CHROMOSOME ABNORMALITIES AND ONCOGENESIS IN CAT
LEUKEMIAS

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ABSTRACT

Chromosome abnormalities are found in feline leukemia virus (FeLV) infected tumor cells as well as in tumor cells free of the virus. On the other hand, normal feline cells infected with the virus do not show chromosomal abnormalities. Here I ask whether chromosome abnormalities and viruses can each cause or play a role in the causation of tumors.

Cytogenetic analyses on three cell lines derived from tumors in the domestic cat (Felis catus), two of thymic origin and one of multicentric lymphoma origin, have been carried out in an attempt to determine whether the FeLV virus was associated with chromosomal abnormalities in these tumors. One thymic tumor and the multicentric lymphoma were FeLV-infected. The other thymic tumor cell line was FeLV-free.

The normal diploid number in domestic cat is 38. All three cell lines had numerical chromosome abnormalities with modal numbers of 37, 38 (pseudodiploid), and 39 respectively. All three cell lines also had structural (marker) chromosome abnormalities that were consistent within cell lines. Three of the markers found in the virus-free cell line (U markers) were shared with either of the virus-positive cell lines. The two FeLV-positive cell lines did not have U markers in common.

Since both the virus-infected and the virus-free cell lines have chromosomal abnormalities, this suggests that these abnormalities may be an

important factor in oncogenesis. Although the FeLV virus may cause chromosome abnormalities, it could not be considered to be the only causative agent of the abnormalities observed.

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INTRODUCTION

The search for the cause of cancer has been underway for many years. In spite of intense efforts, however, our understanding of the etiology of cancer has progressed very slowly and relatively little is known about the molecular basis of this disease.

The transforming (onc) genes of retroviruses are, as yet, the only known genes capable of causing cancer with single hit kinetics; i.e., every cell infected by the transforming (onc) retrovirus will be transformed (Duesberg, 1987 a; Duesberg, 1985; Duesberg, 1983). These genes may arise by rare truncations and illegitimate recombinations between retroviral and cellular genes (Duesberg, 1987 a).

Chromosomal rearrangements, which are consistently found in tumor cells, and which may be indicators of cellular cancer genes, are also produced by illegitimate recombination (Zhou and Duesberg, 1988; Duesberg, 1985). Chromosomal abnormalities are found in most malignancies, with the exception of some tumors caused by retroviruses with onc genes (Sadamori, 1991; Duesberg, 1987 b; Weiss et al., 1985; Weiss et al., 1982). The so called cellular cancer genes or proto-oncogenes are expressed in normal cells where they are postulated to play a role in cell growth and DNA synthesis. However, there is no functional evidence that cellular onc genes or proto-onc

genes have transforming function in normal diploid cells (Haluska et al., 1987; Croce, 1987; Studzinski et al., 1986; Duesberg, 1985; Rabbitts, 1985). Furthermore, it has been found that viral products are not needed for maintenance of tumors (Essex, 1988; Neel et al., 1981).

The proto-onc gene may be "activated" to assume oncogenic function by chromosomal translocations such as proto-myc in Burkitt's lymphoma (Haluska et al., 1987; Forrest et al., 1987; Onion et al., 1987). However, these "activated" proto-onc genes are not capable of transforming normal diploid cells (Haluska et al., 1987; Duesberg, 1987 c; Cichutek and Duesberg, 1987; Duesberg, 1985). Furthermore, in some Burkitt's lymphomas the proto-myc gene remains in its original chromosomal position while a region 3' of the proto-myc gene is translocated (Gough, 1985; Davis et al., 1984; Hollis et al., 1984; Gelmann et al., 1983). However, site specific recombination between proto-myc and avian retroviruses and proto-myc and FeLV virus does generate recombinant genes capable of transforming diploid cells (Zhou and Duesberg, 1988; Onions et al., 1987). Such genes may be cellular cancer genes. Analogous recombinant retrovirus proto-myc genes have been shown to exist in feline leukemias (Norley and Kurth, 1989; Essex, 1988; Onions et al., 1987; Neil et al., 1987; Mullins et al., 1984; Levy et al., 1984).

Many chromosomal abnormalities represent illegitimate recombinations involving translocations of part of one chromosome to another chromosome. These abnormalities may be either random or nonrandom (Maruyama et al., 1990; Haluska et al., 1987; Rowley, 1984; Wolman, 1983). Examples of non-random, characteristic abnormalities

include translocation t(8;14) or variants thereof, all of which involve a break at band 8q24, in Burkitt's lymphoma (BL); translocation t(15;17) in acute promyelocytic leukemia (APL); translocation t(9;22) or variants, almost always involving chromosome 22 (the Philadelphia chromosome), in chronic myelogenous leukemia (CML) (Rowley, 1990).

In Burkitt's lymphoma, the truncation and translocation usually involve an area adjacent to the cellular proto-myc gene on one chromosome and the immunoglobulin heavy chain gene (IgH) of another chromosome. The kappa light chain gene may be involved as well. These translocations may occur during the time of Ig gene rearrangement (Haluska et al., 1987; Croce, 1987; Duesberg, 1985; Rabbitts, 1985; Gough, 1985). Although the translocation breakpoints can vary, they are usually restricted to the specific region of band 8q24 (Rowley, 1990; van Lohuizen et al., 1989; Morishita et al., 1988; Gough, 1985; Rowley, 1984). The chromosomes involved in the translocation are usually tumor-specific as well, though here too some variation can occur (Haluska et al., 1987; Wolman, 1986; Wolman, 1983; Larson et al., 1981). It is important to note that what may be consistent by cytogenetic analysis may be found to be not totally consistent by molecular analysis; i.e., the former only shows gross abnormalities at the band level, whereas molecular analysis can refine the dissection considerably (Wurster-Hill, personal communication).

Chromosomal abnormalities are found both in tumors infected by retroviruses without onc genes as well as in uninfected tumors (Grindem and Buoen, 1989; Duesberg, 1987 b; Duesberg, 1987 c; Rowley, 1984; Trent, 1984;

Wolman, 1983; Levan, 1956). Thus, otherwise indistinguishable tumors may be retrovirus-positive or retrovirus-negative. In fact, chromosomal abnormalities are, at present, the only detectable genetic determinants of tumors (Duesberg, 1987 c; Rowley, 1984; Trent, 1984; Wolman, 1983; Levan, 1956). Therefore, it has been proposed that both virus-positive and virus-negative tumors may be initiated by chromosomal abnormalities, and that transformation can be a virus independent event; i.e., it is not the presence of the retrovirus without onc gene that causes cancer but perhaps the processes set forth by the chromosomal abnormalities (Duesberg, 1987 c; Gough, 1985). The virus may induce hyperplasia which enhances the risk but is not sufficient for tumorigenesis to occur (Duesberg, 1987 c; Gough, 1985).

Feline leukemia virus (FeLV) is a retrovirus transmitted mainly horizontally in a contagious manner through saliva and other excretions between outbred cats (Lutz, 1990; Essex, 1982; Hardy et al., 1977; Hardy et al. 1973; Jarrett et al., 1973). FeLV can have a high infectious incidence of 30% or more among cats and it is involved in the causation of many of their diseases (Lutz, 1990; Donahue et al., 1988; Reinacher and Theilen, 1987; Jarrett, 1984). It is claimed to be the most prevalent infectious cause of death among cats (Lutz, 1990; Lutz and Jarrett, 1987; Hardy et al., 1977). However, there is some controversy among investigators of how often and how much the FeLV virus is really involved in the causation of disease and death in cats (Reinacher, 1989).

The FeLV virus was discovered by Jarrett in a household cluster of cats in 1964 (Jarrett et al., 1964). This retrovirus belongs to the distinct group of

type C viruses. FeLV virus has a long latent period before transformation occurs and also lacks the genetic information for transformation (Levesque et al., 1990; Miura et al., 1987; Essex, 1982). Only four species of Felidae; F. catus, F. sylvestris, F. margarita, and F. chaus, have FeLV virus related sequences in their genes (Benveniste and Todaro, 1975; Benveniste and Todaro, 1974). The sequences are postulated to have originated from a rodent, rather than a primate as in the case of RD-114 which is another feline endogenous virus, and were horizontally transmitted to the ancestors of these felid species during the Pliocene or early Pleistocene somewhere in the region of the Mediterranean basin (Benveniste and Todaro, 1975).

Naturally occurring FeLV virus infection in cats ranges from 0.3% to 9.4% with 2% being the average prevalence of viremia in a cat population in the United States (Romatowski, 1989; McMichael et al., 1986; Gerstman, 1985). On the other hand in multicat households, clustering of the FeLV-positive infections is known to occur and prevalence rates can be as high as 24.2%, 30%, and even 60% (Lutz, 1990; Romatowski, 1989; Lutz et al., 1983; Dorn et al., 1968). Seventy percent of the leukemias and lymphomas occurring in outbred cats are associated with FeLV virus viremia (Rojko et al., 1989; Essex, 1982; Hardy, 1980; Koshy et al., 1980; Hardy et al., 1980; Francis et al., 1979 a). The other 30% do not have detectable virus or serologically detectable antigens (Koshy et al., 1980; Essex et al., 1979; Hardy et al., 1969). On the other hand, there are healthy FeLV-infected cats that excrete high titers of FeLV virus. Furthermore, these cats usually have higher virus titers than leukemic animals (Essex, 1982; Francis et al., 1979 b).

It is postulated here that the FeLV virus may not have a direct role in tumorigenesis. Therefore, it is asked whether chromosome abnormalities and virus integration sites can each cause or play a role in the causation of tumors. A recent study that appeared during the course of this investigation showed chromosomal abnormalities in eight out of nine primary leukemias investigated, lending support to the view that chromosomal changes may cause or play a role in the causation of leukemias (Grimden and Buoen, 1989).

MATERIALS AND METHODS

Reagents

McCoy's 5A and RPMI 1640 media, lyophilized penicillin, streptomycin, neomycin, newborn calf serum, phytohemagglutinin (PHA), demecolcine (colcemid), anhydrous potassium chloride, and Dulbecco's phosphate buffered saline (modified) without calcium and magnesium were obtained from Sigma Co. (St. Louis, MO). Leibovitz's L-15 medium and L-glutamine (L-Gln) (200 mM, 100 x) were obtained from Gibco Laboratory (Grand Island, NY). Concanavalin A (conA) from Canavalia ensiformis was obtained from Boehringer Mannheim (Mannheim, W. Germany). Trypsin (# 0153-59) was obtained from Difco Laboratory (Detroit, MI). Gurr's Giemsa and Gurr's buffer were obtained from Bio/Medical Specialties (Santa Monica, CA). Glacial acetic acid, methanol, sodium phosphate dibasic, potassium phosphate monobasic were obtained from Fisher Scientific (Fair Lawn, NJ). Technical pan film ester-AH base TP 135-36, developer D-19, dektol, photo-flo 200, hypoclear, fixer, paper III kodabromide F 3 D were obtained from Eastman Kodak Co. (Rochester, NY). Cytoseal 60 and Resolve high viscosity immersion oil (1250 cs) were obtained from Stephens Scientific (Denville, NJ). Assure/FeLV (feline leukemia virus antigen test kit) was obtained from Synbiotics Corporation (San Diego, CA).

Lymphoma Cell Lines

Line 3272 was established from a FeLV-positive thymic lymphoma in a naturally infected cat (Felis catus). Line 3201 B was established after in vivo passage through a specific pathogen-free kitten of a naturally occurring FeLV-negative thymic lymphoma. The 3201 B cell line was indistinguishable by allelic isoenzyme signature from the donor tumor (Rojko et al., 1989). Line FL 74 was established from a renal lymphoma (multicentric lymphoma) after in vivo passage of the original Kawakami-Theilen FeLV virus isolate (Rojko et al., 1989). All three cell lines were provided by Dr. William Hardy, Jr. of the Sloan-Kettering Institute for Cancer Research (New York, NY).

Cell Culture

The three cell lines were cultured in T-cell medium made of a 1:1 mix of RPMI 1640 and L-15, 20% newborn calf serum, 1% penicillin-neomycin-streptomycin cocktail, and 1 x L-Gln (200 mM, 100x). Stock cultures were split 1:10 every four days for 3201 B FeLV-free and FL 74 FeLV-positive, and once a week for 3272 FeLV-positive cell lines.

Cultures for chromosome banding were set up from the stock cultures at high cell concentration in the T-cell medium. Aliquots of 1.5 ml of stock cells at a concentration of 1.6×10^6 cells/ml were cultured in 5 ml of T-cell medium for the FL 74 FeLV-positive cell line and 3201 B FeLV-free cell line.

The cells were cultured for four days at 37 °C in a humidified incubator and 5% CO₂ atmosphere. For the 3201 B FeLV-free cell line, the culture medium was two thirds changed 5 h before harvesting. Fresh medium, to a final volume of 5 ml, plus three drops (51 µl) of L-Gln (200 mM) were added at that time. For the FL 74 FeLV-positive cell line, the culture medium was two thirds changed, and 51 µl of L-Gln (200 mM) was added to 5 ml of fresh medium 14 to 16 h before harvesting . For the 3272 FeLV-positive cell line, 3 ml aliquots at a concentration of 7×10^6 cells /ml were cultured in either 7 ml or 10 ml of T-cell medium plus 85 µl of L-Gln (200 mM) for seven days at 37 °C in a humidified incubator and 5% CO₂ atmosphere. T-cell medium (4 ml) plus 51 µl of L-Gln (200 mM) were added to the culture 78 h before harvesting. The culture was completely changed with fresh medium plus 85 µl of L-Gln (200 mM) 24 h before harvesting.

Testing for FeLV

All three cell lines were checked for the presence or absence of the FeLV virus. The Assure/FeLV feline leukemia antigen test kit was used for this purpose. A plastic stick with a bulbous end was coated with an antibody directed against a specific antigen, p27. This antigen is one of the virus core proteins. The test was performed according to manufacturer's instructions (Synbiotics Corporation).

Harvesting Cell Culture for Chromosome Banding

All three cell lines were grown in suspension. They were harvested for chromosome banding without use of a mitotic arrestant such as colcemid. This was done to obtain better chromosome length; i.e., longer chromosomes, thus facilitating better chromosome banding and more accurate analysis.

The cells were centrifuged at 800 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended in warm hypotonic (0.7 % potassium chloride) and incubated in a water bath at 37 °C for 6 minutes for 3201 B FeLV-free and FL 74 FeLV-positive cell lines, and for 12 minutes for 3272 FeLV-positive cell line. They were centrifuged again at 800 rpm for 5 minutes, resuspended in a freshly made fixative of 5 vol. methanol to 2 vol. of acetic acid, and allowed to stand for 30 minutes at room temperature. They were again centrifuged and resuspended in fixative as above. After a further 10 minutes at room temperature, they were centrifuged again and resuspended with enough fixative to give a translucent suspension.

Slide Preparation

Pre-cleaned slides were wiped clean with alcohol and gauze and put in a beaker with alcohol. The clean alcohol soaked slides were dipped ten times in ice water. Two drops of cell suspension were dropped with a pasteur pipet on the slides from a height of 8 cm. The slides were covered with fixative and

dried by gently waving the slides in the air. The slides were checked by phase contrast microscopy for number and quality of metaphase spreads.

Banding

Slides were heat-dried at 45 °C for 24 h before banding. The Giemsa-trypsin banding technique was used as described previously (Seabright, 1971). The slides were mounted with cytoseal 60 (Stephens Scientific) and cover slip for permanent record.

Microscopy

The slides were observed and photographed with an oil immersion lens (100x/1.30) using a Zeiss axiovert 405 M inverted microscope.

Photography

A 35 mm camera was used with the forementioned microscope to take pictures of well spread intact cell metaphases. The film used was a Kodak technical pan film estar-AH base TP 135-36, ASA 25. The film was developed for 4 minutes with Kodak D-19 developer. The process was arrested with a stop bath (diluted acetic acid) for 30 seconds and fixed with Kodak fixer for 5

minutes, followed by 1 minute in hypoclear. Kodak's photo-flo 200 was used for 30 seconds to avoid drop marks on the film.

Printing

Prints were made using a paper III kodabromide F 3 D. The prints were developed as follows: 1:1 Kodak dektol:water for 1 minute, stop bath (diluted acetic acid) 30 seconds, Kodak fixer 5 minutes, hypoclear 2 minutes, wash in running water 5 minutes, air dry.

Karyotyping

Chromosome prints of well spread intact cell metaphases were karyotyped according to the standard of Wurster-Hill and Gray for cat chromosomes (Figure 1) (Wurster-Hill and Gray, 1973). The marker chromosomes were unidentified or unspecified abnormal chromosomes (Read, 1989). The numerical designations used for the markers were unique for each cell line and cannot be compared between cell lines. On the other hand, the chromosomal abnormalities designated as U markers were shared by two or more cell lines and thus can be compared between cell lines.

Figure 1: Normal karyotype of a female cat, *Felis catus*. The diploid number is 38. The karyotype is made of 18 autosomal chromosomes and 2 sex chromosomes. This picture shows G-banding pattern (picture courtesy of Dr. D. Wurster-Hill).

RESULTS

Cell Growth

All three cell lines were sent from Dr. Hardy's laboratory in McCoy's 5A medium supplemented with calf serum; but, attempts to continue the cell cultures in this medium met with failure. Successful maintenance was possible with a medium developed by Dr. P. Donahue (Biomedical Research Institute, St. Paul, MN). The medium formulation, called T-cell medium, was a 1:1 RPMI 1640 medium:L-15 medium, 20% fetal (or newborn) calf serum, 1 x L-Gln (200 mM, 100x initial concentration), and 1% penicillin, streptomycin, and neomycin.

The FL 74 FeLV-positive cell line grew in suspension and stayed in cumuli (Figure 2 A). The cells did not disaggregate easily upon shaking them. When disaggregation was achieved, they would aggregate again fairly quickly. The cells showed a "rough" surface with much cell budding and a round cell morphology (Figure 2 B). It was noticed that cytoplasmic extrusion occurred during cell division. Therefore, budding may have been the result of this event.

The 3272 FeLV-positive cell line grew in suspension but did not have the tendency to form aggregates, and grew very slowly. The cells showed a

Figure 2: Morphology of the FL 74 FeLV-positive cell line. A. The cells grew in suspension and formed cumuli. The cell cumuli did not break up easily upon shaking the culture flask. They reaggregated quickly after cessation of shaking. This photograph taken before splitting the culture 1:10 and shows two cell cumuli. Individual cells were rare in the culture and were usually seen right after cell division had taken place. This picture does not show any isolated cells. B. The cell surface appeared rough as a result of much cell budding. It was noticed that cytoplasmic extrusion also occurred during cell division. Maximum cell budding was seen right after the culture was split and 51 μ l of L-Gln (200 mM) was added to 7 ml of medium. This picture shows rare isolated cells with buds on their surfaces and was taken right after T-cell medium renewal and addition of 51 μ l of L-Gln (200 mM). The cells also show a round morphology.

A

B

"semi-rough" surface with cell budding and an ameboid as well as a round cell morphology (Figure 3).

The 3201 B FeLV-free cell line also grew in suspension forming some aggregates. However, the majority of the cells grew dispersed, the cumuli were easy to break, and the cells did not readily reaggregate (Figure 4 A). The cells showed a smooth surface with no budding and an ameboid, as well as a round cell, morphology. The cells' ameboid morphology was most apparent after addition to 7 ml of medium of 51 μ l of extra L-Gln (200 mM) which acted as a mitotic agent (Figure 4 B).

The Fl 74 FeLV-positive cells treated with phytohemagglutinin (PHA) did not show a greater mitotic index than cells without PHA. However, the cells showed a great increase in mitotic index when extra L-Gln (200 mM) was added to the T-cell medium. For example, a vial of FL 74 FeLV-positive cell line was thawed, washed, and resuspended in T-cell medium plus 51 μ l of L-Gln (200 mM). After 24 h, the cells had to be split 1:10; only 10% of the cells were dead. This culture had to be split again 4 days later (Figure 5 A and B). These results were not obtained when extra L-Gln (200 mM) was not added, as indicated above, to the thawed cells. In that case, the cells took about a week to come to confluence and split.

The 3272 FeLV-positive and 3201 B FeLV-free cell lines also showed mitogenic response to L-Gln and lack of mitotic stimulation by PHA. However, 3201 B FeLV-free cells responded more quickly to the mitotic stimulus than 3272 FeLV-positive cells. The latter also needed a greater concentration of extra L-Gln (200 mM), 85 μ l, than was added to the other two

Figure 3: 3272 FeLV-positive cell line morphology and cell budding. The cells appeared semirough as a result of cell budding. There was less budding per cell than in FL 74 FeLV-positive cells (see Figure 1 B). The presence of the virus could be detected in the culture supernatant after a longer culture period than for FL 74 FeLV-positive cells. The cells were also slow growing and needed a higher initial cell concentration and a longer cell culture time than for the other two cell lines to achieve a similar cell concentration at harvest time. Note the semirough cell surface and change in cell morphology from round to ameboid, the latter morphology was even more apparent after splitting the culture and adding 85 μ l of L-Gln (200 mM) in 7 ml of medium.

Figure 4: 3201 B FeLV-free cell line mode of growth and cell morphology.

A. The cells grew in suspension forming some aggregates. This picture shows most of the cells growing separated, their most common mode of growth, and some in aggregates. Note also the smooth appearing surface without budding and some cytoplasmic extrusion (arrows). B. The cells presented two kinds of morphology, round and ameboid. The ameboid morphology was most prominent right after addition of extra L-Gln (200 mM) to 7 ml of culture.

A

B

Figure 5: FL 74 FeLV-positive cells mitotic response to addition of extra L-Gln (200 mM). Picture A shows the cells 24 hours after having been thawed, washed, and resuspended in 7 ml of T-cell medium plus 51 μ l of L-Gln (200 mM). The culture flask was full of cell cumuli like the ones shown in the picture and had to be split 1:10. No extra L-Gln (200 mM) was added to the split culture. Picture B shows the same culture 5 days later after having been split for a second time 1:10.

A

B

cell lines to obtain the same mitotic index over a longer period of time.

All three cell lines were also treated with conA as a mitogen. In these experiments, none of the three lines showed an increase in mitotic index over cells treated without conA. Furthermore, all three cell lines were treated with a combination of PHA and conA and showed no increase in mitotic index in comparison to untreated cells. Therefore, all cultures set up for karyotyping were treated with L-Gln as a mitogenic agent instead of the traditional PHA or conA.

Assure/FeLV Test

Even though the FeLV virus infectious status for the cell lines was known, the cell lines were tested for the presence of the virus. An antibody highly specific against FeLV virus antigen p27 was used for this test (Synbiotic Corporation). Antigen p27 is one of the proteins coded by the gag gene (Lutz, 1990). A blue color development was indicative of the presence of the virus. Lack of color development indicated its absence. All three cell lines tested as expected. Fl 74 and 3272 were positive, and 3201 B was negative.

Cell Culture Harvest

The best mitotic index for the FL 74 FeLV-positive cell line was

obtained with 51 μ l of L-Gln (200 mM) in 7 ml of culture 14 to 16 h after its addition to just changed medium. For the 3272 FeLV-positive cell line, the best mitotic index was obtained with 85 μ l of L-Gln (200 mM) in 7 ml of culture 24 h after addition to changed medium. For the 3201 B FeLV-free cell line the best mitotic index was obtained with 51 μ l of L-Gln (200 mM) in 7 ml of culture 5 h after adding it to just changed medium.

For all three cell lines a hypotonic concentration greater or less than 0.7% potassium chloride caused cell agglutination. It was found that any change in the proportion of potassium chloride to sodium citrate would cause cell agglutination too.

The best cell metaphase spreads were obtained with a fixative of 5 vol. methanol: 2 vol. acetic acid. The standard fixative of 3 vol. methanol: 2 vol. acetic acid gave metaphases that were usually not countable in any of the three cell lines.

Colcemid was used for 30 minutes prior to harvest to arrest mitosis at the metaphase stage. It was found that the chromosomes had a tendency to have short arms, especially in the 3272 FeLV-positive cell line, making chromosome banding and karyotyping more difficult or impossible. When colcemid was not used, longer chromosome arms were obtained and better karyotyping achieved. Consequently, colcemid was not used and banding was done without arresting the chromosomes.

Cat Karyotype

The normal diploid ($2n$) chromosome number in the domestic cat (*Felis catus*) is 38. The karyotype is composed of eighteen autosome pairs grouped from A to F, based on chromosome size and centromere location, and within each class sequentially numbered from 1 according to the system of San Juan (1965) Conference (Jones, 1965).

The A group comprises three large submetacentric pairs, the B group four large subtelocentric pairs, the C group two large metacentric pairs, the D group four medium small subtelocentric pairs, the E group three small metacentric pairs, and, the F group two small acrocentric pairs. The X is a medium-sized submetacentric and the Y is acrocentric (Figure 6). The chromosomes have a single centromere that appears as a nonstaining constriction. This position is consistent for a given chromosome. Homologous chromosomes are paired together (Hare et al., 1966).

FL 74 FeLV-positive Karyotype

FL 74 had a modal number of 37 with nine consistent markers; i.e., markers appearing in two or more cells (Figure 7). A total of fifty intact metaphases were counted. Karyotype analyses showed hypodiploid or pseudodiploid cells with monosomies, trisomies, and absence of pair chromosomes (Figure 8). Group A, B₁, B₃, group D, group E, group F, X, and Y

Figure 6: Diagrammatic representation of the different types of chromosomes as seen at metaphase. (A) metacentric; (B) submetacentric; (C) subtelocentric; (D) telocentric (acrocentric). (Hare, et al., 1966).

Figure 7: FL 74 FeLV-positive cells modal number and cell count percentage distribution. The modal number was 37. Most of the metaphases counted were hypodiploid (less than 38), with a few being pseudodiploid, as all the cells analyzed showed chromosomal abnormalities.

Figure 8: Karyotype from FL 74 FeLV-positive cell line. This karyotype shows A_1 , D_4 , E_2 , and F_2 monosomies; D_2 and E_1 trisomy; and the E_3 as well as the F_1 chromosome pair missing. Five of the seven consistent markers can be also observed in this cell.

chromosomes showed at least one of the anomalies mentioned above. D_1 and D_4 monosomies, and the absence of both F_1 were the most common occurrence in the karyotypes analyzed (Figure 9).

Markers 1, 2, 3, derivative (der) (D_1 p), and deletion (del) (D_2 q) had an occurrence of 86%, 92%, 64%, 98%, and 66% respectively. The other markers were found in less than 50% of the cells (Figure 10). The only time der (D_1 ;?)(p ;?) was not found, the cell was monosomic for D_1 . Also, 4% of the cells had a double deletion for D_2 (data not shown). Marker U4 (U for "universal" in an attempt to indicate that the marker was shared with at least one other cell line analyzed) was found in 3201 B FeLV-free cell line too (see Figure 19). Marker U4 was present in 2% of FL 74 FeLV-positive cells.

The only identifiable markers for FL 74 FeLV-positive cells in comparison to normal cat chromosomes were mar1, mar 2, der (D_1), and U4. Marker 1 was a possible translocation $?t(E_1;F_1)(q;?)$. Marker 2 was a possible Robertsonian translocation $?t(F_2;F_2)$. A Robertsonian translocation takes place when two acrocentric chromosomes fuse at or near the centromere. The short arms are lost but this does not cause any phenotypic effect (Read, 1989). Der (D_1) was a translocation $t(D_1;?)(q;?)$. Marker U4 was a translocation $t(A_2 ;?)(p;?)$. No cellular onc genes or immunoglobulin (Ig) genes have been mapped so far to the translocation region of the identified markers, del (D_2 q), or der (D_1 p).

Figure 9: FL 74 FeLV-positive chromosome anomalies percentage distribution. Group A, chromosome B₁, B₃, group D, group E, group F, chromosomes X and Y showed monosomies, trisomies, or pair absence. The most common occurrences were D₁ and D₄ monosomies as well as F₁ pair missing.

Figure 10: FL 74 FeLV-positive markers percentage distribution. The cells showed eight consistent markers. Der (D_1), mar2, and mar1 showed the highest percentage of occurrence. The one time when der (D_1) was not found in the cell, the cell was monosomic for D_1 . The U designation stands for "universal" in an attempt to indicate that the marker is shared at least with one of the other cell lines analysed. Marker U4 was shared with 3201 B FeLV-free cells.

3272 FeLV-positive Karyotype

Karyotype 3272 had a modal number of 38 with six consistent markers (Figure 11). A total of fifty intact metaphases were counted. Karyotype analyses showed hypodiploid, pseudodiploid, and hyperdiploid cells with monosomy, trisomy, and chromosome pair absence (Figure 12). All six chromosome groups as well as the sex chromosomes showed at least one of the above mentioned anomalies. A_2 , A_3 , B_1 , D_2 , E_1 , E_3 , and F_1 monosomies were the prevailing anomalies, with F_1 monosomy occurring in 80% of the cells (Figure 13).

Markers 1 and U3 had an occurrence of 55.% and 71% respectively. The other markers were found in less than 50% of the cells (Figure 14). Markers U1 and U3 were shared with 3201 B FeLV-free cell line. Markers U1 and U3 were present in 3272 FeLV-positive cells 26.3% and 71% respectively.

The only identifiable markers for 3272 FeLV-positive cells were U1 and del (B_2q). Marker U1 was a translocation $t(B_1;?)(q;?)$. No cellular onc genes or immunoglobulin genes have been mapped so far to the breakpoint regions of either marker.

3201 B FeLV-free Karyotype

Karyotype 3201 B had a modal number of 39 with fourteen consistent markers (Figure 15). A total of 50 intact metaphases were counted. Karyotype

Figure 11: 3272 FeLV-positive cells modal number and cell count percentage distribution. The modal number was 38. Most of the metaphases counted were pseudodiploid, followed by hyperdiploid and hypodiploid cells. All of the metaphases counted showed chromosomal abnormalities.

Figure 12: Karyotype from 3272 FeLV-positive cell line. This karyotype shows A_2 , B_1 , B_4 , E_1 , E_3 , and F_1 monosomies; D_1 trisomy; and D_2 pair missing. Four of the six consistent markers can also be seen in this cell.

Figure 13: 3272 FeLV-positive cells chromosome anomalies percentage distribution. All six chromosome groups showed monosomies, trisomies, or pair missing. The most common occurrences were A₂, A₃, B₁, D₂, E₁, and F₁ monosomies, the latter being the highest of all.

Figure 14: 3272 FeLV-positive cell markers percentage distribution. The cells showed six consistent markers. Markers 1 and U3 showed the highest percentage of occurrence. Markers U1 and U3 were shared with 3201 B FeLV-free cell line.

Figure 15: 3201 B FeLV-free cells modal number and cell count percentage distribution. The modal number was 39. Most of the metaphases were hyperdiploid. There were also some pseudodiploid and hypodiploid cells. All of the metaphases analyzed showed chromosomal abnormalities.

analyses showed hypodiploid, pseudodiploid, and hyperdiploid cells with monosomy, trisomy, and absence of pair chromosomes (Figure 16). All six chromosome groups as well as the sex chromosomes showed at least one of the above mentioned anomalies. A_1 , B_2 , B_3 , D_1 , E_1 , E_3 , and F_1 monosomies were the prevailing ones with A_1 occurring 100% of the time (Figure 17).

Markers 1, 3, U4, der (A_1 p), and del (B_2 q) had an occurrence of 76%, 100%, 57.4%, 85%, and 63.8% respectively. The other markers were found less than 50% of the time (Figure 18). The 15% of the time when der (A_1 p) was not found, the cell was either monosomic for A_1 or the A_1 pair was missing. Also, 6.4% of the cells had a double deletion for B_2 ; and, 61.5% of the cells were monosomic for B_2 .

The only identifiable markers for 3201 B FeLV-free cells were mar3, U4, der (A_1), and del (B_2). Marker 3 was a translocation $t(D_1;?)(q;?)$. Marker U4 was a translocation $t(A_2;?)(p;?)$. Der (A_1) was a translocation $t(A_1;?B_2)(p;?)$. Marker B_2 had a del (B_2 q).

No cellular onc genes or Ig genes have been mapped so far to the translocation or breakpoint regions of any of the identified markers (O'Brien, 1990). However, the distal half of the q arm of the B_2 chromosome houses the major histocompatibility complex (MHC) class I and class II antigens (O'Brien, 1990). Based on morphological observation, the two MHC genes may have been deleted in all cases of del (B_2 q). However, it cannot be said whether the B_2 q deletion was terminal or interstitial. Furthermore, the exact band location of the MHC on the q arm is not known (Yuhki and O'Brien, 1988).

Figure 16: Karyotype from 3201 B FeLV-free cell line. This karyotype shows B₃, D₁, E₃, F₁, and F₂ monosomies. Nine of the fourteen consistent markers can also be observed in this cell, including der (A₁) and del (B₂). The X chromosome is missing.

Figure 17: 3201 B FeLV-free chromosome anomalies percentage distribution.

All six groups showed monosomy, trisomy, or pair missing. The most common occurrence were A_1 , B_2 , B_3 , and D_1 monosomies, A_1 monosomy being the highest of all. The sex chromosomes were sometimes missing.

Figure 18: 3201 B FeLV-free markers percentage distribution. The cells showed fourteen consistent markers; mar1, mar3, U4, der(A₁), and del (B₂) showed the highest percentage of occurrence. When der (A₁) was not found, the cells were monosomic for A₁. Markers U1 and U3 were shared with 3272 FeLV-positive cells, and U4 was shared with FL 74 FeLV-positive cells.

"Universal", U, Markers

The 3201 B FeLV-free cell line had two markers in common with the 3272 FeLV-positive cell line, U1 and U3. The latter was found in a high percentage in both cell lines, 48.9% and 71%, respectively, in a 1.5:1 relationship between them. The U1 marker was found in a low percentage in both cell lines, 2.1% and 26.3% respectively, in a 13:1 relationship (Table 1).

Also, the 3201 B FeLV-free cell line shared one marker with the FL 74 FeLV-positive cell line, U4. The U4 marker was found in a high percentage in the former and a low percentage in the latter, 57.4% and 2% respectively, giving a relationship of 28.5:1 (Table 1).

The two FeLV-positive cell lines did not share a common marker (Figure 19). Thus, the greatest sharing of chromosomal abnormalities was found between the virus-free cell line and one of the virus-positive cell lines, 3272. This was in spite of the fact that each cell line had a different cell origin, the virus-free cell line originating from a thymic lymphoma whereas the FeLV-positive cell line represents a multicentric lymphoma.

Karyotype Analysis in all Three Cell Lines

Karyotype analysis from all three cell lines showed some monosomies, trisomies, and chromosome pairs missing. The FL 74 FeLV-positive line was the only cell line which had some consistent normal pairs of chromosomes;

	U1	U3	U4	Cell origin
3272 FeLV-positive	26.3%	71.0%		multicentric
FL 74 FeLV-positive			2.0%	thymic
3201 B FeLV-free	2.1%	48.9%	57.4%	thymic
% relationship	13:1	1.5:1	28.5:1	

Table 1: Occurrence of U markers and cell origin. The U ("universal") markers are markers shared between cell lines. Their number identifies the same chromosome marker in each cell line. None of the U markers were clonal. However, the cells may be changing fast (rapid evolution) and a recognizable clone by standard definition cannot be observed. The 3201 B FeLV-free cells had the most U markers. The relative relationship shows the significance of association for each U marker between cell lines. The highest association in chromosome abnormalities was found between the virus-free cells and 3272 FeLV-positive cells. Each cell line had a different cell origin.

Figure 19: Chromosome markers in all three cell lines. All the consistent markers in all three cell lines are seen here. The marker numbers of the chromosomes designated mar are not related to the numbers of the other cell lines. The markers shared between the cell lines are called U, from universal, and their numbers correlate between cell lines. The highest chromosome abnormality association was found between 3272 FeLV-positive cells and 3201 B FeLV-free cells.

MARKERS

3201 B FeLV(-)

3272 FeLV(+)

FL 74 FeLV(+)

i.e., some chromosome pairs never showed any kind of anomaly, namely chromosomes B_2 and B_4 . In all three cell lines, E_3 monosomy occurred with about the same percentage of frequency, approximately 50%. D_1 monosomy was greater in FL 74 FeLV-positive cells than in 3201 B FeLV-free cells, 83% and 69% respectively. F_1 monosomy was greater in 3272 FeLV-positive cells, 80%, than in FL 74 FeLV-positive cells, 16%, and 3201 B FeLV-free cells, 46%. F_1 pair was missing in 83% of the cells of FL 74 FeLV-positive line and only in 7.7% of 3201 B FeLV-free cells. Even though all chromosome pairs have been numerically altered in all three cell lines (with exception of FL 74 FeLV-positive cell line), the only chromosome pairs affected in both positive cell lines were E_3 and F_1 with a percentage of occurrence less than or equal to 50% (data not shown).

DISCUSSION

Chromosomal abnormalities are pervasive in cancerous tumors (Heim et al., 1988). They are found in most malignancies that are retrovirus-positive as well as retrovirus-negative (Grindem and Buoen, 1989), with the exception of some tumors caused by retrovirus with onc genes; i.e., genes encoding for transformation (Duesberg, 1987 b). Furthermore, chromosomal abnormalities are diagnostic of neoplastic disease (Wolman, 1983). Chromosomal abnormalities are also seen in benign tumors and in non-tumorous cells exhibiting abnormal growth as well (Wurster-Hill et al., 1988). In spite of this, not all tumors analyzed showed chromosomal aberrations (Grindem and Buoen, 1989; Wolman, 1983).

Even though many human tumors have specific chromosomal abnormalities, it seems that there are cases where chromosomal aberrations are not visible. It is not unlikely that some fraction of cells in a tumor may be diploid due to tumor heterogeneity; however, about 33 % of all breast tumors are totally diploid (Wolman, 1986; Wolman, 1983). Tumor heterogeneity may occur as a result of genetic instability, and this may be a consequence of the many intervening steps between the first initiating alteration in a normal cell and the total loss of growth control (Wolman, 1986). One such step is the varying environmental conditions for cell growth, especially in solid tumors,

due to the presence of dead and dying cells, lack of or an insufficient blood supply, as well as oxygen, nutrients, and inefficient elimination of cell toxic wastes (Wolman, 1986). However, leukemias must have different selective factors for heterogeneity because leukemic proliferations develop as infiltrates rather than as solid tumors and therefore are not subject to the same kind of environmental restrictions. There is evidence that leukemic cells produce factors that suppress normal stem cells proliferation in the bone marrow as well as replacement of normal stem cells by tumor cells (Wolman, 1986).

However, if chromosomal abnormalities were found in all tumors, this would not be proof of tumor causation (Wolman, 1983). Their appearance may be a late event in tumorigenesis as tumors are usually examined at a far-advanced stage (Wolman, 1983). The chromosomal abnormalities may indicate a subpopulation of cells that was selected by the environment from the initiating population (Wolman, 1983; Nicolson, 1979). Furthermore, in the case of retinoblastoma in humans, a small systemic deletion of the retinoblastoma gene appears to foster malignant transformation (Wolman, 1983). Chromosomal abnormalities may indicate tumor progression instead of initiation ; i. e., causation.

As has already been noted, the only transforming autonomous genes are the retroviruses with transforming onc genes. They are capable of transforming a permissive host cell with one-to-one kinetics; i.e., each cell infected becomes transformed. Therefore, tumors arising from transforming retroviruses are polyclonal as more than one cell gets infected by the retrovirus and its progeny (Duesberg, 1987 c). Usually they do not show

chromosomal abnormalities though they may do so later on during tumor progression. These abnormalities can occur frequently and be specific (Wolman, 1986).

On the other hand, clonal chromosomal abnormalities are commonly recognized in tumors that are not caused by retroviruses with transforming onc genes (Duesberg, 1987 b; Wolman, 1983). This characteristic is shared also with non-viral tumors of the same lineage (Duesberg, 1987 c). However, not all tumors show the high degree of specificity of chromosome abnormality demonstrated in chronic myelogenous leukemia (CML). Furthermore, even in cases like CML, there is not complete uniformity within the tumor; CML involves a translocation $t(9; 22)(q34;q11)$ in about 90% of the cases. The other cases are variant translocations; but, $del(22)(q11)$, which is the Philadelphia chromosome (Ph^1) is a consistent feature (Wolman, 1986; Larson et al., 1981). However, the Ph^1 chromosome is not specific for CML. It is also found in acute lymphoblastic leukemia (ALL) and in acute myeloblastic leukemia (AML) (Larson et al., 1981). Besides, there are cases of primary tumor polyclonalities in breast cancers and carcinomas (Heim et al., 1988; Wolman, 1986). Thus, even in non-viral cases, there are some degrees of variability in clonality and chromosome specificity.

Proto-onc genes, also called cellular onc genes, have been postulated to be latent cellular cancer genes based on the sequence homology between them and viral onc (transforming) genes (Bishop, 1981). The onc gene hypothesis predicts a) both cellular and viral onc genes to be isogeneic; b) expression of cellular onc gene causes tumors; c) transfection of activated cellular onc genes

into diploid cells causes their transformation; d) there are diploid tumors (Duesberg, 1987 b). However, none of these predictions have been proved true yet. On the contrary, a) cellular and viral onc genes are not isogenic; b) cellular onc genes are usually expressed during cell growth and development; c) when cells are transfected with activated cellular onc genes, transformation does not occur, except in recombinants where the myc gene with a retroviral long terminal repeat (LTR) promoter is located between a native c-myc promoter and the second c-myc exon (Zhou and Duesberg, 1988); d) there are not diploid tumors isolated today with activated c-myc (Duesberg, 1987 b). Thus at present, the evidence shows that a completely normal cellular gene cannot cause cancer (Woolford et al., 1988; Rowley, 1984).

Therefore, cellular cancer genes may be rare recombinants of normal cellular genes with proto-onc genes. This would be in agreement with the fact that abnormal rearranged chromosomes are the most consistent transformation-specific markers in tumor cells (Duesberg, 1987 b; Trent, 1984). As a matter of fact, the clonality of these markers would indicate that tumor initiation and probably causation are due to these chromosomal abnormalities (Duesberg, 1987 b).

Specific retrovirus integration site requirements have been proposed as another mechanism for tumorigenesis. Proviral integration occurs near or in a gene critical for cell differentiation or normal cell growth, disrupting its normal functioning (Varmus, 1988). However, the proviral integration site is random. Therefore, the likelihood of a proto-onc gene activation by proviral

integration would be in the order of 1 out 10^6 infected cells as eukaryotes contain about 10^6 kb of DNA per cell (Duesberg, 1987 b).

However, not all proviral integrations activate proto-onc genes even if integration occurs within 1 to 10 kb of the putative proto-onc gene.

Activation of the proto-onc genes, for example the p-myc, happens when the provirus integrates between the native p-myc promoter and the second exon of the p-myc as already mentioned (Zhou and Duesberg, 1988).

Of twenty-one naturally occurring feline lymphomas studied by Levesque and her group (Levesque et al., 1990), only four lymphomas had FeLV virus integrated within a unique 2.4 kb locus; i.e., 19% of the tumors analyzed. The other integration sites were not in any of the commonly known integration loci for other proviruses. Seven of the tumors analyzed were within the same phenotypic subgroup. Of these seven, only the four already mentioned had integration sites within a unique unknown locus. If a specific integration site were the mechanism of tumorigenesis for these lymphomas, the same unique integration loci would have had to appear in all seven of them. Furthermore, no other relationship of integration site to tumorigenesis was drawn from their study. Thus, the integration site hypothesis does not offer a satisfactory explanation for the malignant potential of retroviruses without onc genes, though it may explain a few cases.

It was postulated here that the FeLV virus may not have a direct role in tumorigenesis and was asked whether chromosome abnormalities and virus integration sites can each cause or play a role in the causation of tumors.

It was expected that if the FeLV virus were the cause of tumors and chromosomal abnormalities in FL 74 FeLV-positive and 3272 FeLV-positive cell lines, they might at least have shared one marker in common. Furthermore, 3201 B FeLV-free cells should not have had chromosomal abnormalities, much less have any aberration in common with any of the two positive cell lines. The opposite was true. The FeLV -free cell line had fourteen consistent markers. It also had the best markers occurrence percentage of all three cell lines with 100% for $\text{mar3 } ?t(D_1;?)$ and 85% for $\text{der}(A_1 p)$. This also indicated clonality of chromosomal abnormality. The two FeLV-positive cell lines did not have a marker in common and their karyotypes were quite different from each other. The FL 74 FeLV-positive cells showed the best markers' occurrence for the FeLV-positive cell lines, and was second to 3201 B FeLV-free cells, with 98% for $\text{der}(D_1 p)$ and 92% for mar2 a possible Robertsonian translocation $t(F_2;F_2)$. This was also an indication of clonality of chromosomal abnormality. As for the 3272 FeLV-positive cells, they showed the lowest frequency of markers of all three cell lines with 71% for U3 being the highest occurring marker. This seemed to indicate a lack of chromosomal abnormality clonality of the cell line. Furthermore, 3272 FeLV-positive cells shared two markers in common with 3201 B FeLV-free cells, U1 and U3. The latter cell line also shared one marker with FL 74 FeLV-positive cells, U4. This marker occurred in only 2% of cells in this cell line. Thus, the highest chromosomal abnormality association was found between 3201 B FeLV-free and 3272 FeLV-positive cells (Table 1). Note that each cell line had a different cell origin.

It could be argued that it was not the virus per se; i.e., not a viral product, that caused tumorigenesis and chromosomal abnormalities, but rather the proviral integration site. If this were the case, it would be expected that an integration site within the same locus would cause the same phenotypic tumor, and the chromosomal abnormalities, being a consequence of this event, would be common to the same phenotypic tumors. It has already been noted that the two cell lines that shared markers in common were of different cell origin; and therefore, the tumors were not phenotypically the same. Furthermore, one of the thymic tumors was virus-free while the other was virus-positive. If the virus had been integrated and suppressed in 3201 B FeLV-free cell line, as it is often claimed for virus-negative tumors (Koshy et al., 1980), it could have been expected to have a clonal chromosome marker in common with FL 74 FeLV-positive cells, the other thymic tumor. This was not the case; U4 was present in 2% of cells in FL 74 FeLV-positive cells, hardly a clonal marker, and in 57.4% of cells in 3201 B FeLV-free cells which also cannot be said to be clonal.

It could be hypothesized, on the other hand, that chromosomal abnormalities were the cause of tumorigenesis. In that case, both thymic tumors would be expected to have one chromosome marker in common, and this one to be clonal. Furthermore, the multicentric lymphoma should not share a common marker with either thymic tumor. As already mentioned, this was not the case. It could be argued that a common chromosomal abnormality is the cause of tumorigenesis; but, the phenotype depends on the cell origin and not on the kind of chromosome aberration. If this were true,

the same clonal chromosomal abnormality should have appeared in all three cell lines. This was not the case as no single clonal chromosomal abnormality was found common to all three cell lines. Even though 3201 B FeLV-free and FL 74 FeLV-positive cell lines were clonal within their own cell lineage, this could not be taken as a proof of tumor causation because the relative place in time of the chromosomal abnormalities in tumor initiation and progression was not known nor could it be determined from the available data. The chromosomal abnormalities could be a fairly late event in tumor development, showing progression within the tumor instead of initiation.

Based on genetic mapping done so far for the domestic cat genome, the only chromosomal abnormality with any significant bearing on carcinogenesis was the del(B₂q). The feline leukocyte antigen-1 (Major Histocompatibility Complex, class I) and feline leukocyte antigen-2 (MHC, class II) are located on the distal half of the B₂ q arm (O'Brien, 1990). Based on morphological observation, the two MHC genes might have been deleted in all cases of del(B₂q). However, molecular analysis is needed to determine the real location of this deletion.

The MHC influences virtually every immunological activity that involves the interaction of cells. The ability of an animal to respond to certain antigens is also controlled by the MHC. Also, the ability of most T-cells to carry on with their normal function depends upon their interacting with a self histocompatibility antigen. Deletion of the gene(s) would cause lack of the recognition signals that enable T-cells to discriminate between normal cell surfaces within the body and those that have become altered by

events such as virus infection, the ingestion of foreign molecules, and even possibly neoplastic transformation. T_C cells function to eliminate any altered cell while T_H cells respond to antigens expressed on specialized antigen-presenting cells.

It is interesting to note that a del ($B_2 q$) is found in the 3201 B FeLV-free cell line 63.8% of the time. Also 61.5% of the cells were monosomic for B_2 . It can be speculated that even though deletion of the MHC in this cell line was not the initiating step in tumorigenesis, it may have played a very important role in tumor progression by altering cell mediated and humoral immune response. It is noteworthy too that chromosome 6 in humans, which houses the HLA, is involved quite often in chromosome aberrations in solid tumors (Wolman, 1983).

Since both virus-infected and virus-free cell lines have chromosomal abnormalities, it can be concluded that, although the FeLV virus may cause chromosomal abnormalities, it could not be considered to be the only causative agent of the abnormalities observed. These abnormalities may, however, be an important factor in the development of oncogenesis. On the other hand, a third unknown factor may be the causative agent of the chromosomal abnormalities or cancer and therefore, the initiating step of tumorigenesis. Chromosomal abnormalities may not be, in that case, necessary for tumor development and progression, as it is suggested by the normal karyotype found in 33% of breast cancers and 22% of meningiomas among others (Wolman, 1986).

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