physical chemical measurements.[184]

The precise binding sites of helical nucleic acids recognized by specific antibodies are not known with certainty. Inferences are drawn from the comparisons of polymers for which X-ray diffraction data provides information on three-dimensional structure and from the effects of varying conditions such as ionic strength. The size of a single recognition site must be confined to a few base pairs in a helix or fewer than six stacked bases in a single-stranded nucleic acid, as these dimensions match the limits of known combining regions of antibodies. It is reasonable to conclude that antibodies recognizing helical polynucleotides combine with the outer features, predominantly the sugar-phosphate backbone, and that the precise spacing of the phosphates of both strands can determine the specificity of interaction with antibody. A precise definition of the interaction will require the crystallization of antibody-nucleic acid complexes, a prospect that becomes more possible as more monoclonal antibodies are developed. Homogeneous antibodies defined with that precision will be reliable probes for unique short-range conformational variations in DNA.

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