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ANALYSIS OF 5'-ENDS OF SHORT DNA FRAGMENTS EXCRETED BY PHYTOHEMAGGLUTININ STIMULATED LYMPHOCYTES

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Human peripheral blood lymphocytes were stimulated with phytohemagglutinin and the excreted DNA was isolated from the medium after four days of incubation of cells. The excreted DNA was labeled at the 5'-end with [γ-^{32}P]ATP and polynucleotide kinase. Analysis of the end-labeled material revealed a size distribution with a chain length of 6 - 60 nucleotides. These short DNA fragments did not contain ribonucleotides at their 5'-termini. P1 nuclease digestion did not release specific deoxyribonucleoside monophosphates from the 5'-end of the excreted DNA fragments. These results point to the non-specific degradation of DNA excreted by stimulated lymphocytes.

Peripheral blood lymphocytes show a rather low level of spontaneous DNA synthesis in resting stage. Lymphocytes stimulated by plant lectins release DNA into culture medium (1-3). Freshly isolated human tonsillar lymphocytes being in stimulated stage excrete DNA without phytohemagglutinin stimulation (4). This phenomenon has been extensively studied with respect to the biological function, origin and properties of the released DNA (reviewed in ref. 5,6).

The loss of DNA of stimulated lymphocytes is regarded as an active mechanism connected with gene amplification and homeostatic equilibrium between DNA in cells and that in the culture medium (7). The activation of endogenous C type viruses are suggested by others (8). Other data point to the possibility that the release of DNA is involved in the immune response (1,3,9). Other authors suggest that excreted DNA comes from cell integration followed by degradation of cellular material (10). Another

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possible explanation of DNA release is that cells remove the "extra" DNA simply by reverting from proliferating to resting state (4).

Since the nature of the excreted DNA fragments has not been defined yet, we have designed experiments to distinguish between the nascent and non-nascent DNA fragments. We employed polynucleotide kinase to label the 5'-OH ends of DNA molecules and analyzed the 5'-ends after treatment with alkali or P1 nuclease. We show here that the excreted DNA molecules do not contain specific sequences or ribonucleotides at their 5'-ends ; in fact they are degradation products rather than nascent fragments.

MATERIALS AND METHODS

Chemicals and enzymes. Bio-Gel P-4 was obtained from Bio-Rad, deoxyribonucleotides from Calbiochem, phytohemagglutinin from Wellcome, bacterial alkaline phosphatase was purchased from Worthington, nuclease P1 from P-L Biochemicals, pronase from Serva and polynucleotide kinase from T4 infected *Escherichia coli* was from Boehringer. Alkaline phosphatase was further purified to remove contaminating nucleases by the procedure of Weiss et al.(11). [γ-^{32}P]ATP (200 Ci per mmol) was prepared according to the method of Post and Sen (12).

Isolation of DNA. Lymphocytes were separated from heparinized human peripheral blood by sedimentation through Ficoll/Uropolinum gradient according to Bøyum (13). Cells stimulated with phytohemagglutinin (2 µg/ml) were cultured in Eagle's minimal essential medium. At the fourth day the cultured cells were collected, washed twice with PBS and subjected to DNA isolation according to Rogers (14). Briefly, cell lysate in 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM EDTA, 0.6 % SDS was deproteinized with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. Precipitate was redissolved in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl and digested with pronase (10 µg/ml) for 2 hours at 37 °C. A control experiment without phytohemagglutinin stimulation was carried out. We could not detect DNA without stimulation.

Endgroup labeling of DNA. The DNA to be end-labeled with ^{32}P was first deproteinized by phenol extraction, followed by ether extraction to remove trace amounts of phenol. DNA was incubated in the presence of bacterial alkaline phosphatase to remove any 5'-terminal phosphate moietes (11). The reaction mixture (30 µl) contained 10 mM Tris-HCl, pH 8.0, 20 mM NaCl, 0.8 units of alkaline phosphatase from *E. coli* and 20 µg DNA. After incubation at 37 °C for 60 min, the mixture was subjected to phenol extraction. 5'-OH DNA was desalted on Bio-Gel P-4 column as earlier described (15). The desalted material was evaporated to dryness and incubated at 37°C for 30 min in a volume of 10 µl containing 70 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 16.7 mM 2-mercaptoethanol, 1 mM spermidine, 10 mM [γ-^{32}P]ATP and 0.2 units of polynucleotide kinase (16). The reaction was terminated by heating at 65 °C for 10 min and the mixture was diluted with 0.1 ml 0.1 M triethylammonium bicarbonate buffer, pH 7.5, and subjected to gel filtration on Bio-Gel P-4 for removal of unreacted [γ-^{32}P]ATP.Fractions containing 5'^{32}P-labeled DNA were pooled, evaporated in vacuum and redissolved in an appropriate buffer.

Alkaline hydrolysis. Alkaline hydrolysis of 5'^{32}P-DNA was carried out by incubation in 0.15 M NaOH at 37 °C for 20 hours as described by Ogawa et al. (16).

Separation of mononucleotides. Deoxyribonucleoside 5'-monophosphates produced by P1 nuclease digestion of terminally ^{32}P-labeled DNA fragments were separated by thin layer chromatography (15).

Polyacrylamide gel electrophoresis. The size distribution of 5'^{32}P-DNA was analyzed by gel electrophoresis in vertical polyacrylamide gel slabs (14x32x0.03 cm) as described by Maxam and Gilbert (17). Electrophoresis was performed for 16 hours at 300 V in Tris-borate, pH 8.3, 7 M urea and 1 mM EDTA. Bromphenol blue and xylene cyanol were used as marker dyes, which migrated of chains length 6 and 21, respectively (15). Autoradiography was done at -50 °C using Medifort Rp film, and autoradiograms were scanned on a Joyce-Loebl microdensitometer.

RESULTS

Products of end-group labeling and alkaline hydrolysis of excreted DNA. The nature of 5'-ends of DNA was examined after end-group labeling of alkaline phosphatase treated DNA with [γ-^{32}P]ATP and polynucleotide kinase as described above. Upon gel filtration on Bio-Gel P-4 (Fig. 1A) ^{32}P comigrated in three

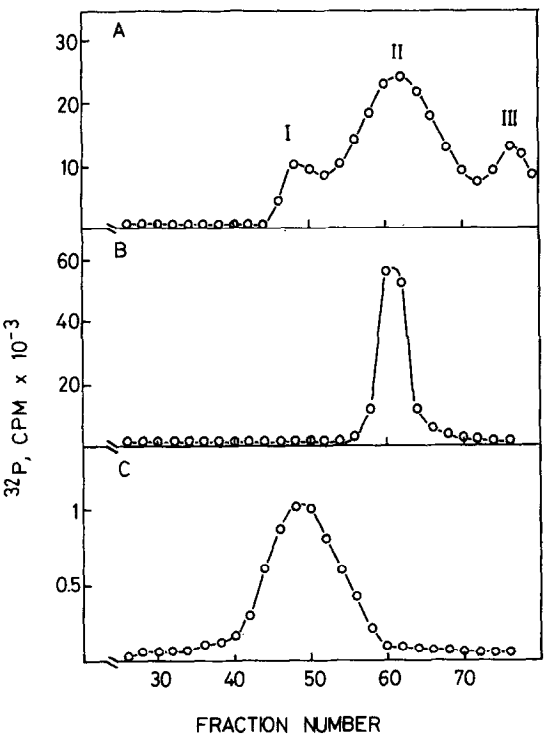

Fig. 1. Gel filtration of 5'^{32}P-labeled DNA fragments before and after alkaline hydrolysis. A. Isolated DNA was terminally labeled as described in "Materials and Methods" and was subjected to gel filtration on Bio-Gel P-4 column as earlier described (15). Fractions (0.15 ml) were collected and analyzed for radioactivity. B. Gel filtration of [γ-^{32}P]ATP as a control. C. Gel filtration of terminally labeled material after alkaline hydrolysis.

peaks, the first being the labeled polynucleotide, the second one the unreacted [γ-^{32}P]ATP and the third one inorganic phosphate. To exclude the possibility that the first peak in Fig. 1A is a polyphosphate or other impurity of ATP, [γ-^{32}P]ATP was run in a control experiment (Fig. 1B). [γ-^{32}P]ATP eluted as a single peak, confirming that peak I in Fig. 1A was a polynucleotide. The labeled polynucleotide fractions (41 - 51) were combined. When the terminally labeled material was subjected to alkaline hydrolysis and rechromatographed on Bio-Gel P-4, ^{32}P remained associated with a high molecular weight species, whereas no ^{32}P migrated with small molecules (Fig. 1C). These observations indicated that virtually none of the DNA chains carried ribonucleotides at their 5'-ends.

Upon hydrolysis of the alkali stable material by P1 nuclease and thin layer chromatography on polyethyleneimine impregnated cellulose nearly equal amounts of ^{32}P were found to be associated with dAMP, dGMP and dCMP (Fig. 2). The separation of dCMP from dTMP was not complete in this system.

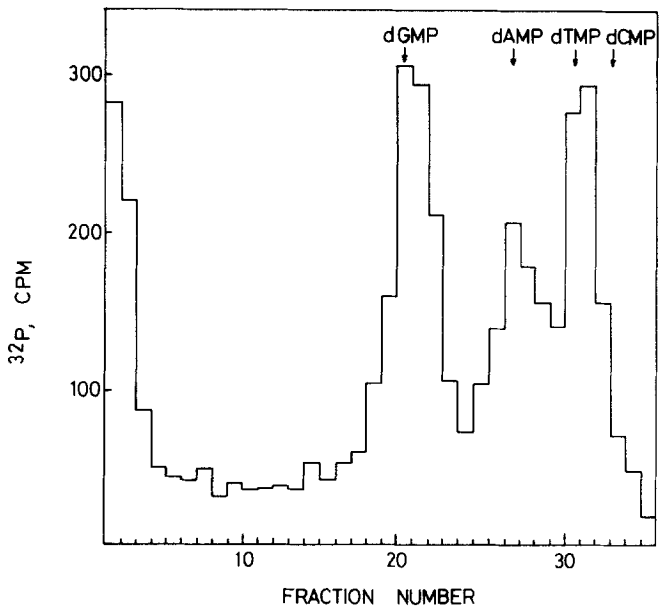

Fig. 2. Thin layer chromatography of the products of P1 nuclease digestion of 5'-^{32}P-labeled DNA. The low molecular weight material produced by P1 nuclease digestion was subjected to thin layer chromatography on polyethyleneinine impregnated cellulose with 0.8 M LiCl as a developing solvent, together with the four deoxyribonucleoside monophosphates as internal standards. The chromatogram was cut into 5 mm stripes and their radioactivity was determined.

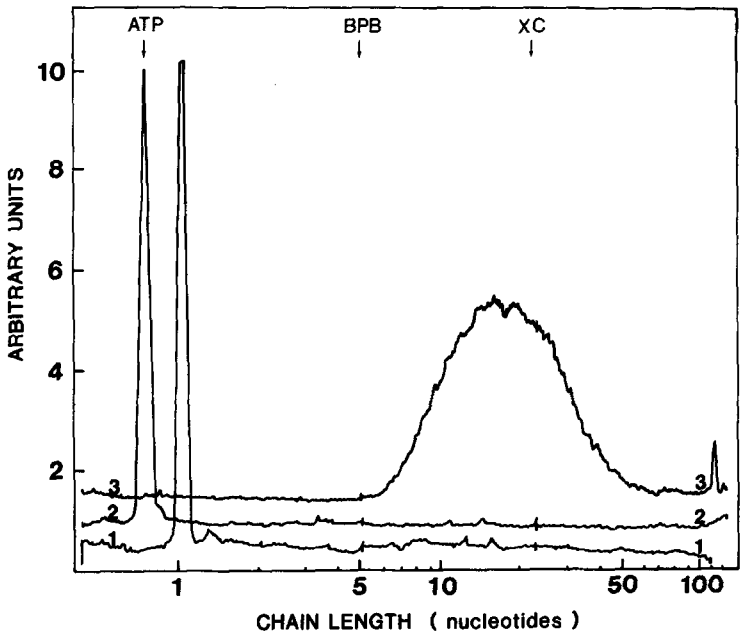

Fig. 3. Size distribution of 5'^{32}P-labeled DNA fragments. ^{32}P-labeled DNA was subjected to electrophoresis in 20 % (w/v) acrylamide and 0.67 % (w/v) methylene bisacrylamide slab gels (31x14x0.03 cm). Bromphenol blue (BPB) and xylene cyanol (XC) were used as marker dyes. Molecular weight standards were the same as earlier described (22). Electrophoresis was at 300 V for 12 hours in 50 mM Tris-borate, pH 8.3, 1 mM EDTA without cooling. Detection of radioactivity was by autoradiography at -80 °C using Medifort RP X-ray film and by densitometry of the autoradiogram. Densitometric profiles of : 1. P1 nuclease digested DNA. Terminally labeled DNA was digested at 37 °C for 2 hours in the presence of 10 units of P1 nuclease in a reaction mixture (5 µl) containing 20 mM Tris-HCl, pH 7.5. 2.[γ-^{32}P]ATP and 3. Labeled DNA.

The frequencies of purine nucleotides at the 5'-ends of DNA fragments were higher than those of pyrimidine nucleotides.

Size distribution of DNA fragments. The 5'^{32}P-DNA was subjected to electrophoresis on 20 % polyacrylamide gels. Most of the labeled material migrated as a heterogenous peak with a size distribution between 6 and 60 nucleotides in length. Fig. 3 shows the position of [γ-^{32}P]ATP and the reduction of size of ^{32}P-DNA after P1 nuclease hydrolysis, as well.

DISCUSSION

In order to facilitate the analysis of DNA excreted by phytohemagglutinin stimulated human blood lymphocytes, 5'-termini of DNA were unmasked with bacterial alkaline phosphatase and labeled with [γ-^{32}P]ATP and polynucleotide kinase. The chain length of 5'^{32}P-DNA varies within

narrow limits (6 - 60 nucleotides). The estimation of length of DNA isolated from culture media showed a broad distribution of size from 4 - 10 S (2) and 7 - 8 S (9) for human blood lymphocytes and 9 - 10 S (3) for rat lymphoblasts.

Accumulation of short DNA fragments is of particular interest, since these species may represent either intermediates formed in the course of DNA replication or may be degradation products generated by nucleases. Short fragments (3 - 5 S i.e. 100 - 300 nucleotides) are formed during early stage of replication. This process proceeds discontinuously at least on one strand of DNA, initiated by short RNA primers covalently linked to the 5' terminus of DNA (18). Upon treatment of excreted DNA with 0.15 M NaOH there was no material convertible to ribonucleotides. This fact indicated that none of the DNA chains carried RNA primer at the 5' terminus.

The absence of any RNA moiety at the 5'-end of the excreted DNA also indicates that the replication origin of these short DNA pieces is unlikely. Taking into account a short lifetime of nascent DNA (15,19,20) it is not possible to rule out the replication origin of the excreted DNA only because the absence of 5'-terminal ribonucleotides. The only available information about RNA presence in nucleoprotein complex detected in culture medium of human lymphocytes referes to the existence of RNA molecules (2.5-4 S), attached to DNA by means of hydrogen bonds (21).

The other series of experiments have shown that P1 nuclease does not release any specific deoxyribonucleoside monophosphate from the 5'-end of DNA fragments, which would indicate a specific sequence. However, partial overlapping homology cannot be excluded by the methods used and described above. All these lines of evidence indicate that the stimulation of cultured lymphocytes is paralled by a nucleolytic process generating short DNA fragments, excreted into the culture medium.

The main difficulty in elucidating the origin of excreted DNA pieces is that there is no specific method available to isolate these fragments

immediately after their generation. Therefore, artefacts arising from the nucleolytic degradation may obscure the picture of the original events. DNA synthesis in permeable lymphocytes followed by affinity chromatography of nascent DNA is a new approach recently developed by us (22). This method may help to isolate some of the excreted DNA fragments in the early stage of their appearance and to obtain further information concerning their biological function.

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