PROTO-ONCOGENE EXPRESSION IN MURINE LEUKEMIA INDUCED BY MAZURENKO VIRUS

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The slow leukemogenic viruses, unlike viruses of acute leukemias, induce a disease with a long latent period, they do not induce morphological transformation of fibroblasts in vitro, and they do not contain oncogenes. The mechanism of their leukemogenic action has not yet been adequately studied, although it is known that long terminal repeats (LTR) contain sequences responsible for activation of some proto-oncogenes on integration of these viruses in cell DNA [5, 8]. For instance, specific intensification of activity of the c-myc gene was first demonstrated for B-cell lymphomas [7], and of the c-erb gene for erythroblastoses of hens [6]. Having suggested that this mechanism, which has been called insertion mutagenesis, occurs also in murine leukemias, we decided to study expression of a number of oncogenes in leukemia induced by Mazurenko virus (MMLV). This virus which belongs to the group of slow or true leukemia viruses, was isolated by N. N. Mazurenko in 1957 from CC57Br mice with leukemias arising after injection of vaccinia virus [1]. The unusual activating action of the latter is evidently connected with vaccinia virus growth factor (VGF), which is structurally and functionally similar to transforming (TGFα) and epidermal (EGF) growth factors [15]. MMLV is a B-tropic, ecotropic virus free from the MCF component, it does not induce erythroleukemias and does not grow well in culture in vitro; it proliferates best in cells infected with vaccinia virus [3]. From 2 to 6 months after receiving an injection of MMLV, more than 90% of mice develop leukemia, which follows a course either of a generalized lymphoma or a thymoma. In rats, MMLV also induces thymomas more than 30% of cases [1].

It was therefore decided to study activity of intracellular proto-oncogenes in mice with leukemias induced by MMLV, and to pay particular attention to the comparison of two forms of leukemia.

EXPERIMENTAL METHOD

MMLV was subjected to passage through CC57Br mice, by injecting a 25% cell-free viral suspension from the organs of leukemic animals subcutaneously into newborn mice. Numbers of T and B lymphocytes in the mice were determined by the fluorescent antibodies method. Lymphocytes were isolated in a Ficoll–Verografin density gradient. Surface immunoglobulins of lymphoid cells were determined by the direct immunofluorescence test, using fluorescein isothiocyanate (FITC)-labeled rabbit antibodies against mouse IgG, exhausted beforehand on normal mouse thymocytes. T-associated receptors were determined by the indirect immunofluorescence test, using monoclonal a/Thy 1,2 antiserum (generously provided by B. D. Brondz) and FITC-labeled polyclonal antiserum to mouse IgG. To determine oncogene expression, total nRNA was isolated from individual organs of newly killed mice, by lysis of homogenized tissues in guanidine–isothiocyanate buffer followed by centrifugation through a CsCl cushion [4]. The RNA was denatured in 1 M glyoxal, fractionated by electrophoresis in 1% agar gel, and transferred to nitrocellulose [14]. Quality control of the preparations was based on the ratio of 28S and 18S RNAs, stained with methylene blue in a parallel gel. RNA immobilized on the filter was hybridized in 50% formamide with 10% dextran sulfate at 42°C for 40 h with ^{32}P-DNA probes with specific activity of $2 \cdot 10^8$ cpm/μg toward oncogenes: myc (pKH_{47}), Ha-ras (pEJ-ras), Ki-ras (pBR322 clone Hihi 3), fos (pBR 322, pfos 1), myb (pVmyb) erb (pV erbB), B-lym (pHuB-lym1), abl (pAB_1 sub 9) [2]. The filters were then washed with

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TABLE 1. Number of T and B Lymphocytes in Lymphoid Tissue of Mice with Mazurenko Leukemia

Clinical form of Mazurenko leukemia	Latent period, months	No. of observations	Mean number of fluorescent cells		T lymphocytes	B lymphocytes
			T	B	%	
Thymomas	3—4	3	111,5±4,5	39±1	55,2	18,9
Gen. lympnatic leukemia	4,5—5	3	93,5±2,5	79,5±3,5	47,3	43,25
Early leukemia	1,5—2	1	96	76	48	38
Control (intact mouse)	—	3	109±7	99,9±3,05	48,5	49,2

TABLE 2. Proto-Oncogene Expression in Hematopoietic Organs of Mice with MMLV-Induced Leukemias

Mouse No.	Clinical form of disease	Tissue	Expression of oncogenes							
			myc	Ha-ras	abl	myb	Ki-ras	fos	erb	B-lym
137	Thymoma	LN, thymus	++	+	—	—	—	—	—	—
			+	+	—	—	—	—	—	—
141	Thymoma (early leukemia)	LN, spleen, thymus	++	++	—	—	—	—	—	—
			+	+	—	—	—	—	—	n.d.
			+	+	—	—	—	—	—	n.d.
142	The same	LN, thymus, liver	++	++	—	—	—	—	—	—
			+	+	—	—	—	—	—	—
			+	+	—	n.d.	—	—	—	—
147	Thymoma	LN, thymus, spleen, liver	++	++	—	—	—	—	—	n.d.
			++	++	—	—	—	—	—	n.d.
			+	+	—	—	—	—	—	—
			+	+	—	—	—	—	—	n.d.
148	»	LN, thymus, liver	+	++	—	—	—	—	—	n.d.
			+	+	—	—	—	—	—	n.d.
			+	+	—	—	—	—	—	n.d.
150	»	LN, thymus	+	+	—	—	—	—	—	n.d.
			+	+	—	—	—	—	—	n.d.
136	Generalized leukemia	LN, liver	—	+	—	n.d.	—	n.d.	—	—
			—	+	—	n.d.	—	n.d.	—	—
132	The same	LN + thymus, spleen	—	+	—	n.d.	—	n.d.	—	—
			—	+	—	n.d.	—	n.d.	—	—
144	» »	LN, spleen	—	+	—	—	—	—	—	n.d.
			—	+	—	—	—	—	—	n.d.
149	» »	LN + thymus	—	+	—	—	n.d.	—	—	n.d.
154	Generalized leukemia	LN + thymus	—	+	—	—	—	—	—	n.d.

Legend. ++) Strong expression, +) moderate expression, −) expression absent, n.d.) expression not determined.

2 × citrate–salt buffer (SSC) with 1% sodium dodecylsulfate (SDS) and in 0.1 × CSB with 0.1% SDS at 50°C, and exposed with Kodak S1 film at −70°C with an intensifying screen. Since each nitrocellulose filter was hybridized 4 times with probes for different oncogenes, the hybridized ^{32}P-DNA was washed in a 65% solution of formamide in 2 × CSB at 65°C for 1 h.

EXPERIMENTAL RESULTS

By now the MMLV has gone through 73 consecutive passages in CC57Br mice. Thymomas developed under these circumstances in about 30% of cases, and generalized leukemias in approximately 65% of cases. Early leukemias, whose clinical picture is marked by enlargement of the thymus, very slight enlargement of the lymph nodes (LN), and absence of splenomegaly, were developed by 3-5% of the infected animals; the animals began to die with severe toxic manifestations 35-50 days after infection.

To characterize the leukemic process, T- and B-cell populations were analyzed (Table 1). This showed marked predominance of T over B lymphocytes in thymomas, whereas the ratio between them in the case of the generalized lymphoma was almost indistinguishable from normal. In cases of early development of leukemias the difference in the ratio of T- and B-cells was very small, with some tendency for T-cells to predominate. In thymomas, a T-cell leukemia thus developed.

The results of hybridization of 25 RNA preparations from different hematopoietic organs of 11 mice with probes with 8 oncogenes are given in Table 2. Increased expression of c-myc-

specific RNA measuring 2.3 and 1.8 kilobase pairs (kbp) was found in the organs of mice with thymomas, but not those with generalized lymphomas. Expression of c-myc RNA likewise was not found in a control RNA preparation from the spleens of ten healthy mice. On hybridization with a probe for the Ha-ras oncogene expression of Ha-ras-specific RNA measuring 1.4 kpb was found in both forms of the disease. The highest expression of Ha-ras- and myc-specific RNA occurred in the thymus and LN, and less high expression occurred in the spleen and liver of the sick animals. On hybridization of RNA with probes for myb, abl, fos, erb, Ki-ras, and B-lym oncogenes, in no case was increased expression of specific RNA found, although on hybridization with a probe for the abl oncogene, diffuse hybridization bands were found in thymomas.

Thus of eight oncogenes, Ha-ras is expressed in both forms of leukemia but c-myc only in the thymic form. The increase in expression of c-myc RNA found in thymomas occurred in all the hematopoietic organs tested, and its highest level was observed in the thymus and LN, which correlates with the degree of propagation and accumulation of the virus in them [1]. Besides the 2.3 kbp band characteristic of c-myc RNA, an RNA measuring 1.8 kbp also was found. This shortened c-myc RNA has been found in muscle plasmacytomas, where chromosomal translocation splits the c-myc gene within the first exon [13], and also in murine erythroleukemia [11], caused by Friend virus. Increased expression of c-myc-specific RNA in thymomas induced by MMLV and a change in size of the transcript indicate that this gene is evidently involved in the process of MMLV-induced leukemogenesis, probably, due to insertion of the virus near to or even inside the c-myc gene.

MMLV receptors are evidently located on the surface of both types of cells, but specific enhancement of c-myc RNA takes place only on infection of T lymphocytes with the virus, leading to the development of a thymoma. In generalized lymphoma the virus is perhaps integrated into other regions of the genome.

These data are confirmed by reports of activation of the c-myc gene in leukemias induced by other murine viruses [9, 12]; in particular, Moloney murine leukemia virus integrates close to the c-myc gene in 45% of T-cell lymphomas, and this is accompanied by a 30-fold increase in expression of this gene [12]. Meanwhile the study of the mechanism of this process has shown that primary integration of the virus may occur in different sites, and early leukemias are polyclonal, whereas the thymomas which develop are monoclonal and are accompanied by activation of the c-myc gene [10].

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