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Isolation and Characterization of DNA from the Plasma of Cancer Patients

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Abstract—*Ten out of 37 patients with advanced malignant diseases were found to have extractable amounts of DNA in their plasma whereas no DNA could be detected in 50 normal controls. After its purification from the original nucleoprotein complex, DNA plasma levels ranging from 0.15 to 12 μg/ml were measured, the lowest concentration detectable with our method being 0.1 μg/ml. Knowing from recovery experiments performed with ^{32}P-DNA that the loss of DNA during the extraction procedure is about 65%, the real concentration of DNA in the plasma corresponds to about 3 times the given figures. The purified DNA was shown to be double-stranded and composed of fractions ranging from 21 kb to less than 0.5 kb, as determined by agarose gel electrophoresis. All these fractions hybridized with a ^{32}P-labelled human DNA probe indicating the human origin of the bulk of the circulating DNA. In conclusion: the finding of extractable amounts of DNA in the plasma of 27% of the investigated cancer patients, and its absence from the controls, suggests some correlation with malignancy.*

INTRODUCTION

INCREASED plasma levels of DNA have been observed in various chronic diseases such as lupus erythematosis [1], viral hepatitis [2] and cancer [3–5]. While DNA has recently been isolated and characterized in lupus and hepatitis [1, 2], this has not been the case for malignancies. In cancer patients indeed, only indirect techniques such as radioimmunological [3] or actinomycin-D-binding tests [5] have shown increased DNA plasma levels. These procedures bear 2 major disadvantages. First, the DNA present in the serum is mainly in the form of a nucleoprotein complex and might thus escape the anti-DNA antibody or the actinomycin-D used in the assay. Secondly, lack of purification of plasma DNA precludes any characterization of this material whose exact nature and origin still remains unknown.

Using a modification of a technique developed in our laboratory in order to extract DNA from the nucleoprotein complex released by cultured lymphocytes [6], we isolated, purified and characterized DNA from the plasma of cancer patients.

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METHODS

Patients and material

Thirty-nine 50 ml blood samples were collected on heparin from a total of 37 patients with various advanced malignancies who were not receiving any kind of oncological treatment during this period. The plasma was immediately separated from the blood cells, and kept at −30°C until use. Specimens were obtained from patients with lung cancer, breast cancer, acute leukemia, lymphoma and multiple myeloma, various abdominal tumors and metastases from unknown primary carcinoma. Plasma samples were collected from 50 healthy controls. By separating immediately the plasma from the blood cells, we know that any DNA found in the plasma cannot be due to spontaneous cytolysis or to manipulation of the cells. Indeed, experiments from our laboratory show that detectable amounts of DNA appear in the plasma of blood-bank whole blood samples only after a storage of at least 15 days at 4°C. We took advantage of this finding to extract DNA from the plasma of whole blood stored for 30 days in order to develop the method of DNA purification and to test its reliability.

Plasma DNA purification

The plasma was diluted at 60% with a solution of 0.9% NaCl and 1% SDS and was shaken for

20 min in the presence of an equal volume of phenol at 60°C saturated with water. After centrifugation for 20 min at 27,000 ***g***, the aqueous phase was collected, mixed with 2 volumes of ether and centrifuged in order to remove residual phenol. The aqueous phase was collected, shaken with an equal volume of a mixture of chloroform and isoamylic alcohol (10 : 1 vol/vol), and centrifuged as before. After dialysis and concentration the aqueous phase was passed over a sepharose-bound concanavalin-A column to remove the high amount of polysaccharides present in the solution [7]. The eluted material was then centrifuged at 90,000 ***g*** on a Cs_2SO_4 gradient for 75 hr at 20°C. Fractions were collected and their DNA content determined by spectrophotometry at 260 nm. The sharp peak of eluted DNA was kept and dialyzed.

Characterization of the DNA

The amount of DNA measured by spectrophotometry was controlled by diphenylamine staining. Efficiency of DNA recovery was checked by reference to specimens of control plasma samples to which known amounts of ^{32}P-DNA labelled by nick-translation [8] had been added. Reproducibility of the DNA extraction method was tested on plasma obtained from 30-day stored normal blood. Double-stranded nature of the DNA was established by its elution pattern through hydroxyapatite columns and by its density on a Cs_2SO_4 gradient. Purity of DNA extracted from the plasma was confirmed by digestion with DNase I (100 μg/ml), pancreatic RNase (100 μg/ml) and pronase (100 μg/ml). Two μg samples of DNA were electrophoresed on 1% agarose gels for 3 hr at 100 V and stained with ethidium bromide. Plasma DNA molecular weights were determined by reference to molecular weight markers. Various DNA specimens were digested with Eco R1, electrophoresed for 16 hr at 100 V on agarose gel (1%) and transferred to nitrocellulose according to Southern. After denaturation, they were hybridized with human lymphocyte cellular DNA which had been labelled by nick-translation [8].

RESULTS

Reliability of the method of DNA extraction

Table 1 shows that the method of extraction used to purify DNA from the plasma is quantitatively reliable as shown by reproducibility of the amount of DNA recovered after separate extractions performed on the same sample. The loss of DNA during the extraction procedure is about 65% as shown by the recovery experiments performed with ^{32}P-DNA added to the plasma before extraction.

Table 1. Reproducibility of the method used to determine DNA plasma levels

Number of samples from the same plasma	Amount of DNA (μg) extracted from 20 ml plasma samples		
3	24	25.6	25.1
2	9.1	9.4	
2	14	12	
2	16	13	
2	13	12.2	
2	10	9.8	
2	58	60	

Plasma samples used for these determinations were obtained from blood-bank blood units stored for 30 days at 4°C.

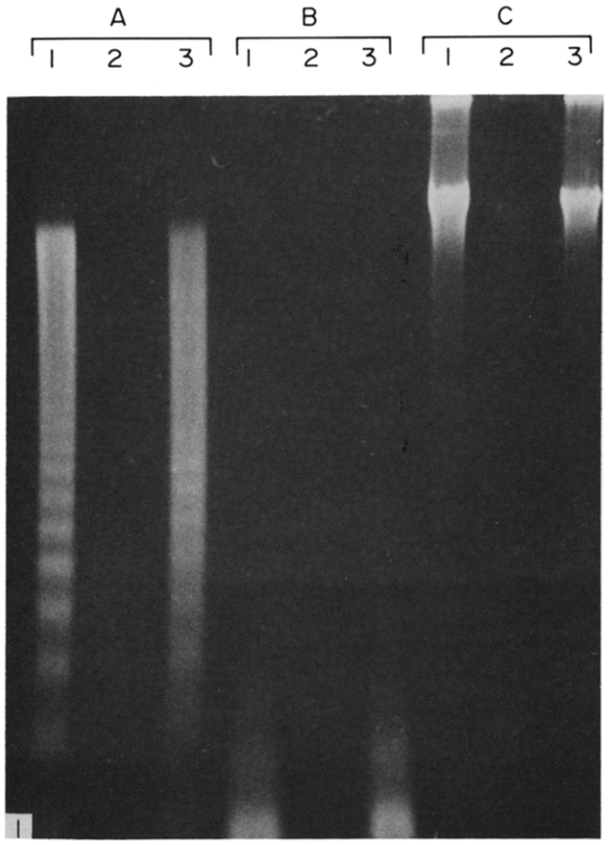
Amount of DNA found in plasma of cancer patients

Extractable amounts of DNA were found in 10/37 patients presenting the following malignancies: acute leukemias (2/3), plasmocytomas and lymphomas (1/9), lung cancers (1/7), various abdominal tumors (2/12), breast cancers (1/3), metastases from unknown primary carcinoma (3/3). As shown in Table 2, concentrations ranging between 0.15 and 0.6 μg/ml were found in 6 cases whereas in the other 4 cases much higher values with a maximum of 12 μg/ml were measured. In 2 patients, a second plasma sample obtained at a later time during tumor progression was tested and showed in both cases an increased DNA concentration. No DNA could be extracted in 20 ml plasma collections from 50 normal control subjects.

Characteristics of the DNA purified from the plasma of 10 cancer patients

The purified DNA is double-stranded as shown by its elution characteristics on hydroxyapatite columns and its density after Cs_2SO_4 ultracentrifugation. It is resistant to RNase and pronase, but sensitive to DNase I. Most of the DNA fractions recovered from the plasma of cancer patients, being of rather low molecular weight, one could have suspected to be in presence of some RNA. Figure 1 shows that even the lowest molecular weight fragments are insensitive to RNase but sensitive to DNase I. Agarose gel electrophoresis shows that the plasma DNA of cancer patients is most of the time of low molecular weight, composed of fractions of different sizes ranging from 21 kb to less than 0.5 kb (Fig. 2).

Southern blot procedure (Fig. 3) reveals that all fractions appearing after agarose gel electrophoresis hybridize with a ^{32}P-labelled human DNA probe indicating the human origin of the bulk of the plasma DNA. Absence of hybridization with ^{32}P-labelled mouse DNA confirms the authenticity of the labelled human DNA.



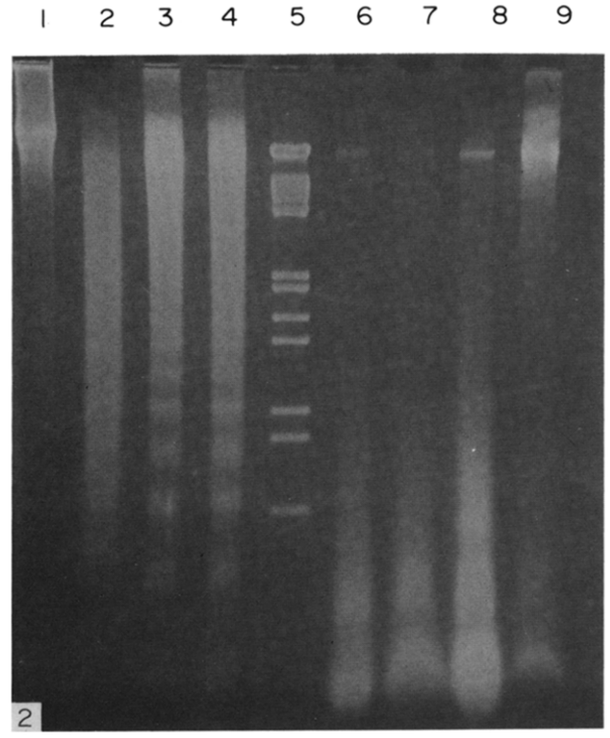


Fig. 1. *Effects of DNase and RNase on DNA samples of various origins.*
A. Plasma DNA from normal blood stored for 30 *days.*
B. Plasma DNA from a cancer patient.
C. Cellular DNA (human lymphocytes).
1. *Untreated DNA sample.*
2. *DNA treated with DNase I* (100 μg/ml) *during* 2 hr *at* 37° C.
3. *DNA treated with RNase* (100 μg/ml) *during* 2 hr *at* 37° C.
Two μg *aliquots of DNA were electrophoresed on agarose gel* (1%) *for* 1 hr *at* 100 V, *in a Tris–borate (EDTA) buffer. DNA was stained with ethidium-bromide.*

Fig. 2. *Molecular weight of several different DNA fragments after electrophoresis on* 1% *agarose gel.*
1. *Cellular DNA extracted from blood mononuclear cells.*
2. *DNA released by cultured lymphocytes.*
3–4. *DNA extracted from the plasma of* 30*-day old stored blood.*
5. *Molecular weight markers* (λ *phage DNA digested with EcoR, and Hind III); bands from bottom to top represent the following base pair numbers*: 564, 831, 985, 130, 1584, 1904, 2027, 3530, 4277, 4973, 5148, 21226.
6–9. *DNA extracted from the plasma of cancer patients.*
Experimental conditions as in Fig. 1.

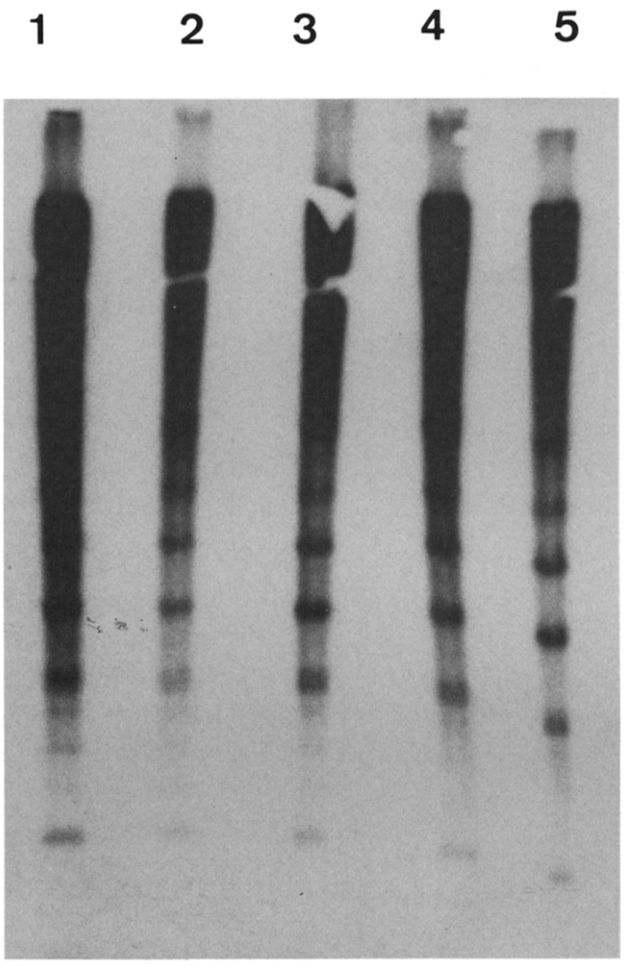


Fig. 3. Hybridization of various DNA samples with ^{32}P*-labelled cellular DNA, according to Southern.*
Lane 1. *DNA extracted from the plasma of* 30*-day old stored blood.*
Lane 2–5. *Plasma DNA samples from cancer patients.*
The various DNAs were digested with EcoRI and electrophoresed on agarose gel (1%) *for* 16 hr *at* 100 V. *After transfer on nitrocellulose they were hybridized with human cellular DNA labelled with* ^{32}P.

Table 2. Plasma levels of DNA in various malignant conditions

Diagnosis	DNA concentration (μg/100 ml)	
Acute lymphoblastic leukemia in relapse	425	
Acute lymphoblastic leukemia in relapse	64	
Plasmocytic lymphoma	23.5	
Epidermoid bronchial carcinoma	130	(Sep.17.84)
with liver metastases	267.5	(Dec.12.84)
Metastatic adeno-cortical carcinoma	38.5	
Metastatic rectum	170	(Sep. 3.84)
carcinoma	1230	(Sep.24.84)
Metastatic breast carcinoma	32.5	
Metastatic condition from unknown origin	15.5	
Metastatic condition from unknown origin	26	
Metastatic condition from unknown origin	62.5	

DISCUSSION

In 10 out of 37 cases of advanced malignant diseases, double-stranded DNA was isolated and purified from the plasma. The finding of extractable amounts of DNA in the plasma of 27% of the investigated cancer patients, and its absence from the controls, suggests some correlation with malignancy. Concentrations ranging from 0.15 to 12.3 μg/ml were determined. With our method it is not possible to extract less than 2 μg per sample of 20 ml of plasma. No DNA could be found in 20 ml plasma collections from 50 control subjects. Measuring circulating DNA levels with a radioimmunoassay capable of detecting 0.025 μg/ml, Shapiro *et al.* [4] observed values ranging from 0 to 10 μg/ml in 199 cancer patients and from 0 to 0.1 μg/ml in 88 controls. DNA plasma concentrations determined with the actinomycin-D binding test in leukemic patients [5] have also been shown to be of the same order of magnitude.

The method described in this paper is, to our knowledge, the first one to allow DNA extraction and purification from the plasma of cancer patients. However, the relatively low yields of the extraction procedure and Southern blot analysis do not rule out the existence of some viral DNA.

What is the cellular origin of circulating DNA in cancer patients? Does it originate in the malignant cells or in the host lymphocytes reacting against the tumor? The biochemical characteristics of our purified DNA being of no help to solve this problem, we have to rely on direct data. We observed that detectable amounts of circulating DNA were found only in patients with advanced malignancies bearing a large tumor cell burden and that in the 2 cases with progressive cancer where a second determination could be performed later in the course of the disease, an increased plasma DNA concentration was measured, suggesting a relation between this parameter and tumor evolution.

In a statistically significant study, Leon *et al.* [3] found higher DNA values in patients with metastatic than localized disease, and decreasing values with tumor regression. Furthermore, several *in vitro* studies showed that higher amounts of surface DNA could be detected on malignant cells than on normal cells [9, 10]. Testing several tumor lines, Rosenberg [11] observed that all of them had nucleic acids on their surface which was recognized as being DNA, RNA or both, and which could be released in their culture medium.

Although these clinical and laboratory observations point to a tumor rather than to a lymphocyte origin of the circulating DNA, the mechanism of its release from the tumor cells remains a controversial issue: is it a product of dying necrotic tumor cells or of actively secreting live tumor cells? An argument in favour of an active DNA secretion has been reported by Leon *et al.* [3] who observed that patients responding to radiotherapy usually showed decreasing plasma DNA levels whereas increasing levels should be expected if tumor necrosis were responsible for the phenomenon.

If most of the *in vitro* studies on active DNA release have been carried out on resting [6, 12] or stimulated lymphocytes [13], such a phenomenon has also been observed in other normal cell varieties [14–16] and various malignant cells [11]. Previous work from our laboratory on resting human lymphocytes [6] and on frog auricles has shown that DNA release takes place within a 2–4 hr period after which an equilibrium between extra- and intracellular DNA is reached, with no further increase in its extracellular concentration. However, after medium renewal the same excretory phenomenon takes place again in suspension of live cells. Using cultured

malignant cell lines, Juckett and Rosenberg [17] removed the surface nucleic acids by nuclease treatment and demonstrated their reappearance after about 2 hr. Moreover, several studies [6, 14–16] on spontaneous release of DNA by cultured cells show that the DNA is shed in the form of a nucleoprotein complex, containing also lipids and polysaccharides, which is resistant to DNase whereas DNA material released from killed cells is quickly digested by the extracellular nucleases [6, 16]. A final argument in favor of an active secretion has been brought up by studies on circulating RNA in cancer patients by Wieczorek *et al.* [18]. The same RNA–proteolipid complex which was found in the plasma of the cancer patients was also identified in the supernatant of cultured malignant cells; addition of metabolic inhibitors like cytochalasin-B or monensin to the cultures considerably reduced the secretion of the RNA-complex, demonstrating that its production is dependent on a well-preserved cellular metabolism. Killing the tumor cells with CN^- abolished completely the secretion.

In conclusion, increased levels of a DNA–lipoprotein complex seems to be associated with extensive malignant conditions. The bulk of plasma DNA which is of human origin seems to be excreted by active malignant cells and may represent a valuable parameter of tumor evolution.

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