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Leukemia-Specific DNA Sequences in Leukocytes of the Leukemic Member of Identical Twins

(virogene hypothesis/RNA-directed DNA polymerase)

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ABSTRACT **The discovery in human leukemic cells of particulate elements encapsulating 70S RNA and RNA-directed DNA polymerase made possible the synthesis of a [^{3}H]DNA probe that could detect leukemia-specific sequences in the DNA of normal and leukemic individuals. In an earlier study of a series of unrelated leukemic patients, we established that the nuclear DNA of their leukemic cells contain particle-related sequences that cannot be detected in leukocytes of normal individuals. This result is inconsistent with the virogene concept that demands the inclusion of one complete copy of oncogenic information in the genome of every normal cell.**

The present study carries this analysis one step further by showing, with two sets of identical twins, that the leukemic member contains particle-related sequences in the DNA of his leukocytes that cannot be detected in the leukocytes of his healthy identical sibling. This finding implies that the additional leukemia-specific information found in the DNA of the leukemic individuals must have been inserted subsequent to fertilization. This outcome argues against the virogene hypothesis or any other etiologic concept that invokes vertical transmission through the germ line of the particle-related information found uniquely in the DNA of leukemic cells.

We have shown by molecular hybridization that human adenocarcinomas of the breast (1), leukemias (2), sarcomas (3), and lymphomas (4) contain RNA molecules possessing a small but significant homology to RNAs of tumor viruses that cause the corresponding malignancies in mice. More telling for a viral involvement was the demonstration with the simultaneous detection test (5) that the RNA detected in these human cancers was 70 S in size and encapsulated with RNA-directed DNA polymerase in a particle possessing a density between 1.16 and 1.19 g/ml (6–9). Furthermore, DNA synthesized endogenously by these RNA–enzyme complexes exhibited evidence of complementarity to RNAs of the analogous murine viruses (6–9).

Taken together, these experiments documented the existence in human neoplasias of particulate elements possessing four features diagnostic of animal RNA tumor viruses. The data did not of course establish that the particles identified were causative, but the evidence for their involvement was sufficiently convincing to encourage further exploration of their significance.

In our studies of human leukemias we developed the methodology required to separate the particles containing the RNA-dependent DNA polymerase and its 70S RNA template. These particulate elements were then used to generate ^{3}H-labeled DNA probes suitable for detecting corresponding sequences in genomes of any cells of interest. We found (9) that RNA of human leukemic particles shared sequences with the DNA of *normal* cells, a feature observed (10) *with animal RNA tumor viruses and the normal DNA of their indigenous hosts*. Sequences common to both normal DNA and the [^{3}H]-DNA synthesized by leukemic particles were removed by exhaustive hybridization to a vast excess of normal DNA followed by hydroxyapatite chromatography to separate duplexes from unpaired [^{3}H]DNA. The residue was then used to obtain an answer to the the following question: "*Does the DNA of leukemic cells contain sequences that are not found in the DNA of normal cells?*" The data obtained with eight leukemic patients were satisfyingly clear cut. In all cases it was possible to demonstrate (9) that the DNA of leukemic cells contained particle-related sequences that could not be found in DNA of leukocytes of normal individuals. The sensitivity of the assay was such that 1/400th of an equivalent per normal genome would have been detected. We have shown (Kufe, D., Peters, W. P. & Spiegelman, S., in manuscript) that a similar situation exists in human lymphomas, including Burkitt's tumors and Hodgkin's disease, and in feline sarcomas (10).

The fact that specific particle-related sequences are unique to the DNA of leukemic cells possesses obvious implications for the etiology and possible control of this disease. It is evident that a specific segment of the viral-related sequences detected earlier by hybridization has in some manner been inserted into the genome of the leukemic cells. Our inability to detect these specific sequences in the DNA of normal leukocytes argues against the virogene-oncogene hypothesis (11, 12), which stipulates that a complete copy of the information required for virus production and malignant transformation is vertically transmitted and is therefore present in the DNA of all cells in all animals prone to cancer.

The question of whether or not the virogene concept is applicable to the human disease is central to the development of a rational approach toward its control and cure. The availability of two sets of identical twins, each containing a leukemic individual, provided an extraordinary opportunity to pursue this issue further. The data derivable from this situation could serve to test the validity of our earlier findings, and, if confirmatory, sharpen the biological implications of our conclusions concerning the etiology of this disease.

Since identical twins derive their genomes from the same fertilized egg, any vertically transmitted information must be

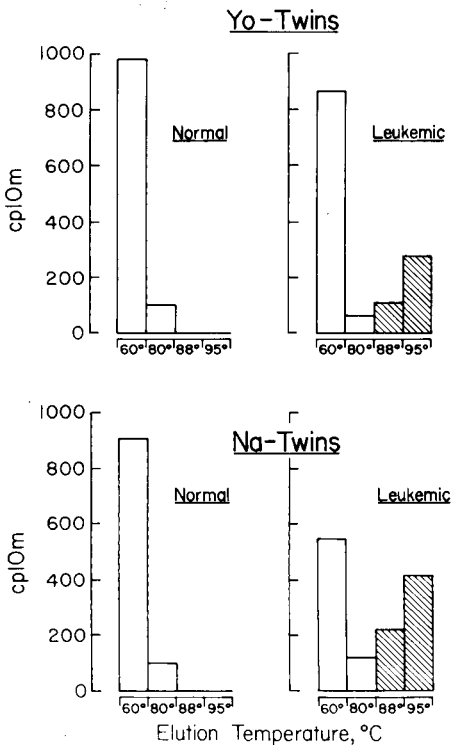

FIG. 1. Hydroxyapatite elution profile of a hybridization reaction of recycled [^{3}H]DNA probe of the leukemic twin to nuclear DNA from normal leukocytes, normal twin leukocytes, and leukocytes from the same leukemic twin. The annealing reaction mixtures contained 20 A_{260} units of cellular DNA and 4 fmol of [^{3}H]DNA, in a final volume of 0.1 ml. The reaction was brought to 98° for 60 sec, and 40 μmol of NaCl was added. The reaction mixture was then incubated at 60° for 50 hr. The reaction was stopped by addition of 1 ml of 50 mM $NaHPO_4$ (pH 6.8). The sample was then passed over a column of hydroxyapatite of 20-ml bed volume at 60°. The column was washed with 40 ml of 0.15 M $NaHPO_4$ (pH 6.8) at 60°, 80°, 88°, and 95°. Fractions of 4 ml were collected, the A_{260} of each fraction was read, and the DNA was precipitated with 2 μg/ml of carrier yeast RNA and 10% trichloroacetic acid. The precipitate was collected on Millipore filters, which were dried and counted. In all cases, more than 80% of the nuclear DNA reannealed. A background count of 8 cpm was subtracted in all instances.

present in both. The virogene hypothesis would therefore predict that if the leukemic member of the pair contains specific particle-related DNA sequences, these should also be found in the leukocyte DNA of his healthy sibling. The data obtained and described here do not agree with this prediction. We found the same difference between the healthy and leukemic identical siblings as we had earlier found between unrelated leukemic patients and random healthy blood donors. In the two sets of identical twins examined, the leukemic member possessed particle-related sequences in his myeloblast DNA that could not be detected in the leukocyte DNA of his healthy sibling.

MATERIALS AND METHODS

Clinical. The Yo male twins were 24 years old when one of them (DYo) was diagnosed as having acute myelocytic leukemia. The patient was leukophoresed with an IBM cell separator when his peripheral leukocyte count was 27,500 (90% myeloblasts). Evidence for the monozygosity of the Yo pair came from concordance in fingerprint analysis, HL-A (1 and 2) typing, and of the erythrocyte major and minor antigenic groups.

The Na twins were 26 years old when one of them (PNa) was diagnosed as having acute myelocytic leukemia. The patient was leukophoresed with an IBM cell separator when his peripheral leukocyte count was 28,000 and yielded a leukocyte population containing 40% myeloblasts. Evidence for the monozygosity of the Na pair came from concordance in fingerprint analysis, HL-A (groups 1 and 2) typing, and of the erythrocyte major and minor antigenic groups.

In both cases, leukocytes from the healthy sibling were obtained from the buffy coats separated from a unit of whole blood.

Preparation of [^{3}H]DNA. Leukemic leukocytes were gently opened with a Dounce homogenizer. The extract was centrifuged at 3000 rpm for 15 min at 2° to remove nuclei and then at 10,000 rpm for 15 min at 2° to remove mitochondria. The resultant supernatant was layered on 25% sucrose–10 mM Tris·HCl (pH 8.3)–0.15 M NaCl–10 mM EDTA and centrifuged in a Beckman SW-41 rotor at 40,000 rpm for 1 hr at 2°. The resulting pellet was suspended in 1 ml of 10 mM Tris·HCl (pH 8.3) and used in a typical endogenous RNA-directed DNA polymerase reaction to generate [^{3}H]DNA (6). The product was brought to 0.3 M NaOH and incubated at 37° for 2 hr to destroy RNA. To provide acceptable backgrounds for such probes, it is imperative that all self-complementary material be removed before use. To this end, the alkali-treated material was neutralized with 40 mM HCl and 1 M Tris·HCl (pH 7.4) and then self-annealed, corresponding to C_0t values of 0.01–0.25 as defined by Britten and Kohne (13), at 60° for 48 hr. It was then passed over an HTP Biorad hydroxyapatite column with a 4-ml bed volume at 60°. Single-stranded [^{3}H]DNA was eluted with 4 ml of 0.15 M $NaHPO_4$ (pH 6.8), which recovers 90% of the input. This was then layered upon a Sephadex G-50 column with a bed volume of 100 ml (coarse). The DNA region of the column was pooled, and the [^{3}H]DNA was precipitated with 2 μg/ml of yeast carrier RNA and two volumes of 95% ethanol. The pellet was dissolved in 3 mM EDTA and stored at 2°. [^{3}H]DNA banded in Cs_2SO_4 gradients at densities between 1.42 and 1.45 g/ml.

Preparation of Nuclear DNA. The nuclear pellet (3000 rpm for 15 min) was suspended in 10 mM Tris·HCl (pK 8.3)–0.15 M NaCl–10 mM EDTA and lysed by addition of Na dodecyl sulfate to a concentration of 1% in the presence of 1 M $NaClO_4$. After repeated extraction with chloroform–isoamyl alcohol (4%), the DNA was precipitated with alcohol and spooled out of the solution. The DNA was dissolved in 10 mM Tris·HCl (pH 8.2)–3 mM EDTA and sheared to 6–8 S, as measured in a 10–30% glycerol gradient. Shearing was accomplished with a Bronwill Biosonic IV sonifier with the microtip at maximum power for two 30-sec intervals. The sample was then incubated with 0.3 M NaOH for 16 hr at 37°. After neutralization, DNA was precipitated with alcohol and dissolved in 10 mM Tris·HCl (pH 8.2)– 3 mM EDTA. The DNA preparations had an A_{260}/A_{280} ratio greater than 1.8. More than 90% renatured as measured by hydroxyapatite chromatography.

Annealing Conditions. Annealing reactions contained >60 A_{260} units of nuclear DNA, 0.1–1.0 pmol of [^{3}H]DNA, in a final volume of 0.1 ml. The reaction was brought to 100° for

60 sec, and 40 μmol of NaCl was added. The reaction mixture was then incubated at 60°. The reaction was stopped by addition of 2 ml of 0.15 M $NaHPO_4$ (pH 6.8). The sample was then passed over a column of HTP hydroxyapatite with a 30-ml bed volume (15, 16) at 60°. The column was washed with 20 ml of 0.15 M $NaHPO_4$ (pH 6.8) at 60°, 80°, 88°, and 95°. Fractions of 4 ml were collected. A_{260} of each fraction was read, and the DNA was precipitated with 2 μg/ml of yeast carrier RNA and 10% trichloroacetic acid. The precipitate was collected on Millipore filters, which were dried and counted as described (6).

The method identifies unpaired strands that elute at 60° and poorly paired duplexes that disassociate at 80°. *Only the duplexes disassociating and eluting at 88°–95° are counted here as hybridized.*

Recycling of Probe on Normal DNA to Separate Leukemic from Normal Sequences. Reactions were done with normal DNA and the eluates were collected in 1-ml fractions at 60°. The peak fractions were combined and the resulting pools were passed over a Sephadex G-50 column with a 20-ml bed volume. Regions containing DNA were collected, and the DNA was precipitated with 2 μg/ml of yeast carrier RNA and 2 volumes of 95% ethanol. The material eluted at 60° was used as the recycled product unable to hybridize to normal DNA.

RESULTS

The strategy of the experiments to be described may be outlined as follows: (*1*) Isolate from the leukemic cells of each patient the particles encapsulating the RNA-directed DNA polymerase, and its 70S RNA template (8). (*2*) Use this fraction to generate [^{3}H]DNA endogenously synthesized in the presence of high concentrations (400 μg/ml) of actinomycin D to inhibit host and viral DNA-directed DNA synthesis (14–16). (*3*) Purify the [^{3}H]DNA by hydroxyapatite and Sephadex chromatography with care being exercised to remove all self-complementary material. (*4*) Sequences shared with normal DNA are removed by exhaustive hybridization in the presence of vast excess of normal DNA followed by hydroxyapatite chromatography to separate paired from unpaired [^{3}H]DNA. In this step the normal DNA used came from leukocytes of healthy unrelated blood donors and not from the normal twin. To have used the latter would have obviously prejudged the issue. (*5*) The residue of [^{3}H]DNA probe that does not pair with normal DNA is then used to test for the presence of sequences in the leukocyte DNA of the patient and in that of his healthy identical sibling. In such experiments it is imperative to adjust DNA concentrations and durations of hybridizations so that sequences present only once per genome equivalent will be readily detected. In the experiments to be described, this was done by running the annealing reactions to C_0t (concentration of DNA nucleotides in mol/liter times sec) values of 10,000 and above.

In carrying out the fourth step for removal of normal sequences from the [^{3}H]DNA, annealing reactions were set up containing 60 A_{260} units of normal cellular DNA, 0.1 pmol of [^{3}H]DNA (3000 cpm), and 15 μmol of $NaHPO_4$ (pH 7.2) in a final volume of 0.1 ml. The reaction was brought to 98° for 60 sec and 40 μmol of NaCl was added.

Annealing was done at 60° for 50 hr. The reaction mixture was then added to 1 ml of 0.15 M $NaHPO_4$, passed over a 30-ml hydroxyapatite column at 60°, and washed with 0.15 M $NaHPO_4$; eluates were collected in 1-ml fractions and yielded 25% of the input [^{3}H]DNA. The peak fractions were combined, and the resulting pools were passed over a Sephadex G-50 column of 20-ml bed volume. The DNA regions were collected, and the DNA was precipitated with 2 μg/ml of carrier yeast RNA and 2 volumes of 95% ethanol.

This material, which cannot hybridize to normal DNA, was then used to examine the nuclear DNA from leukocytes of the normal twin and from that of his leukemic sibling. The results obtained with the two sets of twins are shown in Fig. 1 and reveal a response pattern indistinguishable from our previous findings with eight unrelated leukemic patients. In that study we showed that after recycling on normal DNA the particle-derived [^{3}H]DNA showed no ability to form well-paired duplexes (requiring 88° and above to melt) with normal DNA but did form stable duplexes with the DNA of the original patient. In the present instances, we see that the same situation holds between the members of each twin pair. After removal of normal DNA sequences with unrelated normal DNA, the remaining [^{3}H]DNA can still form stable duplexes with the DNA from the leukemic individual but not with the DNA from his healthy identical sibling.

DISCUSSION

Using lymphocyte toxicity tests, Levine and his collaborators (17) examined 10 sets of identical twins and found antigenic differences in seven instances between the leukemic and healthy members of each pair. Although consistent with the results reported here, such phenotypic dissimilarities do not necessarily imply a difference in genome content since they could reflect rather a disparity in gene expression. It is worth explicitly emphasizing that experiments that focus on phenotype or search for specific RNAs in tumor cells are limiting their attention to active genes in the form of transcripts or their translated products. In contrast, the experiments described here and in our previous study of leukemias (9) center on DNA sequences and, as such, include all genes, active or silent.

The fact that we could establish a sequence difference between identical twins implies that the additional information found in the DNA of the leukemic members was inserted after the zygote was formed. This outcome agrees with the results and conclusions of our previous study (9) of unrelated leukemic patients and is consistent with the original provirus concept (18). The data argue against the validity of the virogene hypothesis (11), which demands that if leukemia-specific sequences are found in the DNA of the individual with the disease they must surely also exist in the genome of his identical healthy sibling. We have shown (Baxt, W., & Spiegelman, S., manuscript in preparation) that human leukemia-specific sequences are partially homologous to RNA of Rauscher leukemia virus. This latter fact, along with the outcome from the monozygotic twins, permits a biologically more informative conclusion with respect to the virogene hypothesis and the etiology of human leukemia. One could have argued from the study of the unrelated leukemia patients that they had the disease because, in fact, each inherited the necessary malignant information in his genome. The results with the monozygotic twins contradict this possibility. The data imply that even those who come down with this malignancy do not inherit the required information in their germ lines.

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