Characterization of Free DNP Induced by X-irradiation in Rat Spleen

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For the characterization of free DNP (saline-soluble deoxyribonucleoprotein) increasing in rat spleen after wholebody exposure to ionizing radiation, the estimation of the molecular weight of this DNP and the determination of the base composition of the DNA held in this DNA-protein complex were attempted with several analytical methods such as gel filtration, buoyant density centrifugation and paper chromatography. The molecular weight of free DNP as a complex of DNA and protein was in the range of millions. In the present course of experiment, a large contamination of RNA rich in guanine was found in the DNA fraction prepared and purified according to the methods of Swingle and Cole. However, this contaminated RNA resistant to pancreatic RNase could be removed completely by the additional treatment with RNase-T1. The base composition of the DNA held in free DNP or residual DNP, DNP other than free DNP, was not so different from that of DNA prepared from normal spleen of rat. These results were discussed in terms of sub-structure of chromatin.

INTRODUCTION

The degradation of DNA from splenic cell nucleus of mouse after whole-body X-irradiation was reported by Cole and Ellis in 1956. The degradation product was said to be the polydeoxyribonucleotides free from nuclear proteins and called "free DNA". However, this product was recently found to be in a form of DNA-protein complex and termed "free DNP". It is of some interest, because the normal DNP is known to be insoluble in saline, whereas the free DNP is soluble in it, and furthermore, almost all the degradation products of DNA from living cells besides lymphoid cells are sorts of polydeoxyribonucleotides or the derivatives of nucleotides opposite to the complexing nature of this free DNP. It is therefore suspected that the unique degradation mechanisms may be involved in the lymphoid cells. The free DNP is thought to be a very large molecule and the molecular weight of the DNA held in free DNP was suspected to be greater than $10^9$. On the other hand, the recent work on the structure of DNA has pointed to the sequence arrangements and spatial organization of DNA in chromatin. There have been several reports concerning the DNA or chromatin having a certain high

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molecular weight suggesting the existence of its subunit.\textsuperscript{17-19)}

So the experiments have been carried out to characterize the free DNP in its molecular weight and base composition, and to see if the experimental results give any information on the relation between the nature of free DNP and the basic structure of the DNA or chromatin in mammalian cell nucleus.

\textbf{MATERIALS AND METHODS}

\textit{Preparation of Free DNP}

The male Wistar strain rats 6-8 weeks old were exposed to 600 R of whole-body X-irradiation. Animals were sacrificed at 6 hours thereafter and then the spleens were excised quickly and homogenized with nine-volumes of ice-cold 0.14 M NaCl. The homogenate was centrifuged at 1500 g for 15 min. The clear supernatant fluid containing the free DNP (free DNP fraction) was carefully pipetted off and subjected to the following analytical procedures.

\textit{Molecular Exclusion Chromatography}

A rough estimation of the molecular weight of free DNP was attempted by gel filtrations on the columns of Sephadex G-200 (Pharmacia, Ltd., Uppsala, Sweden) and a more open gel, 2\% agarose in gel beads. Prior to gel-filtration of the free DNP, both gels were equilibrated with 0.14 M NaCl, 20 mM phosphate buffer, pH 7.2, and the void volumes of both columns were determined by the use of Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden). Small portions of the “free DNP fraction” were then loaded on a column of Sephadex G-200 (2.8 x 70 cm) or 2\% agarose in gel beads (2.8 x 60 cm), and eluted with 0.14 M NaCl, 20 mM phosphate buffer, pH 7.2, at a flow rate of about 4 ml/hour or 7 ml/hour respectively at 4°C. The fractions of 5 ml were collected and subjected to the determination of DNA held in free DNP.

\textit{Isolation of DNA from Free DNP}

The DNA was isolated from free DNP for the determination of base composition. The procedures described by Swingle and Cole in 1967\textsuperscript{1}) were adapted for the present study as follows: The “free DNP fraction”, about 20 ml, was treated with an equal volume of 12\% (W/V) sodium $p$-aminosalicylate and stirred for 5 min at room temperature. Then 40 ml of 80\% (W/V) phenol containing 0.1\% 8-hydroxyquinoline was added, and stirring was continued for 1 hour. The mixture was centrifuged for 1 hour at 1500 g and about 20°C. The supernatant aqueous layer was removed and treated with an equal volume of 2-ethoxyethanol, and chilled enough at 0°C. The precipitate was collected by centrifugation at 4°C and dissolved in 0.1 M sodium acetate buffer, pH 7.2. This preparation was treated with pancreatic RNase (Nutritional Biochemicals Co., U. S. A.) at a final concentration of 5 $\mu$g RNase/ml 0.1 M sodium acetate buffer, pH 7.2. After incubation at 37°C for 2 hours with occasional mixing, the trypsin (Schwarz Bioresearch Inc., U. S. A) was added to a final concentration of 5 $\mu$g/ml reaction mixture, and incubation continued for another 2 hours. An additional anhydrous sodium acetate was added to make the molarity
0.5, and then an equal volume of ice-cold 2-ethoxyethanol was added and mixed well. The precipitate was collected by centrifugation for 15 min at 1500 g and 4°C and subjected to the analysis of base composition of DNA contained in free DNP.

**CsCl Density Gradient Centrifugation**

The DNA isolated from free DNP and precipitated with 2-ethoxyethanol was dissolved in 2 ml of 20 mM Tris chloride buffer, pH 7.4, and then mixed with CsCl in centrifuge tube to give a density of 1.86 and a volume of 2.5 ml. An equal volume of lower density (1.5 g/cm³) of CsCl solution was layered over it. The centrifugation was performed at 37000 rev/min for 18 hours at 20°C with the Spinco SW 39 rotor. Fractions were then collected from the bottom of the tube and examined for the optical densities at 260 nm. The density of CsCl solution was calculated from the refractive index by the method of Ifft et al.20) When the marker DNA was required for comparison of density of DNA, the radioactive DNA was prepared from rat spleen according to the method of Kirby21) after intraperitoneal injection of ³H-thymidine.

**Paper Chromatography**

When the base composition of DNA was examined by acid hydrolysis followed by paper chromatography, the DNA contained in residual DNP, the DNP other than free DNP, was also subjected to the analysis. The procedure for the isolation of DNA from the residual DNP was similar to that from the free DNP, except that the pelleted residual DNP after free DNP extraction with 0.14 M NaCl was rehomogenized directly with 6% sodium p-aminosalicylate, and that the DNA precipitated with 2-ethoxyethanol was collected by spooling on a rod instead of by centrifugation.

The appropriate amount of each DNA isolated from free DNP or residual DNP was weighed into a test tube made of heavy-walled Pyrex glass (9 mm x 70 mm) and trifluoroacetic acid was added. The tube was then heated in an oil-bath for the hydrolysis of DNA at 155°C for 60 min. The tube was opened carefully, after chilling in floating ice, and the trifluoroacetic acid was evaporated by vacuum pump. The residue was dissolved in 0.5 ml of 0.1 N HCl, and 100 μl of this solution was used for each spot on the paper for chromatography (Whatman paper No. 1). The solvent used was an aqueous solution containing iso-propanol 65% (V/V) and HCl 2 N in the whole volume.22) After development by the descending method for 16 to 20 hours at room temperature, the paper was dried and the spots were marked with the aid of a Chromato-VUE (Ultra-violet Products, Inc., U. S. A.). The bases were eluted from each spot with 5 ml of 0.1 N HCl at 37°C for 72 hours. The eluates were read at their absorption peaks against the corresponding blanks in the Hitachi spectrophotometer, and the amount of each base was estimated.

**Extraction and Determination of Nucleic Acids**

The nucleic acids contained in each fraction were precipitated with ice-cold 0.3 N PCA, and then extracted at 90°C with 0.3 N PCA for 15 min. The determi-
nations of DNA and RNA by colorimetry were based on the diphenylamine method of Burton and the p-bromophenylhydrazine (PBPH) method of Webb respectively.

RESULTS

Molecular Size of Free DNP

The gel-filtration profiles of free DNP on Sephadex G-200 and 2% agarose in gel beads are shown in Figure 1. The free DNP was eluted at the exclusion volume of the column by the gel, Sephadex G-200. From the column of 2% agarose in gel beads, the free DNP emerged somewhat more retarded than the Blue Dextran 2000 which is said to have a weight-average molecular weight of $2 \times 10^6$. Hence, the molecular weight of free DNP appears to be in the range of millions, although the shape of the molecules makes their behavior on gel filtration media difficult to interpret.

Base Composition of DNA

The equilibrium buoyant density banding pattern of DNA prepared from free...
DNP is shown in Figure 2. The density of the main peak of this DNA appears to be somewhat higher than that expected from the normal DNA. The band shape is not of single sharp peak, and a wide zone, even a region of high density corresponding to the density of RNA, was occupied in the density gradients. The buoyant density of the DNA was partially an unusual high value, which could not yet be accounted for the abnormal base sequences richest in G-C contents. So the each fraction was subjected to the estimation of RNA and DNA by colorimetical methods after hydrolysis with 0.3 N PCA at 90°C for 15 min. Since the results obtained by the most commonly employed method for RNA assay of Mejbaum (1939) with orcinol must be corrected for DNA interference, the p-bromophenylhydrazine method was used for RNA assay. The presence of DNA does not interfere with the colour reaction in this method. The buoyant band shapes estimated colorimetrically are shown in Figure 3. From the result, it is obvious that the DNA isolated from the free DNP according to the method of Swingle and Cole is contaminated, to some extent, with RNA.

The contamination of RNA in the isolated DNA was also detected in the analytical process with paper chromatography. A diagram of the bases released from
DNA prepared from free DNP or residual DNP by hydrolysis and subsequent chromatography is shown in Figure 4. The hydrolysate of DNA isolated from residual DNP was separated into four distinct spots of bases, and the base ratios, expressed as mol/100 mol of total bases, of guanine, adenine, cytosine and thymine with standard deviations were 22.6 ± 0.2, 29.5 ± 0.5, 20.7 ± 0.2 and 27.2 ± 0.1, respectively. The base composition of residual DNP was not so different from that of
normal DNA. However, the hydrolysate of DNA prepared from free DNP turned out 6 spots on the paper after chromatography. Four of them were obviously guanine, adenine, cytosine and thymine, and the tailing of the spot of guanine was conspicuous. Others were identical with uracil and cytidylic acid in R_f values and spectra in alkaline or acid solution. These unexpected extra spots and the extreme tailing of guanine were interpreted as the result of insufficient digestion of RNA with pancreatic RNase. Since the main action of pancreatic RNase is to split the linkage joining the phosphate residue at C-3' in a pyrimidine nucleotide to C-5' in the next

| Fraction       | RNA/DNA(%)    |
|----------------|---------------|
| F_1 fraction   | 462.5 ± 34.8  |
| F_2 fraction   | 192.3 ± 18.2  |
| F_3 fraction   | 38.9 ±  1.9   |
| F_4 fraction   |    0          |

F_1 fraction, free DNP fraction; F_2 Fraction, DNA fraction precipitated from aqueous layer with ethoxyethanol after phenol treatment of F_1 fraction; F_3 fraction, DNA fraction prepared from F_2 fraction after treatment with pancreatic RNase; F_4 fraction, DNA fraction purified from F_3 fraction by digestion with RNase-T_1.

Fig. 5. Equilibrium buoyant density banding pattern of normal DNA and DNA isolated from free DNP. —— O. D. 260 of DNA in free DNP; ——— Radio activity of ^3H-normal-DNA; ——— Density of CsCl.
nucleotide in sequence, the RNase-T₁ was used together with pancreatic RNase in further experiments. RNase-T₁ acts specifically on RNA in a manner different from that of pancreatic RNase. It hydrolyses the internucleotide bonds of RNA between 3'-GMP and the 5'-hydroxyl groups of adjacent nucleotides. As shown in Table, the additional use of RNase-T₁ enabled to remove the RNA completely from the DNA prepared from free DNP. The equilibrium buoyant density banding pattern of this RNA-free DNA is shown in Figure 5. There were no essential differences between the buoyant densities of intact DNA and DNA prepared from free DNP, and the buoyant band shapes of both DNA were also similar each other.

DISCUSSION

Since the discovery of the degradation of DNA from X-irradiated lymphoid cells, the degradation product of DNA was believed to be free from nuclear proteins. In 1967, Swingle and Cole reported that this DNA had been isolated and purified for the estimation of its terminal end, and they concluded that the degradation of DNA in lymphoid cells after exposure to ionizing radiation was the result of the action of DNase I. However, it seems somewhat earlier to come to such a conclusion. It was revealed, recently, that this degradation product of DNA was making a complex with protein, and moreover, by the present study, it became obvious that the DNA prepared by their method from the degradation product was contaminated with the RNA. So their preparation method seems to be insufficient for the analysis of terminals of the degraded DNA. The impurity of DNA prepared according to their method may be caused by a large amount of ribosomal RNA contained in “free DNP fraction”, because the ribosomal RNA is known to be rich in guanine, whereas the pancreatic RNase they used is not effective against such nucleotides rich in purines. For the analysis of the productive mechanisms of free DNP, its accurate characterization is necessary. So the procedure established by the present study for the preparation and purification of DNA from the free DNP may contribute to the further study of the free DNP.

By the present study, it was also revealed that the free DNP was of high molecular weight, about 10⁶ or more, and that its base composition was almost identical with that of normal DNA. If the free DNP is produced by an attack of DNase I, it may decompose to a continuous range of molecular weight as having been reported by others. So the DNase I is not expected to be a cause of this type of degradation. The direct action of radiation and the action of radiation-generated free radicals or other active substances are also unexpected, because the 1000 R of X-rays is known to form in the DNA only one doublestrand break/2.8×10¹⁰ daltons. If the free DNP is produced through a random process, it may be more heterogeneous in its molecular weight and the base composition of its DNA. So it seems that the site of the degradation of chromatin in lymphoid cells after X-irradiation is severely limited. According to the recent work on the organization of DNA or chromatin, a simple, basic, repeating structure for chromatin has been proposed.
The DNA fragments of high molecular weight, $4 \times 10^6$ to $20 \times 10^6$, or of 1,400 base pairs have also been obtained using the restriction enzyme EcoRI (Restriction endonuclease).\textsuperscript{19,29} So it is likely that the production and the nature of free DNP are concerned with the chromatin sub-structure as such like this although there may be many interpretations about the present results and the mechanisms of free DNP production have yet to be elucidated.

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