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Antibody against Single-Stranded DNA Useful for Detecting Apoptotic Cells Recognizes Hexadeoxynucleotides with Various Base Sequences[1]

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Our previous study demonstrated that an antibody against single-stranded DNA could detect apoptotic cells [Naruse *et al.* (1994) *Histochemistry* 101, 73-78]. In this paper we describe the development of an improved method for the production of the antibody and investigations into the antigenic determinants of the antibody so that it could be of practical use for detecting apoptotic cells. Rabbits, hyperimmunized with complexes of alkaline-denatured calf thymus DNA and methylated bovine serum albumin, produced an IgG antibody to single-stranded DNA. Analysis by sandwich ELISA using various naturally occurring nucleic acids revealed that the antibody was specific to single-stranded DNA. Furthermore, using synthetic polymers in the assay, it was found that the antibody could recognize single-stranded DNA with various base sequences. Gel electrophoresis retardation assays, with synthetic oligodeoxynucleotides with differing lengths of single-stranded DNA, indicated that a hexadeoxynucleotide constituted the minimum size of the antigenic determinants, and suggested that the antibody probably consists of several antibodies which recognize hexadeoxynucleotides with various base sequences. Western blot analysis demonstrated that the antibody can recognize both a DNA ladder and oligonucleosomes prepared from rat liver nuclei with endogenous endonuclease. The present findings demonstrate that this antibody is a useful tool for detecting apoptotic cells.

Key words: antibody, apoptosis, gel electrophoresis retardation assay, sandwich ELISA, single-stranded DNA.

Programmed cell death (PCD), which is a selective process of physiological cell deletion, plays a key role in developmental biology and in maintenance of the steady state of continuously renewing tissues (*1-3*). PCD is morphologically known as apoptosis (*4*), and is associated with endogenous endonuclease activity (*5-7*). DNA fragmentation can be seen not only in histologically defined apoptotic cells, but also in morphologically intact cells going through the process of PCD. At present, DNA fragmentation is considered the most characteristic feature of apoptosis. Current detection methods for apoptosis involve genomic DNA extraction and subsequent analysis of the ladder of nucleosomal DNA fragments in agarose gels which, however, do not allow distinction between individual cells. Recently, Gavrieli *et al.* developed the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method that visualizes the process of programmed cell death *in situ* at the single-cell level (*8, 9*). This method is based on the specific binding of terminal deoxynucleotidyl transferase to the 3′-OH ends of DNA, and subsequent synthesis of a polydeoxynucleotide polymer. The TUNEL method, however, is complex and expensive to perform because of the complicated processes involved and the use of expensive reagents. In this regard, immunohistochemical methods involving antibodies specific to apoptosis would have several distinct advantages. Tomei (*10*) proposed that single-stranded DNA (ssDNA) modification in the nucleosomal linker region might constitute a critical early step in apoptosis. Therefore, we attempted to use an antibody against ssDNA to detect apoptotic cells *in situ*. In a previous study, we demonstrated that a polyclonal antibody against ssDNA was able to detect both PCD and drug-induced apoptosis (*11*). The production of an antibody against ssDNA was first demonstrated by Plescia *et al.* (*12*), who used a complex of heat-denatured DNA and MBSA. However, a large proportion of the antibody against heat-denatured DNA produced in rabbits was of the IgM isotype, and a high antibody titer was not obtained (*13*). As an IgG isotype antibody is suitable for immunohistochemical staining, we attempted to develop a method for the production of an IgG isotype antibody against ssDNA. Moreover, in order to use this antibody to detect apoptotic cells, we considered it first necessary to elucidate the binding specificity of the anti-ssDNA antibody. Although there have been only a few reports about the antigenic determinant groups of such an antibody, Wakizaka and Okuhara suggested that the base

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; MBSA, methylated bovine serum albumin; RDV-RNA, rice dwarf virus RNA; ssDNA, single-stranded DNA.

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composition and sugar phosphate backbone are important factors in the antigenic determination of heat denatured DNA (*14*).

In this paper, we report an improved method for the production of an IgG isotype antibody against ssDNA, and also the characterization of the antibody by means of ELISA and gel electrophoresis retardation assays. Moreover, we demonstrate that the antibody recognizes an apoptotic DNA ladder and oligonucleosomes.

MATERIALS AND METHODS

Materials—Calf thymus DNA, bakers' yeast tRNA and *Escherichia coli* rRNA were obtained from Sigma Chemical (USA). *Candida utilis* 5S-RNA and RDV-RNA were obtained from Dr. Y. Kitagawa (*15*). Poly(dA), poly(dT), poly(dG), poly(dC), poly(dA)•poly(dT), poly(dG)•poly(dC), poly(dG-dC)•poly(dG-dC), poly(dA-dT)•poly(dA-dT), and poly(dA-dC)•poly(dG-dT) were purchased from PL-Biochemicals (now Pharmacia Biotech, Sweden). RNase A and proteinase K were obtained from Boehringer Mannheim (Germany). MBSA was prepared from crystalline bovine serum albumin fraction V (Miles Laboratories, USA) according to Mandell and Hershey (*16*). Complete Freund's adjuvant was purchased from Iatron Lab. (Tokyo). An alkaline-phosphatase (Boehringer Grade I for ELISA)-conjugated anti-ssDNA IgG antibody was prepared by the one-step glutaraldehyde procedure (*17*). All other chemicals used were of analytical grade.

Preparation of Immunizing Antigen—Calf thymus DNA was purified by digestion with RNase A and proteinase K, followed by two rounds of extraction with SDS-phenol. A solution of purified DNA (1.15 mg/ml in 10 mM Tris, 0.1 mM EDTA, pH 8.0) was brought to a final concentration of 1 N NaOH with 19 N NaOH, and then allowed to stand for 2 h at room temperature. The alkaline solution was neutralized with HCl, immediately after which a 1% solution of MBSA in water (one-tenth volume) was added with mixing, which resulted in the formation of fine particles. A volume of the complex was emulsified with an equal volume of complete Freund's adjuvant.

Preparation and Purification of an Antibody against ssDNA—Female Japanese white rabbits (3-4 kg) were given seven intramuscular injections of freshly-prepared antigen containing 1 mg ssDNA at weekly intervals. The rabbits were bled from a marginal ear vein 7 days after the last injection, and sera were prepared by the usual method. Purification of the antibody was performed according to the procedure of Kitagawa and Stollar with slight modification (*18*). Serum was incubated with an equivalent amount of alkaline-denatured DNA, as determined on quantitative precipitation, for 2 h at 37°C and then allowed to stand for 3 days at 4°C. The resulting precipitate was collected by centrifugation at 1,000×*g* for 10 min, washed three times with cold PBS (10 mM sodium phosphate, pH 7.4, containing 140 mM NaCl), and then dissolved in 20 mM $NaHCO_3$, pH 10.5, containing 5% dimethylsulfoxide (DMSO). The dissociated antibody and antigen were absorbed to a column (2×5 cm) of DEAE-cellulose (DE52; Whatman International, England) equilibrated with the same buffer. The retained antibodies were eluted with 50 mM NaCl, 20 mM $NaHCO_3$, pH 10.5, containing 5% DMSO and then dialyzed against PBS. The antibodies were further separated on a Sephacryl S-200 column (2.5×45 cm) developed with PBS to obtain IgG and IgM antibodies, and then concentrated and stored at 4°C in the presence of 0.02% sodium azide.

S1 Nuclease Digestion—Samples of 50 μg of nucleic acid, dissolved in 100 μl of 30 mM sodium acetate, pH 4.6, containing 3 mM $ZnCl_2$ and 0.3 M NaCl, were digested with 500 units of S1 nuclease (Miles Labs, USA) at 37°C for 30 min. The reactions were stopped by the addition of 10 μl of 0.1 M EDTA. The mixtures were then passed through a column (1×20 cm) of Sephadex G-50 equilibrated with PBS.

Enzyme-Linked Immunosorbent Assay—A sandwich ELISA was used to detect the binding of the antibody against ssDNA to various nucleic acids. Each well of a 96-well microtiter plate (Nunc-Immuno Plate I; Nippon Inter-Med, Tokyo) was coated with 150 μl of purified anti-ssDNA IgG antibodies at a concentration of 3 μg/ml in PBS for 30 min. After washing three times with PBS, the uncoated portions of the wells were saturated by incubation with PBS containing 1% BSA for 30 min. After washing with PBS, each well was incubated with 150 μl of a nucleic acid solution (from 3 μg/ml to 1.37 ng/ml in PBS, containing 0.01% BSA) for 30 min. After washing with PBS containing 0.2% Tween 20 (PBS-Tween), the wells were incubated with 150 μl of alkaline-phosphatase conjugated anti-ssDNA IgG antibodies in PBS-Tween containing 0.01% BSA for 30 min, and then washed three times with PBS-Tween and finally 3 times with PBS. To detect the bound alkaline-phosphatase conjugate, the wells were incubated with 150 μl of an alkaline-phosphatase substrate solution (1 mg/ml *p*-nitrophenylphosphate in 50 mM carbonate buffer, pH 9.5, containing 2 mM $MgCl_2$) for 30 min, the reaction being terminated by the addition of 50 μl of 1 N NaOH. All procedures were carried out at room temperature. The absorbance at 405 nm of each well was measured using a Dynatech MR580 Microplate Autoreader.

Gel Electrophoresis Retardation Assay—A gel retardation assay was used to detect antibody binding to single-stranded oligodeoxynucleotides. Isotope-labeled oligodeoxynucleotide antigens were prepared as follows: Synthetic oligodeoxynucleotide SD-5 (5′-TGCATACTTCTGCCTGCTGGGGAGCCTGGG-3′, 30′mer), a partial sequence of the late promoter of SV40, was labeled with [γ-^{32}P]ATP (Amersham, USA) at its 5′ end using T4 polynucleotide kinase (Takara, Kyoto). Each of the synthetic oligodeoxynucleotides, SD-1 (5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCA-3′, 30′mer), SD-2 (5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCATTTC-3′, 34′mer), SD-3 (5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCACCTTTC-3′, 36′mer), and SD-4 (5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCACTCCTTTC-3′, 38′mer), which have sequences complementary to SD-5, were annealed with the ^{32}P-labeled SD-5 oligomer. The resulting double-stranded oligodeoxynucleotides, which were designated as SS-0, SS-4, SS-6, and SS-8, respectively (Table I), were purified by electrophoresis through a 5% acrylamide (80:1 acrylamide/bisacrylamide) slab gel (14×16×0.1 cm, with 0.6 cm sample wells) with TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) as the buffer system. The SS-4, SS-6, and SS-8 oligodeoxynucleotides thus contained different lengths of single-stranded DNA (4 to 8′mers) at the 3′ ends of the complementary strands (SD-2, SD-3, and SD-4). An appropriate amount of each antigen (SD-5, SS-0, SS-4,

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SS-6, and SS-8) was incubated with the purified anti-ssDNA IgG antibodies (at a final concentration of 0.5 μg/μl) in 4 μl of 25 mM sodium phosphate, pH 7.4, containing 100 mM NaCl at 4°C overnight. Free and antibody-bound oligodeoxynucleotides were resolved by 5% polyacrylamide (80:1 acrylamide/bisacrylamide) gel (14×16×0.1 cm with 0.6 cm sample wells) electrophoresis with 1/4×TBE as the buffer system. The samples were mixed with a 1/5 volume of 6×loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) and then loaded on the top of the gel, followed by electrophoresis at 150 V until the marker dyes had migrated a suitable distance. After electrophoresis, the gel was dried on filter paper (Whatman 3MM), and autoradiography was performed at room temperature for 2 days on X-ray film (Fuji Film, Tokyo).

Western Blot Analysis of Oligonucleosomes and a DNA Ladder—Nuclei were isolated from a rat liver. A rat was killed by decapitation, and its liver was quickly removed and washed in ice-cold 0.25 M sucrose. The liver (10 g) was minced and homogenized, with a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle, in 30 ml of ice-cold 0.25 M sucrose containing 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 0.5 mM PMSF. The homogenate was centrifuged at 1,000×g for 10 min at 4°C. The precipitate (1 ml) was mixed with 2 volumes of 2.3 M sucrose and then the suspension was loaded onto 1 ml of 2.3 M sucrose in a centrifuge tube (10 PC Bottle; Hitachi Koki, Tokyo), which was then subjected to centrifugation at 124,000×g for 30 min at 4°C. Oligonucleosomes were prepared by the method of Hewish and Burgoyne (*19*). The nuclear pellet was suspended in 0.34 M sucrose containing 15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, and 15 mM 2-mercaptoethanol. Incubations were carried out in 0.5 ml lots at 37°C. Reactions were terminated by the addition of 4 ml of 20 mM potassium phosphate, pH 7.4, containing 5 mM EDTA, and then centrifugation at 500×g for 10 min at 4°C. To prepare chromatin, the nuclear pellet was homogenized in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 0.5 mM PMSF, followed by centrifugation at 5,000×g for 5 min at 4°C to remove insoluble materials. Preparation of DNA from the nuclear pellet was carried out by extraction with SDS-phenol. Oligonucleosomes or DNA extracted from oligonucleosomes was separated by electrophoresis through a 1.8% agarose gel (12×12×1 cm with 0.6 cm sample wells) with TAE (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) as the buffer system. After electrophoresis, the gel was stained with ethidium bromide (EtBr) and photographed. Oligonucleosomes or DNA resolved on the gel was blotted on to Immobilon-N membranes (Millipore, USA) using 3 M NaCl, 0.3 M sodium citrate (pH 7.0) as the developing buffer. The membranes were washed with PBS and then blocked with PBS-Tween containing 10% non-fat dry milk. After washing with PBS-Tween, the membranes were incubated in 20 ml of PBS-Tween containing 2% non-fat dry milk and 20 μg of rabbit anti-ssDNA IgG antibodies for 1 h, and then washed five times with PBS-Tween. Subsequently, the membranes were incubated with the second antibody (horseradish peroxidase conjugated goat anti-rabbit IgG antibody) in PBS-Tween containing 2% non-fat dry milk for 30 min and then washed five times with PBS-Tween. The membranes were developed using ECL (chemiluminescence) reagent (Amersham, USA) according to the manufacturer's protocol.

RESULTS

Production of an Antibody against ssDNA—Initially, we immunized five female rabbits, using heat-denatured calf thymus DNA as the immunogen, according to the method of Plescia *et al.* (*12*). However, the antibodies against ssDNA elicited in all rabbits were mainly of the IgM isotype and the titers were low, even if the rabbits were hyperimmunized. These results indicated that heat-denatured DNA exhibits poor immunogenicity, possibly due to the lack of sufficient ssDNA because of inefficient heat denaturation. Therefore, we attempted to use alkaline-denatured DNA for production of the antibody. After seven weekly injections, the IgG isotype antibody was raised in four of five rabbits, the yields being 46.2, 58.5, 113.3, and 249.4 μg per ml of serum.

Characterization of the Anti-ssDNA Antibody by ELISA—To determine the binding of the anti-ssDNA antibody to various nucleic acids, we used the sandwich ELISA method, which allows the direct measurement of antigenic activity. Figure 1 shows the reactivity of various natural nucleic acids with the anti-ssDNA antibody. Of them, only alkaline-denatured calf thymus DNA reacted strongly with the antibody, the strength of the reaction of native DNA being only about one-hundredth that of denatured DNA. Bakers' yeast tRNA, *E. coli* rRNA, *Candida utilis* 5S-RNA and RDV-RNA did not react at all. As native DNA also reacted with the antibody, S1 nuclease digestion was performed to remove single-stranded regions to confirm whether the antibody recognized double-stranded DNA or not. However, the S1 nuclease digested native DNA was no longer able to react with the antibody, indicating that the anti-ssDNA antibody is specific for ssDNA (Fig. 2).

To determine the base sequences involved in the antigenic determinants of the antibody, various synthetic deoxypolynucleotides were used as antigens. As shown in Fig. 3, poly(dT) and poly(dC) reacted strongly with the

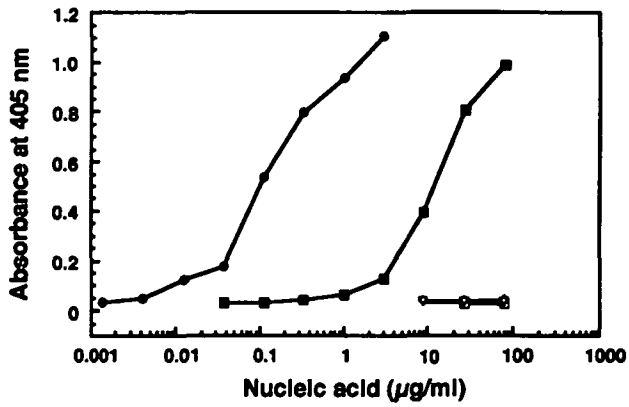

Fig. 1. **Reactivity of the antibody against ssDNA with various naturally occurring nucleic acids on sandwich ELISA.** Alkaline-denatured calf thymus DNA, ●; native calf thymus DNA, ■; bakers' yeast tRNA, △; *E. coli* rRNA, □; *Candida utilis* 5S-RNA, ◇; RDV-RNA, ▽.

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antibody, but poly(dA) and poly(dG) did not. In addition, heat-denatured poly(dG-dT)·poly(dA-dC) reacted with the antibody, although its binding ability was only one-tenth that of pyrimidine homopolymers. On the other hand, double-stranded deoxypolynucleotides, such as poly(dA)·poly(dT), poly(dA-dT)·poly(dA-dT), and poly(dG-dT)·poly(dA-dC), did not react with the antibody, although poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC) reacted slightly with the antibody.

Size of the Antigenic Determinants of the Antibody against ssDNA—It is predicted that oligonucleosomes formed in apoptotic cells contain short lengths of ssDNA at both ends of the double-stranded DNA. Therefore, we considered it important to determine the sizes of the antigenic determinants of the anti-ssDNA antibody, so as to understand the basis of their ability to detect apoptotic cells. We selected gel electrophoresis retardation assays for this purpose and used, as antigens, three kinds of synthetic double-stranded oligodeoxynucleotides, SS-4, SS-6, and SS-8, with 4′mer, 6′mer, and 8′mer ssDNA lengths, respectively, at the 3′ ends of the complementary strand of SD-5 (Table I). The base sequence of SD-5 (30′mer), corresponding to the late promoter region of SV40, was selected because of its high GC content, which stabilizes double-stranded structures, even ones of such short chain (30′mer) lengths. The base sequences of the single-stranded regions of SS-4, 5′-TTTC-3′, SS-6, 5′-CCTTTC-3′, and SS-8, 5′-CTCCTTTC-3′, were of random design, but did not contain purine deoxynucleotides since single-stranded purine polymers, poly(dA) and poly(dG), did not react with the antibody (Fig. 3). Each of the ^{32}P-labeled antigens was incubated with the antibody at 4°C overnight in the buffer system described under "MATERIALS AND METHODS." Free and antibody-bound oligodeoxynucleotides were resolved by polyacrylamide gel electrophoresis and then subjected to autoradiography with X-ray film. As shown in Fig. 4, sharp gel shift bands were detected for SS-6 (lane 5) and SS-8 (lane 7), but not for SS-0 (lane 1) or SS-4 (lane 3). However, a broad gel shift band was observed when the single-stranded oligodeoxynucleotide

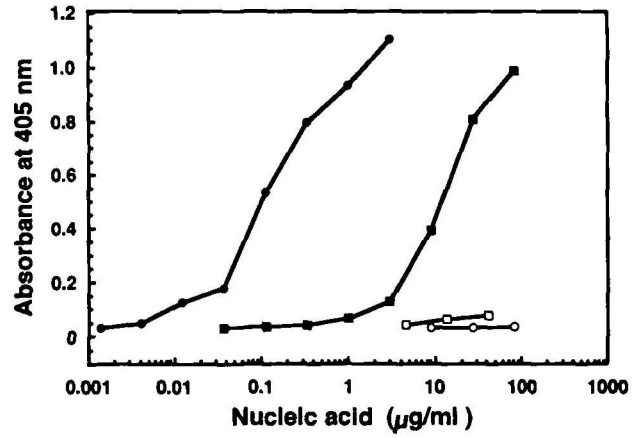

Fig. 2. **Reactivity of the antibody against ssDNA with S1 nuclease-digested ssDNA and native DNA on sandwich ELISA.** Alkaline-denatured calf thymus DNA, ●; native calf thymus DNA, ■; S1 nuclease-digested alkaline-denatured calf thymus DNA, ○; S1 nuclease-digested native DNA, □.

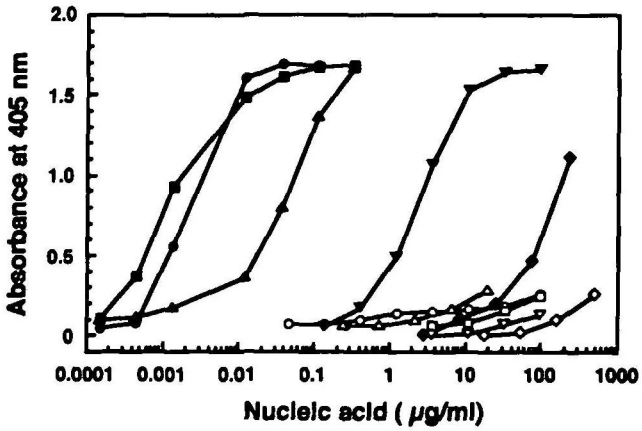

Fig. 3. **Reactivity of the antibody against ssDNA with synthetic polymers on sandwich ELISA.** poly(dT), ●; poly(dC), ■; poly(dA), ○; poly(dG), □; heat-denatured poly(dG-dT)·poly(dA-dC), ▲; poly(dA)·poly(dT), ▽; poly(dG)·poly(dC), ◆; poly(dA-dT)·poly(dA-dT), ◇; poly(dG-dC)·poly(dG-dC), ▼; poly(dG-dT)·poly(dA-dC), △.

TABLE I. **Nucleotide sequences of antigens used for the gel-shift assays.**

Antigen	Nucleotide sequence
SS-0	3′-GGGTCCGAGGGGTCGTCCGTCTTCATACGT-5′-^{32}P 5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCA-3′
SS-4	3′-GGGTCCGAGGGGTCGTCCGTCTTCATACGT-5′-^{32}P 5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCATTTC-3′
SS-6	3′-GGGTCCGAGGGGTCGTCCGTCTTCATACGT-5′-^{32}P 5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCACCTTTC-3′
SS-8	3′-GGGTCCGAGGGGTCGTCCGTCTTCATACGT-5′-^{32}P 5′-CCCAGGCTCCCCAGCAGGCAGAAGTATGCACTCCTTTC-3′
SD-5	3′-GGGTCCGAGGGGTCGTCCGTCTTCATACGT-5′-^{32}P

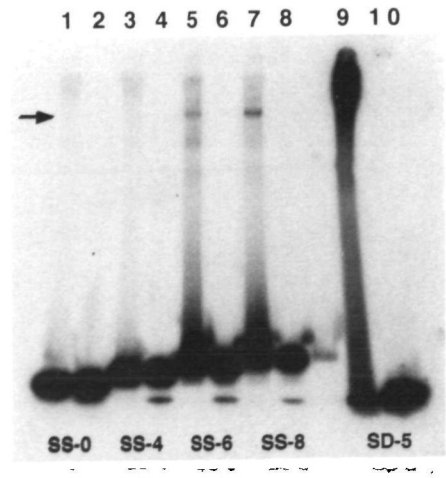

Fig. 4. **Binding of the anti-ssDNA antibody to synthetic oligodeoxynucleotides on gel electrophoresis retardation assaying.** Isotope-labeled oligodeoxynucleotides (Table I) were incubated with the anti-ssDNA antibody (lanes 1, 3, 5, 7, and 9) or control rabbit IgG (lanes 2, 4, 6, 8, and 10). The procedures and conditions for the assay are described in the text. The oligodeoxynucleotides used were: SS-0 (lanes 1 and 2), SS-4 (lanes 3 and 4), SS-6 (lanes 5 and 6), SS-8 (lanes 7 and 8), and SD-5 (lanes 9 and 10).

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(SD-5) was reacted with the antibody (Fig. 4, lane 9).

Detection of an Apoptotic DNA Ladder and Oligonucleosomes by the Antibodies against ssDNA—A biochemical hallmark of apoptosis is internucleosomal chromatin fragmentation resulting from the activation of endonucleases. In a previous study, we demonstrated that polyclonal antibodies against ssDNA are able to detect both PCD and drug-induced apoptosis (*11*). It is, therefore, important to establish whether or not the antibody is really able to recognize oligonucleosomes formed in apoptotic cells. For this purpose, we utilized the ability of an endogenous endonuclease in nuclei to prepare oligonucleosomes (*19, 20*), since the nature of the endonuclease resembles that of an inducible lymphocyte nuclear Ca^{2+}/Mg^{2+}-dependent endonuclease associated with apoptosis (*21*). After incubation of rat liver-derived nuclei for the indicated times at 37°C in the presence of Ca^{2+} and Mg^{2+}, chromatin and DNA were prepared from the nuclei and subjected to agarose gel electrophoresis to produce a ladder. Figure 5A shows the DNA ladder produced from oligonucleosomes and visualized on ethidium bromide staining. The DNA ladder became apparent after incubation of nuclei for 20 min (Fig. 5A, lane 2), and a high level of DNA fragmentation was observed after 40 min (lane 3). Subsequent blotting of the DNA fragments onto an Immobilon-N membrane and Western blot analysis using the anti-ssDNA antibody showed that the DNA ladders in all lanes were recognized by the antibody against ssDNA, and that mononucleosome DNA fragments were the most strongly stained fragments in the ladder (Fig. 5B). Figure 6A shows the ethidium bromide staining patterns of oligonucleosomes obtained from nuclei incubated for the indicated times. Western blot analysis revealed that antibody against ssDNA was able to recognize oligonucleosomes (Fig. 6B). To confirm that the anti-ssDNA antibody really recognizes single-stranded regions in DNA fragments and oligonucleosomes, they were digested with S1 nuclease and then subjected to Western blot analysis. Both DNA fragments and oligonucleosomes digested with S1 nuclease showed

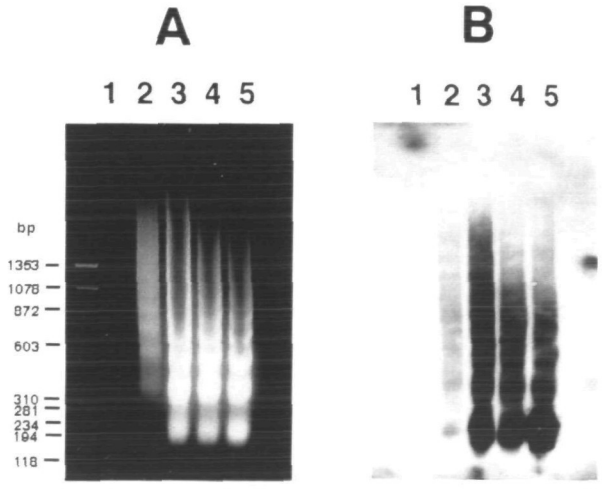

Fig. 5. **Western blot of a DNA ladder derived from rat liver nuclei with the anti-ssDNA antibody.** Rat liver nuclei were incubated in the presence of Mg^{2+} and Ca^{2+} at 37°C for 0 min (lane 1), 20 min (lane 2), 40 min (lane 3), 60 min (lane 4), and 80 min (lane 5). The preparation of DNA and the conditions for Western blotting are described in the text. The X-ray film was exposed for 30 s. A: Ethidium bromide staining of the DNA ladder in the agarose gel. B: Western blot of the DNA ladder stained with the anti-ssDNA antibody.

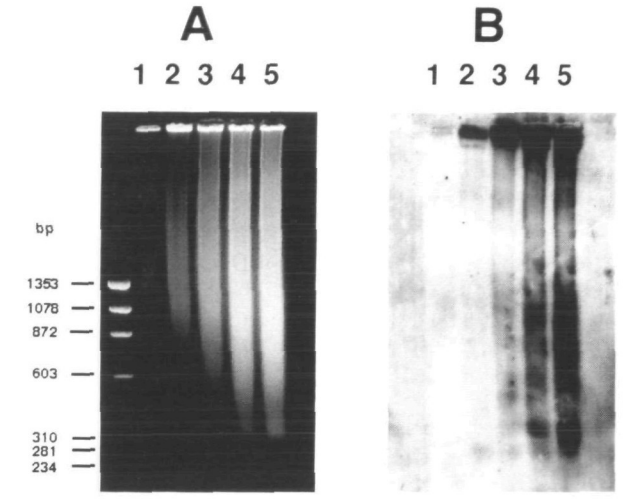

Fig. 6. **Western blot of nucleosomes from rat liver nuclei with the anti-ssDNA antibody.** Rat liver nuclei were incubated in the presence of Mg^{2+} and Ca^{2+} at 37°C for 0 min (lane 1), 20 min (lane 2), 40 min (lane 3), 60 min (lane 4), and 80 min (lane 5). The preparation of nucleosomes and the conditions for Western blotting are described in the text. The X-ray film was exposed for 3 min. A: Ethidium bromide staining of nucleosomes in the agarose gel. B: Western blot of nucleosomes stained with the anti-ssDNA antibody.

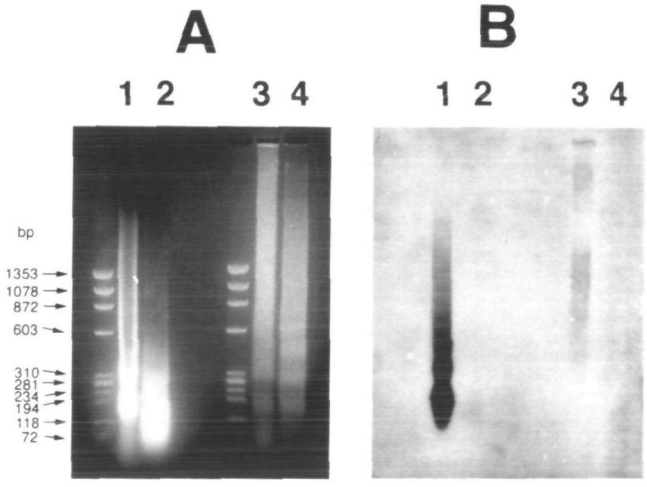

Fig. 7. **Western blot of S1 nuclease-digested DNA and nucleosomes from rat liver nuclei with the anti-ssDNA antibody.** Rat liver nuclei were incubated in the presence of Mg^{2+} and Ca^{2+} at 37°C for 80 min. The preparation of DNA and nucleosomes are described in the text. The conditions for S1 nuclease digestion and Western blotting are described in the text. The X-ray film was exposed for 30 s. A: Ethidium bromide staining of the DNA ladder and nucleosomes in the agarose gel. Lane 1, undigested DNA ladder; lane 2, S1 nuclease-digested DNA ladder; lane 3, undigested nucleosomes; lane 4, S1 nuclease-digested nucleosomes. B: Western blot of the DNA ladder and nucleosomes stained with the anti-ssDNA antibody. Lane 1, undigested DNA ladder; lane 2, S1 nuclease-digested DNA ladder; lane 3, undigested nucleosomes; lane 4, S1 nuclease-digested nucleosomes.

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slight reductions in molecular masses (Fig. 7A, lanes 2 and 4), and completely lost their binding activities as to the antibody (Fig. 7B, lanes 2 and 4).

DISCUSSION

In a previous paper, we reported that an antibody against ssDNA was able to detect apoptotic cells (*11*), however, an important problem that had to be resolved in order to put the antibody to practical use was the difficulty in producing the antibody. The production of an antibody against ssDNA was first reported by Plescia *et al.* (*12*). However, large proportions of the antibodies elicited in rabbits were of the IgM isotype (*13, 22-24*). Initially, we also attempted to raise antibodies against ssDNA in rabbits using heat-denatured DNA, but results similar to those of previous studies were obtained. It is well known that the heat denaturation of high concentrations of double-stranded DNA is inefficient, and that double-stranded DNA shows limited immunogenicity (*25*). Therefore, we assumed that the poor immunogenicity of heat-denatured DNA may be due to the limited quantity of ssDNA rather than to the nature of the ssDNA itself. Consequently, we used an alkaline-denatured DNA to produce the antibody, and we succeeded in eliciting a sufficient quantity of an antibody of the IgG isotype in rabbits. We subsequently attempted to analyze the binding specificity of the antibody in order to understand the basis of the detection of apoptotic cells by the antibody. Among the naturally-occurring nucleic acids used in this study, only denatured-DNA exhibited strong reactivity with the antibody, although native DNA reacted very weakly. To determine whether or not the antibody shows any cross-reactivity with double-stranded DNA, native and alkaline-denatured DNAs were digested with S1 nuclease to remove any single-stranded regions. As a result, both DNA fractions completely lost their binding activities as to the antibody (Fig. 2). These results demonstrated that the antibody specifically recognizes ssDNA, and suggest that the deoxyribose-phosphate backbone of single-stranded DNA may play an important role in the antigenic determinant.

In order to determine the specificities of the base sequences in the antigenic determinant, four kinds of synthetic homopolymers were used in the assay. Of them, poly(dT) and poly(dC) reacted with the antibody, whereas poly(dA) and poly(dG) did not, indicating that the antibody did not recognize homopurine sequences. However, it was unclear why poly(dA) and poly(dG) did not react with the antibody since if these purine homopolymers do form single-stranded structures, so the previous assumption that the sugar-phosphate backbone is important for the antigenic determinant might not hold. As both poly(dG) and poly(dA) are capable of forming four-stranded (quadruplex) structures containing Hoogsteen-base-paired tetrads of adenine or guanine residues (*26-28*), it is possible that the non-reactivity of the purine homopolymers with the antibody may be due to the formation of these multi-stranded structures and not because of the homopurine sequences.

In addition, whereas poly(dG-dT)·poly(dA-dC) did not react with the antibody, the heat-denatured form did, suggesting that the antibody could recognize ssDNA with various base sequences. It was not surprising that poly(dG-dC)·poly(dG-dC) reacted with the antibody, since alternating purine-pyrimidine sequences, such as $d(GC)_n$ or $d(AC)_n$, are able to form not only B-form duplexes but also extruded cruciforms or left-handed Z-type structures (*29*). It is possible that the single-stranded region of the loop structure in a cruciform could be recognized by the antibody.

Finally, a most important problem that was addressed in this study was the minimum size of the antigenic determinant of the antibody, since it seems likely that the length of the single-stranded region in fragmented DNA of apoptotic cells may not be so large. For this purpose, we analyzed the reactivity of double-stranded oligodeoxynucleotides (30′ mer), containing different lengths (4′mer to 8′mer) of ssDNA at the 3′ end, by means of a gel electrophoresis retardation assay. The base sequences of the single-stranded regions (5′-TTTC-3′, 5′-CCTTTC-3′, and 5′-CTCCTTTC-3′) were randomly designed but did not contain purine deoxynucleotides for the reasons described above. Both SS-6 (6′mer single-stranded extension) and SS-8 (8′mer) gave gel-shift bands exhibiting equal migration, whereas no gel-shift band was observed for SS-0 or SS-4 (4′mer). These results suggest that the minimum size of the antigenic determinant may be a hexadeoxynucleotide, consistent with results obtained in radioimmunoassay inhibition studies using oligonucleotides derived on DNase digestion (*30*). Furthermore, it is of interest that sharp gel-shift bands were observed when SS-6 and SS-8 were reacted with the antibody, and that the two bands migrated the same distance. In general, sharp gel-shift bands are observed when a monoclonal antibody is used in this assay. These findings suggest that the co-migrating antibodies may have been derived from one clone which recognizes a specific base sequence (5′-CCTTTC-3′). On the other hand, a broad gel-shift band, which often reflects the presence of a polyclonal antibody, was observed when the single-stranded oligodeoxynucleotide (SD-5), that contains 25 kinds of hexanucleotide sequences, reacted with the antibody. SD-5 gives a very strong band shift in which most of the DNA is retarded. The bands in the case of SS-6 and SS-8, on the other hand, are very weak. Since presumably one antibody is sufficient to retard one DNA molecule, these weak bands may reflect that the quantity of the antibody derived from one clone which recognizes a specific base sequence (5′-CCTTTC-3′) was insufficient to retard most of the antigen under these conditions. We therefore assume that a polyclonal antibody specific for ssDNA may consist of many monoclonal antibodies, each of which recognizes a hexadeoxynucleotide with a specific base sequence.

On the basis of these findings, we conclude that the antibody is suitable for detecting apoptotic cells, since such cells appear to contain ssDNA at the ends of fragmented oligonucleosomes. Indeed, we were able to confirm our conclusion by demonstrating that we could detect both a DNA ladder and oligonucleosomes, prepared from rat liver, with the antibody on Western blot analysis.

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