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SEQUENCES RELATED TO MOUSE MAMMARY TUMOR VIRUS GENOME IN TUMOR CELLS AND LYMPHOCYTES FROM PATIENTS WITH BREAST CANCER

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Cloned murine mammary tumor virus (MuMTV) sequences allowed us to search for murine mammay tumor virus related sequences in the DNA of surgically removed human breast tumors. Out of 28 tumors so far examined two were found to contain an Eco RI DNA fragment homologous to the long terminal repeat-group antigen (LTR-Gag) and the Envelope (Env) sequences of MuMTV. We have taken the lymphocytes of these patients and cultured them. Rapid growth of lymphocytes, mostly of T origin, occurred in the presence of T cell growth factor (TCGF). Whereas DNA extracted from fresh lymphocytes is negative, that extracted from the 3-day cultured lymphocytes showed MuMTV related sequences. Long term cultures of T cells and a similar culture derived from a healthy person donor were negative at all stages. DNA extracted from the Ebstein Barr Virus-transformed B cells of the patient does not contain the MuMTV related sequences.

In the last few years, considerable interest has been devoted to the identification of molecular and host response markers for neoplasms of the human breast. Several investigators have reported in the sera of patients with breast carcinoma the presence of antibodies to the Murine Mammary Tumor Virus (MuMTV) (1-5) a retrovirus clearly involved in the high incidence of mouse mammary carcinoma (6). More recently, antigen related to the envelope glycoprotein of MuMTV (gp 52) was detected in some cells of human breast tumors (7) and in a cell line (MCF7) established from a breast carcinoma (8). Recently MuMTV related sequences were found in the DNA of normal human tissues (9). The availability of cloned MuMTV DNA and the increased

ABBREVIATIONS: MuMTV = Murine Mammary Tumor Virus; TCGF = T Cell Growth Factor; LTR-Gag = Long Terminal Repeat - Group antigen; EBV = Ebstein Barr Virus.

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sensitivity of hybridization techniques prompted us to reexamine the possibility of MuMTV related sequences in the DNA of human breast tumors. Using moderatly stringent conditions we have searched for MuMTV related sequences in the DNA of breast tumor and in that of cultured leucocytes from patients with breast cancer.

Material and Methods

Preparation of DNA and analysis of MuMTV DNA

Tumoral tissues frozen in liquid nitrogen were pounded in a cold mortar after dissociation in small pieces with a hammer. The resulting powder was suspended in 5 volumes of extraction buffer: 0.01 M Tris HCl pH 7.9, 0.05 M NaCl, 0.005 M EDTA, homogenized in a Dounce homogenizer (10 strokes with a B pestle). 0.5% of SDS was then added and proteins were digested with 100 µg/ml of proteinase K for 10 hours at 37°C. The resulting solution was gently poured on pure ethanol and DNA fibres were spooled onto a glass rod. They were washed three times in ethanol, rapidly dried and redissolved overnight in sterile water at 4°C. Further deproteinization was achieved by 3 cycles of phenol-chloroform extraction. The final aqueous phase was dialyzed against the extraction buffer. 20 µg of each DNA were digested until completion with 40 units of Eco RI enzyme (Boehringer) and loaded on 1% agarose gel. Pst 1 fragments of the recombinant pBR 322-LTR plasmid were used as molecular weight markers. After electrophoresis, the DNAs were transfered to nitrocellulose filter as described by Southern (10). The filter was hybridized with LTR-Gag and Env MuMTV DNA probes, successively. Both probes were obtained as DNA fragments from Dr. H. Diggelman, reinserted into pBR 322 and amplified in our laboratory. 1.5 Kb and 2.0 Kb DNA fragments corresponding to LTR-Gag and Env respectively, were obtained by restricting the corresponding recombinant plasmids with Pst 1 enzyme and isolated by electroelution from 1% agarose gel. The probes were labeled by nick translation with ^{32}P dCTP and ^{32}P TTP (Amersham). Tumor DNA filter was hybridized 48 hrs with $4x10^6$ cpm of LTR-Gag DNA in 50% formamide at 42°C as described by Wall et al. (11), and then washed 4 times with 0.1 SSC at room temperature. The wet filter in a plastic bag was autoradiographed for two weeks at -70°C using Kodak Royal X O Mat film and an amplifying screen.

Culture of human leucocytes

Leucocytes from fresh blood collected with 100 U:ml of heparin were allowed to sediment by gravity for 2 hrs at 37°C and then centrifuged at 800 g for 10 min. Cells were resuspended in RPMI 16-40 medium (Gibco) supplemented with 10% fetal calf serum (Boehringer), 10^{-5}M β-mercaptoethanol, $5x10^{-7}$ M dexamethasone, 0.1% sheep anti-HU interferon serum (neutralizing titer : 6 U at 10^{-5}), 10 µg/ml protein A (Pharmacia) and 25% (final concentration) human crude TCGF in RPMI 16-40 medium. Cells were incubated in Corning 25 cm^2 plastic flasks, at the concentration of 10^6 cells/ml (7 ml per flask) at 37°C. After 24 hrs, 5 ml of fresh medium were added and the flasks were placed in vertical position. After 72 hrs of culture, about $2x10^7$ cells were removed by centrifugation and kept at -80°C until DNA extraction. Fresh uncultured lymphocytes were separated from another sample of blood on a Ficoll-Hypak gradient and similarly kept frozen at -80°C.

Results and discussion

A 52 year old woman (TEB) was tumorectomized for a comedocarcinoma in her left mammary gland. 18 months later, she presented a nodule on her

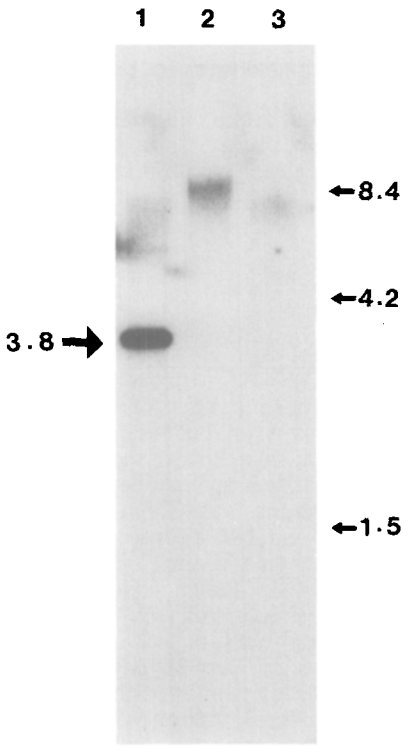

Figure 1 Analysis of Eco RI restricted DNAs from 3 mammary tumors. Slots 1, 2, 3 represent tumor DNA from patient TEB, NOV (adenocarcinoma) and IRT (intragalactophoric tumor) respectively. Arrows indicate migration of some of the DNA markers (the 8.4 Kb fragment arises from a dimer form of plasmid DNA).

right mammary gland, wich was biopsied and then excised. The nodule showed histological signs of premalignant lesion, such as a high variation in nuclear size and staining and lymphocytic infiltration. DNA was extracted from the nodule in a high molecular weight form, digested with Eco RI restriction enzyme and electrophoresed on an agarose gel. DNAs extracted from 28 other breast tumors of various histological types were similarly treated. After transfer onto nitrocellulose filters, the DNA fragments were hybridized with either MuMTV LTR-Gag or Env radioactive probes. Filters were washed under various conditions of stringency. Only the DNA from the TEB patient contained a fragment (3.8 Kb long) hybridizing with both probes (Fig.1). Such hybrids remained stable after washing at room temperature with 0.1 SSC, but disappeared when the filter was incubated at 55°C for 1 hour in

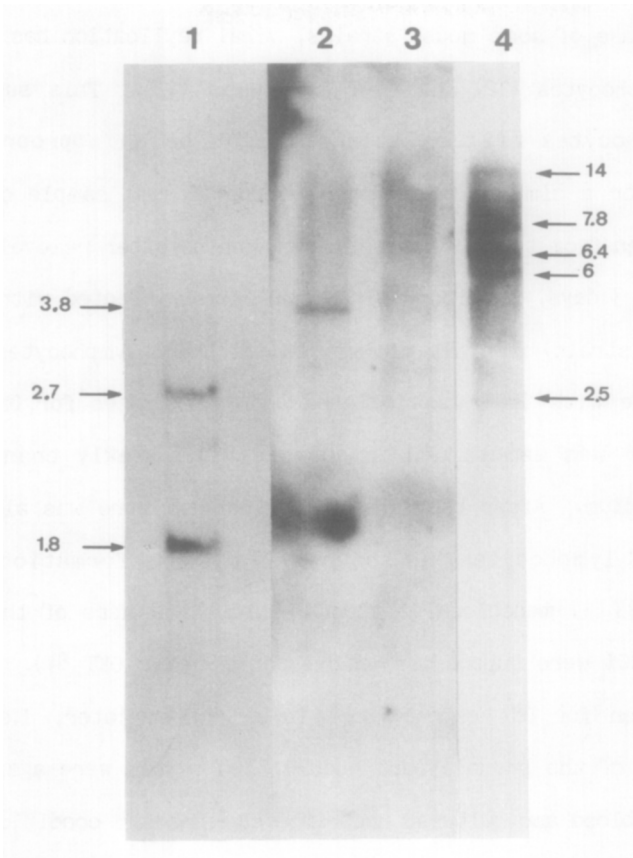

Figure 2 Analysis of leucocyte DNA from patient TEB.
20 µg of Sst 1 restricted DNA from lymphocytes cultured for 3 days were electrophoresed on 1% agarose gel (lane 1). 10 µg of Eco RI restricted DNA from cultured TEB lymphocytes (lane 2), from fresh TEB lymphocytes (lane 3) and from C3H mouse lines (HE strain) as positive control (lane 4) were electrophoresed on 1% agarose gel. The blotted filter was hybridized with MuMTV LTR-Gag under conditions described in figure 1. Exposure time was 3 days.

the same ionic conditions. When another aliquot of the same DNA was digested with Sst 1 enzyme, two bands strongly hybridized under the same conditions with the LTR-Gag DNA probe. Figure 2 shows a band of 3.8 Kb in lane 2, absent in lane 3, and two major bands (2.7 and 1.8 Kb) in lane 1. Restriction pattern of MuMTV DNA in C3H mouse cells is indicated by 5 arrows corresponding to two and a half integrated genomes (20). These Eco RI and Sst1 DNA fragments have never been described in any MuMTV proviral DNA and therefore are unlikely to be a contamination of human cells by the mouse virus.

In the case of some mouse strains, viral replication has been shown to occur in lymphocytes (12) and in T lymphomas (13). This suggested to us that T lymphocytes of the patients might be an appropriate site of replication for a similar human retrovirus. A 30ml sample of heparinized blood was taken from the TEB patient three months after removal of her first nodule. After 3 days, a culture without TCGF was infected with Epstein-Barr virus (B 95/8 strain) in order to immortalize the B lymphocytes. An aliquot of the culture with TCGF was pelleted and kept frozen for DNA extraction. The remainder was maintained in culture with weekly changes of TCGF-containing medium. After the first week, this culture was almost uniquely composed of T lymphocytes, as shown by E rosette formation and surface labeling by OKT 11 monoclonal antibody. About 40% were of the helper type (OKT 4+) and 40% were suppressor or cytotoxic cells (OKT 8+). Samples were regularly taken for DNA extraction. Three months later, i.e. six months after removal of the premalignant nodule, leukocytes were again taken from the patient's blood and cultured under the same medium conditions with TCGF. Samples from this second culture were similarly analyzed for DNA at different times.

Results of both experiments were identical : a distinct band of 3.8 Kb similar in size to that described in tumor DNA, was apparent after blot hybridization with the LTR-Gag probe of Eco RI restricted DNA extracted from the 3 day old leukocytes cultured in the presence of TCGF (Fig.3, lane 4). The band was absent when unrestricted DNA was used, a result which makes it unlikely that the human DNA was contaminated by the MuMTV plasmid (Fig.3, lane 7). The band could not be detected in the initial sample of fresh leucocytes isolated on a Ficoll gradient, nor in the long term cultures of T cells (lane 6) (from the 20th day after the culture was started). The lymphoblastoid line obtained by EBV transformation was also negative.

Similar cultures derived from three healthy female donor were negative at all stages (Fig.3, lane 3). Leucocytes from another patient with evolutive breast cancer (RID) were cultured under the same conditions. At

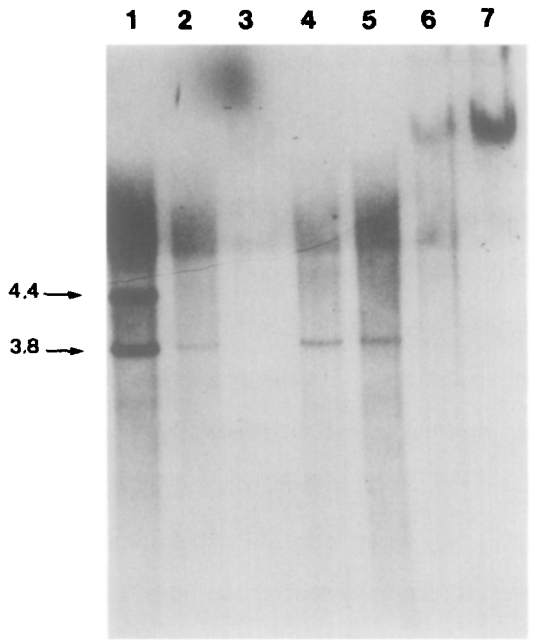

Figure 3 MuMTV related sequences in DNAs of cultured lymphocytes from two patients.

Lymphocytes from fresh blood collected from two patients (TEB, IRD) were cultured and DNA analyzed as described in Material and Methods. 40 µg of each DNA mixed together (lane 1) or 20 µg of TEB DNA were cut with Eco RI and and hybridized with the MuMTV Env DNA probe (5×10^6 cpm). Under the same conditions 20 µg of cultured lymphocyte from a normal person (ROM) were analyzed (lane 3).

6 months after the first lymphocyte culture, lymphocytes from patient TEB were cultured for 3 days, 11 days and 21 days and Eco RI digested. DNAs were analyzed (lanes 4, 5, 6). Unrestricted DNA from cultured lymphocyte was analyzed and hybridized under the same conditions (lane 7).

the 4th day of culture, the RID DNA contained an Eco RI fragment (4.4 Kb) which strongly hybridized with the LTR-Gag DNA probe (Fig.3, lane 1).

The results with TEB lymphocytes suggested that only a subset of the whole leucocyte population of the patient contains MuMTV-like sequences in their DNA. Presumably, this subpopulation was a minor fraction of T lymphocytes, which was initially expanded under TCGF stimulation, but then was rapidly eliminated by the overgrowth of cytotoxic T lymphocytes in the culture. On the other hand, the possibility that other types of blood cells (monocytes, blasts) present at the beginning contain the viral sequences cannot be excluded.

According to the first assumption, the T cell long term culture would include lymphocytes sensitized to MuMTV-like antigens. In order to confirm this, we performed a leucocyte migration inhibition (LMI) test in agarose (14) on TEB lymphocytes taken from the second 30 day old culture. The test was highly positive, using as antigen a purified preparation of MuMTV made from a virus producing C3H tumor.

Taken together, our results indicate that sequences close to the MuMTV genome can be found in humans. However, suggested by the washing at 55°C, the stability of the molecular hybrid formed between the human sequence and the MuMTV probes was not that of a perfect match.

Callahan et al. (9) have recently detected in the human genome endogenous sequences distantly related to MuMTV genes. These sequences hybridize to MuMTV sequences under conditions much less stringent that those used in the present work. Therefore, such weak signals were not apparent in our experiments. It is possible, however, that selective amplification of endogenous sequences may result in a hybridization signal strong enough to be detectable under our conditions. The relationship between Callahan's sequences and ours remains to be determined. It should be mentioned that amplification of MuMTV proviral sequences in murine lymphoid cells and mammary tumor cells has been shown to be associated with the appearance of T cell leukemia (15) and of mouse mammary carcinoma (16,17).

Another possibility is that the sequences detected in the patient's DNA belong to an as yet unrecognized human retrovirus, which would be horizontally transmitted. This virus might replicate in some blood cells and perhaps in other tissues not analyzed here. If this putative virus had the general structure of MuMTV retrovirus, we should have detected two fragments with the LTR probes. The detection of only one fragment could be explained by the deletion of a sequence containing the Eco RI site of a MuMTV-like virus.

The presence of whole viral particles could not be detected in the supernatant of early and late cultures, using the reverse transcriptase

assay of MuMTV and electron microscopy. However, the fact that T lymphocytes sensitized to MuMTV-like antigens emerged in long term culture suggests that some viral expression had occurred in the patient.

The presence of such sequences in only two cases out of 29 analyzed does not necessarily indicate that the virus is rarely associated with breast cancer. The virus may act by "a hit and run" mechanism, perhaps in promoting oncogene expression (18) in normal mammary cells, but other factors may be required for a further progression towards malignancy of the primarily transformed cells. The viral genome may be secondarily lost in these cells through chromosomal rearrangements, under the selective pressure of the host immune reaction raised against viral antigens.

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