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Studies of Proto-oncogene Expression in the Chronic and Blastic Phases of Chronic Myelogenous Leukemia

Harvey D. Preisler, Rajni Agarwal, Hiroshi Sato, Pradeep K. Singh, Ziqui Wang and Avery A. Sandberg

Chronic and blastic phase chronic myelogenous leukaemia cells have been studied by northern and Southern blot analysis. DNA from matched chronic and blastic phase cells obtained from the same patient demonstrated that the rearrangement site within the breakpoint cluster region did not change at the time of blastic crisis. A search for a mutation in a controlling region of the first exon of c-*myc* also failed to demonstrate any new abnormality at the time of blastic crisis. While some differences in the transcript levels for several genes (c-*myc*, *p53*, histone H_3, *MRS*) were detected, these differences could be ascribed to differences in the proportions of immature cells during the chronic and blastic phases. The data suggested that the c-*myc* transcripts in blastic phase cells and in immature chronic phase cells differ in that the latter contain some c-*myc* transcripts that are not polyadenylated. Differences in c-*myc* transcript half-life could contribute to the differences in the behaviour of chronic phase and blastic phase immature cells.
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INTRODUCTION

THE BENIGN chronic phase of chronic myeologenous leukaemia (CML) is characterised by a loosening of the regulation of cell proliferation and by the presence of the *bcr/abl* fusion gene [1, 2]. The malignant acute blastic phase, which eventually occurs in virtually every patient, is characterised by both disordered cell proliferation and by the failure of cells to differentiate beyond a primitive level. Little is known about the changes in gene structure and expression that may accompany or even precede blastic transformation. There is one report that a translocation in the 3′ end of the *bcr* gene is more common in the blastic phase than in the chronic phase of CML, suggesting that a change in the *bcr–abl* rearrangement site may be responsible for blastic transformation [3].

We have compared the chronic and blastic phases of CML at the molecular level. The possibility of blastic crisis resulting from a change in the site of *bcr* rearrangement [3] or from a mutation in a putative regulatory region in the 1st exon of the c-*myc* proto-oncogene [4] was evaluated by Southern blot hybridisation after restriction enzyme digestion. In addition, northern blotting was used to analyse the level of expression of several photo-oncogenes in chronic phase and blastic phase cells. The genes selected for study were those we believe to play a role in cell proliferation and myeloid differentiation (X. Zin, X. Z. Lau, Z. Wang, H. Preisler).

Correspondence to H. D. Preisler, Barrett Center for Cancer Prevention, Research and Treatment, 234 Goodman Ave, Cincinnati, OH 45267-0501, U.S.A.
H. D. Preisler is also at, and R. Agarwal and Z. Wang are at the University of Cincinnati Medical Center, Cincinnati, Ohio, U.S.A.; H. Sato is at the Kanto-Teishin Hospital, Shinagawa-Ku, Tokyo, Japan; P. K. Singh is at the University Hospitals of Cleveland, Ohio; and A. A. Sandberg is at the Cancer Center of Southwest Biomedical Research Institute, Scottsdale, Arizona, U.S.A.

MATERIALS AND METHODS

Cells

Peripheral blood or bone marrow aspirates were anti-coagulated with 6% sodium citrate. Red blood cells were removed by density centrifugation over "Ficoll–Hypaque" (1.077). The low density cells were used. Cells were further purified to compare RNA transcripts in immature chronic phase cells with those in blastic crisis cells. The low density cells underwent E rosetting, the monocyte/macrophages were removed by adherence, the remaining cells were centrifuged over Ficoll–Hypaque 1.063 and RNA was extracted from the light density and high density cells. Over 70% of the cells in the light density subpopulation were immature [5].

Specimens obtained before 1986 had been frozen in liquid nitrogen until study. Informed consent was obtained from each patient.

Preparation of RNA and northern blotting

For specimens obtained after 1986, the cells were dissolved directly in guanidium isothiocyanate (GITC). For specimens which had been frozen in liquid nitrogen, the frozen material was broken into small pieces and dissolved by grinding with GITC in a mortar and pestle.

After dissolution in GITC the RNA was pelleted by centrifugation over 5.8 ml cesium chloride. The RNA was dissolved twice in distilled water and reprecipitated [7,8]. To prepare poly A+ and poly A− RNA, RNA samples were passed over an oligo-dT-cellulose column [9]. The RNA which did not bind to the column as well as that which bound to the column were recovered for study.

5 μg RNA or the poly A+ RNA obtained from 50 μg of whole cell RNA were denatured with glyoxal and electrophoresed through a 1.2% agarose gel. The RNA was transferred onto a nylon mesh ("Zetabind", Cuno Inc.) and cross-linked by

exposure to ultraviolet light. The blots were hybridized [10] and autoradiographs prepared. [5–7].

We thank the following investigators for providing the probes used in these studies: *myc*, J. Battey [11]; *myb*, P. Rothberg [12]; histone H_3, G. Stein [13], triose phosphate isomerase (*TPI*), L. Maquat [14]; myeloid related sequence (*MRS*), W. Mars [15]); *fes*, R. Gallo [16]; and *abl*, S. Collins [17].

Analysis within breakpoint cluster region

DNA isolation. DNA was isolated from peripheral blood cells and/or from bone marrow aspirates with a technique modified from Wyman and White [18]. Blood samples were centrifuged at 400 g for 5 min to settle the red cells, and the buffy coat was removed to a sterile tube and resuspended in 1–5 ml icecold lysis buffer (10 mmol/l Tris–HCL, pH 7.5, 100 mmol/l NaCl, and 1 mmol/l EDTA). Marrow aspirates were centrifuged to pellet cells and the cells resuspended in lysis buffer. An equal volume of lysis buffer containing 2% Na N-lauroylsarcosine and 200 μg/ml proteinase K was added, mixed and the lysate incubated at 4°C overnight. The lysate was twice extracted with phenol and once with chloroform and the DNA recovered from the aqueous phase by ethanol precipitation. The DNA pellet was resuspended in 10 mmol/l Tris-HCL, pH 8.0, 1 mmol/l EDTA (TE) at a final concentration of 1 mg/ml.

Agarose gel electrophoresis of DNA and Southern blotting. 10 μg DNA was digested with *Bgl*II, *Bam*HI, *Hin*dIII, and *Eco*RI or *Xba*I in conditions recommended by the manufacturer (Bethesda Research Labs). Control DNA from a cytogenetically normal lymphoblastoid cell-line was digested in the same manner. The digested DNA was resolved on a 0.7% agarose gel, which was then soaked in 0.5 μg/ml ethidium bromide and photographed. Southern transfers were prepared [19]. The gel was "nicked" in ultraviolet light for 30 s and the blot was set up with a sponge reservoir filled with 0.5 mol/l NaOH and 1.5 mmol/l NaCl. The DNA was transferred to a nylon membrane filter ("Biotrace RP", Gelman).

Hybridization. After transfer, the filters were baked at 80°C in a vacuum oven for 2 h. Filters were washed at 60°C in 0.1 × sodium saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) for 30 min and then placed in sealable plastic bags with prehybridization fluid (50% formamide, 5 × SSC, 0.2% polyvinyl pyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin [BSA], 50 mmmol/l $NaPO_4$, pH 6.6, 500 μg/ml denatured salmon testis DNA and 2.5% dextran sulphate). Prehybridization was done overnight at 42°C, and then the prehybridization fluid was replaced with hybridization fluid (50% formamide, 5 × SSC, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, 0.02% BSA, 20 mmol/l $NaPO_4$, pH 6.6, 100 μg/ml denatured salmon testis DNA and 10% dextran sulphate). Denatured probe (2×10^6 counts per min/ml hybridization fluid) was added to the bag and the hybridization was done at 42°C overnight. Filters were removed form the bag, washed twice in 2 × SSC 0.1% SDS at room temperature and then at 65°C for 30 min. The filters were placed in a plastic wrap and used to expose Kodak "X-Omat AR" X-ray film at −70°C with intensifying screens (DuPont–Cronex Lighting Plus).

Probes. The 3′ probe (pSBRI-1) was a 11 kb *Hin*dIII/*Eco*R1 subclone (iro pGem-1, Promega Biotec) isolated from a lambda phage clone (SBRI-1) of the *bcr* region. The 5′ was a 2 kb *Bgl*II/*Hin*dIII fragment inserted into a pUC18 vector and was provided by C. R. Bartram, University of Ulm, France. To prepare the probes, plasmid DNA was isolated with the alkaline-SDS method followed by centrifugation to equilibrium on CsCl gradients. The insert fragments were labelled directly with ^{32}P by the oligonucleotide primer synthesis method [20].

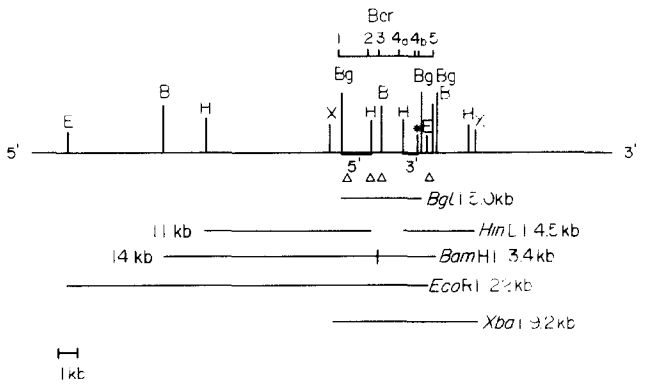

Fig. 1. Restriction map of *bcr* site illustrating regions 1–5 used to define *bcr* rearrangement during chronic and blastic phase of CML.

Detection of mutation in PvuII *site in first exon of c*-myc

Matched peripheral blood chronic and blastic phase samples were obtained from four patients with CML. High molecular weight DNA was extracted from these samples [21]. Samples of DNA were digested with *Pvu*II restriction enzyme. (Boehringer–Mannheim, 10 U/ml). 5 μg DNA was digested for every sample and 2 IU/μg of restriction enzyme was used for digestion at 37°C overnight in a medium salt buffer. Test digests were made to evaluate the degree of digestion. Lambda DNA digested with *Hin*dIII and *Pvu*II was used as a DNA size marker. HL-60 cell DNA and lymphocyte DNA digested with *Pvu*II were used as controls.

The samples were fractionated for size by electrophoresis on 0.8% agarose gels at 30 V for 16 h. The gel was denatured (1.5 mol/l NaCl and 0.5 mol/l NaOH for 45 min) and neutralised (Tris–HCl 1 mol/l and NaCl 1.5 mol/l, pH 8.0 for 45 min). Transfer was done on "Zetabind" transfer membranes with 20 × SSC at pH 7.0.

Hybridization was done with c-*myc* exon 1 DNA labelled with ^{32}P CTP, $2–4 \times 10^8$ disintegrations per min mixed with 50% formamide, at 70°C overnight. The blots were washed with buffers containing decreasing concentrations of SDS (0.5–0.2%) for 2–4 h at 70°C.

RESULTS

DNA studies

Matched DNA specimens were available from 10 chronic and blastic phase patients. Figure 1 maps the *bcr* sites that were characterized. During the chronic phase of the disease the *bcr* rearrangement in 6 of the 10 specimens was in the 3′ end of the region: two rearrangements in region 4a, two in 4b, one in 4b or in the 3′ end of 4a and in one there was a deletion in 4a. Of the remaining specimens, there were three rearrangements in region 3 and one in region 2. At the time of blastic crisis no new *bcr* rearrangements were seen, except for that in the only patient whose cells had deletion in region 4a during the chronic phase of CML. In this case a break in region 3 with the deletion in 4a characterised the blastic phase cells.

Matched chronic and blastic phase DNA specimens from 4 patients were studied for a possible mutation in the *Pvu*II site

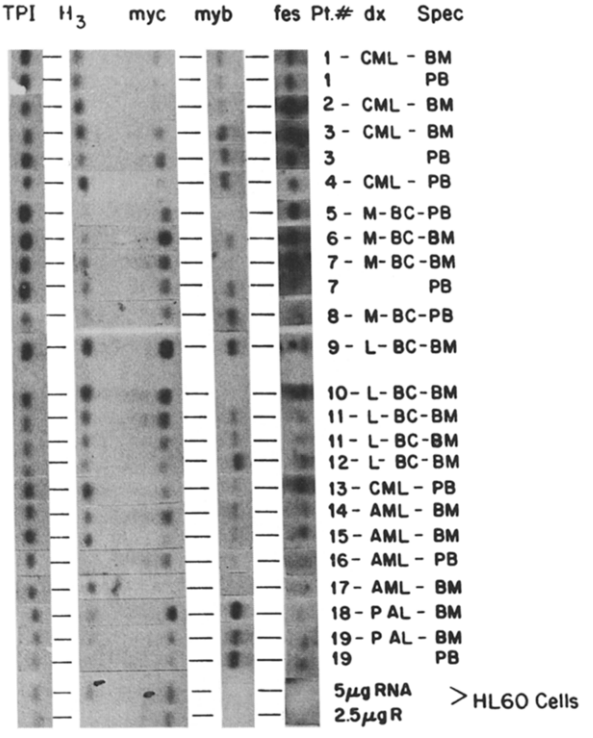

Fig. 2. Northern blot analysis of whole cell RNA from patients with CML or acute myelogenous leukaemia (AML). M-BC = myeloid and L-BC = lymphoid blastic crisis. P AL = Philadelphia chromosome positive acute leukaemia. BM = bone marrow and PB = peripheral blood. Pt = patient number, dx = diagnosis and spec = specimen.

in the first exon of c-*myc*. No mutation was detected in any of the specimens.

Gene expression with whole cell RNA

Figure 2 presents a comparison of gene expression in RNA obtained from 5 chronic phase CML patients (1–4 and 13), 4 patients in myeloid blastic crisis (5–8), 4 patients in lymphoid blastic crisis (9–13), 4 conventional AML patients (14–17) and 2 Ph′ positive acute laukaemia patients (18 and 19). Similar amounts of RNA were present in each lane and there was no evidence of RNA degradation. c-*myc* transcript levels were low in chronic phase cell populations and high in blastic phase cells. In contrast, histone H_3 RNA levels were high in chronic phase and low in blastic phase cells. c-*myb* was detectable in 10 of the 13 CML specimens but there was no consistent pattern of transcript level in either the chronic or blastic phase specimens nor was there any relation between c-*myc* and c-*myb* RNA levels.

The transcript levels of c-*fes* and *TPI* were higher in chronic phase CML cells and in myeloid blastic crisis cells than in lymphoid blastic crisis cells. RNA from 4 AML cell populations were also studied with the level of expression of c-*myc*, c-*myb*, c-*fes*, *TPI* and histone H_3 varying from very high to very low levels.

Figure 3 illustrates RNA transcript levels in 10 matched chronic and blastic phase specimens. In these studies RNA levels for a myeloid-related sequence (*mrs*), for *p53*, c-*myc* and *TPI*, were measured. In 6 matched specimens *mrs* transcript levels were higher in chronic phase than in blastic phase cell populations, equivalent in 2 and higher in the blast cell populations in 2. *mrs* transcripts were not detectable in the single AML specimen studied (patient 5). *mrs* transcripts were detectable in both lymphoid and myeloid blastic crisis cells.

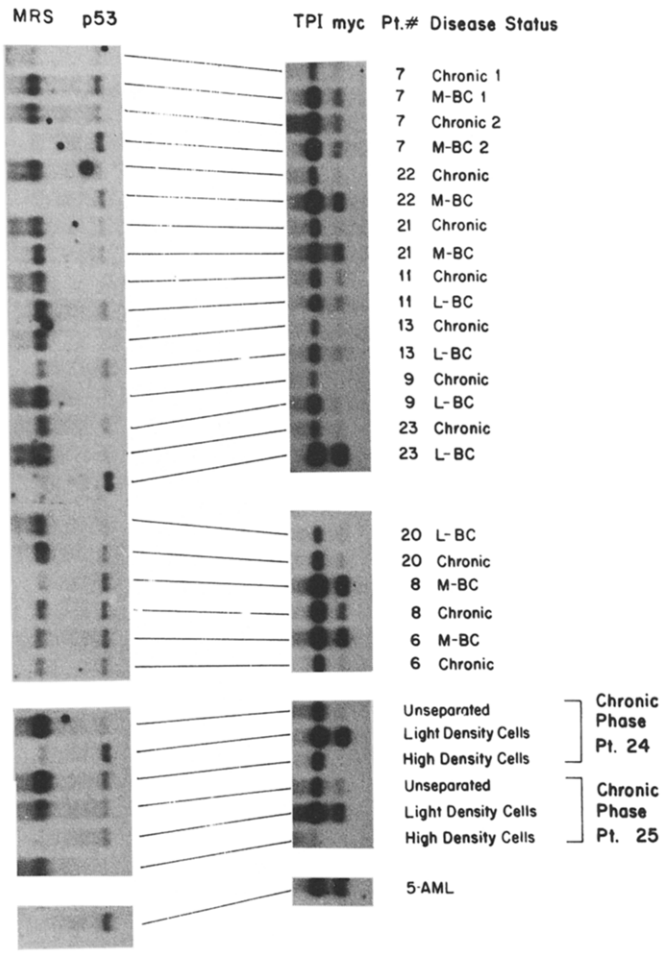

Fig. 3. Matched northern blots of whole cell RNA from patients during chronic phase CML and at blastic crisis. RNA from chronic phase low density cells (patients 24 and 25) was also analysed since the proportion of immature cells in this subpopulation is similar to that in blastic phase specimens. Patient 7 was studied four times: during two chronic phases and two blastic phases.

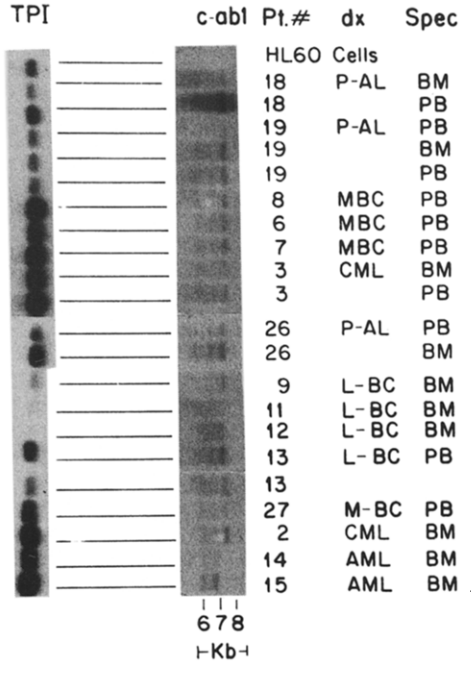

Fig. 4. Northern blots of poly A+ RNA obtained from CML and AML cells.

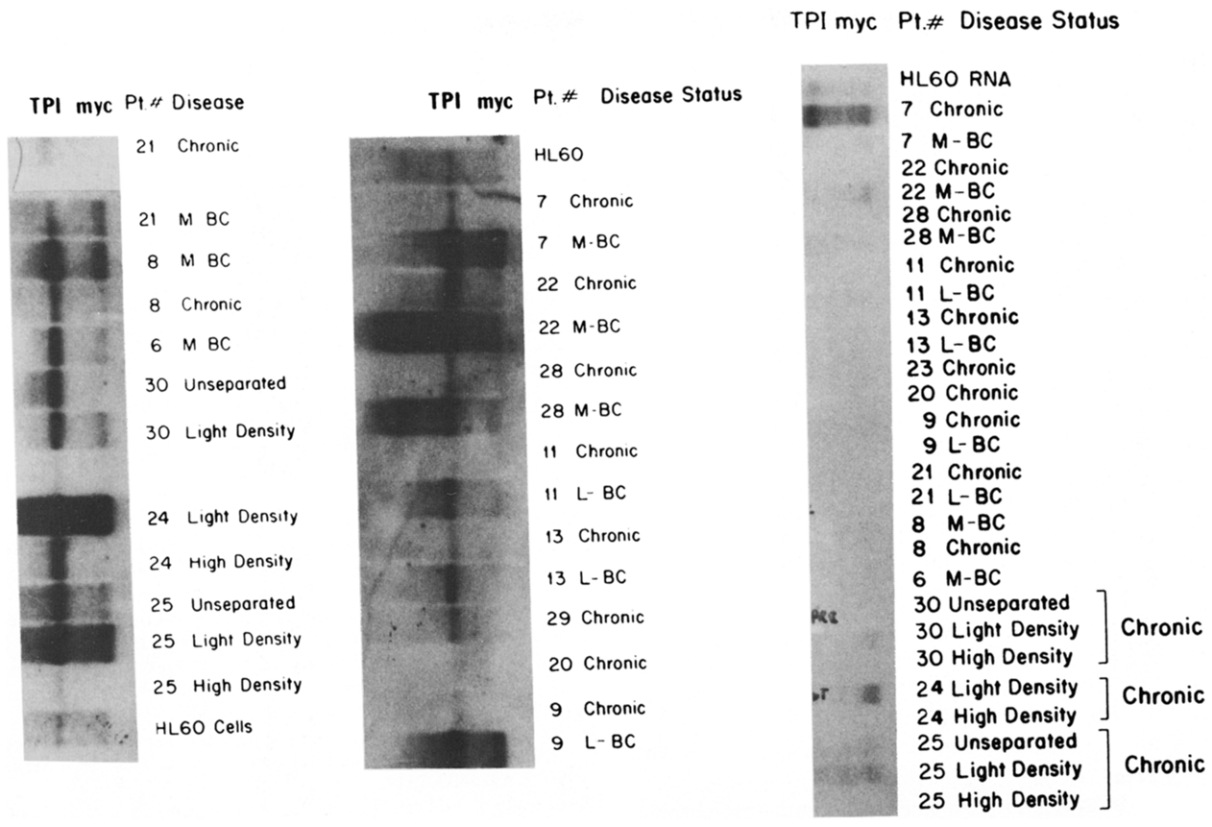

Fig. 5. Northern blots of poly A+ and poly A− c-*myc* RNAs from chronic and blastic phase CML specimens. Left = poly A+ RNA. Right = RNA that did not bind to oligo-dT column was recovered and northern blotted. Blot was probed for both *TPI* and c-*myc*, with *TPI* being used as indicator of presence of poly A+ RNA that passed through column.

In 8 of the 10 matched chronic and blastic phase specimens, both c-*myc* and *p*53 levels were higher in the blastic phase cell population than in the chronic phase cell population. These observations confirmed the studies illustrated in Fig. 2 and demonstrate the parallel relation between c-*myc* and *p*53 expression.

Subpopulations of cells

To assess whether the differences in the level of c-*myc*, *p*53 and *mrs* expressions between chronic and blastic phase CML cells were a reflection of a change in the disease state *per se* or merely of differences in the proportion of immature cells in the different stages of CML, RNA was obtained from chronic phase cell populations that had been enriched for immature cells (light density population) or depleted of immature cells (high density cells). In each study the immature chronic phase cells contained higher c-*myc* and p53 RNA levels and lower *mrs* transcript levels than either the original cell population containing the immature cells or the cell subpopulation depleted of immature cells (Fig. 3).

Studies of bcr/abl

Given the relation between the presence of the *bcr/abl* fusion gene and CML, we also studied *abl* expression in chronic and blastic phase CML cells. Poly A+ RNA was used in these studies. Figure 4 shows a northern blot of poly A+ RNA studied with c-*abl* and *TPI* probes. In every CML RNA, whether obtained from the chronic or blastic phase cells, 8, 7 and 6 kb, RNA transcripts were present which hybridised with c-*abl* and were the same in all specimens. In the two Philadelphia chromosome (Ph) positive acute leukaemic specimens, 8 kb RNAs complementary to c-*abl* were present and in one of the specimens a higher molecular weight species was also detected. In the two non-Ph' positive AML specimens only 6 and 7 kb *abl* complementary RNAs were present.

Poly A+ and poly A− transcript levels

Figure 5 (left) shows a northern blot of poly A+ RNA hybridised with probes for TPI and c-*myc*. As in the whole cell RNA studies (Fig. 3), blastic phase cell populations and immature chronic phase cell populations contained more c-*myc* RNA than unseparated chronic phase cell populations. Figure 5 (right) shows a northern blot of the RNA that did not adhere to the oligo-dT column used to prepare the RNA studied in Fig. 5 (left): c-*myc* transcripts but not TPI transcripts were detectable in the poly A− RNA obtained from all 3 immature (low density) chronic phase cell populations studied. In contrast TPI transcripts were not detectable in the same specimens (patients 24, 25 and 30). Among the 9 matched chronic and blastic phase poly A− RNA specimens studied, c-*myc* transcripts were detectable only in the RNA extracted from two of the blastic phase specimens (patients 7 and 22). Since TPI transcripts were also detectable in the RNA of patient 7, it is likely that in this case the oligo-dT column failed to remove all the A+ RNA.

DISCUSSION

The molecular events at the DNA level responsible for the transformation of CML into an acute leukaemia are unknown. There are at least three possibilities: an alteration in the *bcr/abl* rearrangement site [3], the abnormal expression of a proto-

oncogene coding for a nucleoprotein such as c-*myc* [22] (perhaps because of a mutation in a controlling region of the c-*myc* gene [4]) and a mutation in the N-*ras* gene [23]. Our studies failed to confirm the first two possibilities. The *bcr/abl* rearrangement is not altered when CML enters the acute phase nor is the *Pvu*II site mutated. Further evidence for non-involvement of the *bcr/abl* fusion gene in blastic crisis was suggested by our failure to demonstrate a detectable change in either the quantity or size of the *bcr/abl* transcript at the time of blastic crisis.

Additionally, we have shown that while N-*ras* mutations are common in CML, they are usually detectable in less than one half of the cells that are present and may appear and disappear during the course of the disease [24]. Hence N-*ras* mutations do not appear to be responsible for blastic transformation. Therefore, to date, our studies at the DNA level have not detected any changes that might be responsible for blastic transformation.

Our studies at the total cell RNA level failed to detect differences between chronic and blastic phase cells. While c-*myc* and *p*53 RNA levels were higher in blastic phase cells and histone H_3 and *mrs* RNA levels lower, these differences are attributable to differences in the level of maturity of the cells present during these two phases [25]. Hence if these genes are involved in the genesis of blastic crisis [22], the abnormalities would have to involve abnormal regulation of expression or coding mutations rather than simply an elevation in the level of expression.

The presence of poly A− c-*myc* RNA in immature chronic phase cells but not in most blastic phase specimens appears to be the only clear difference between chronic and blastic phase cells that we have detected. We have reported the presence of poly A− c-*myc* RNA in HL60 cells and that this mRNA has a prolonged half-life compared with poly A+ c-*myc* RNA [26]. It is possible, therefore, that the stability of c-*myc* RNA is altered at the time of blastic crisis. Whether this change is merely a reflection of the blastic state or whether the change *per se* contributes to the evolution of the blastic phase is unknown.

Our other studies are providing insight into the possible pathogenesis of both the chronic and blastic phases of CML. Cells from most patients with CML contain (IL1β) transcripts [27] which is reminiscent of a report that the transformation of rat embryo fibroblasts by the Abelson murine leukaemia virus is associated with the release of a transforming growth factor [28]. Perhaps the overproduction of myeloid cells during the chronic phase of the disease results from a similar effect of the *bcr/abl* fusion gene in stimulating ILIβ production.

As regards the blastic phase of CML, studies of chronic and blastic phase cells *in vitro* have demonstrated that the differentiation of chronic phase immature cells to granulocytes is associated with a fall in c-*myc* and *p*53 transcript levels, while the failure of blastic crisis cells to differentiate under the same conditions is associated with the persistence of high levels of expression of c-*myc* and *p*53 expression [29]. Perhaps the inability to downregulate the expression of these genes prevents differentiation. Studies demonstrating that the failure to downregulate *myc* expression can prevent the differentiation of leukaemia cells [30] are compatible with this possibility.

Thus we could not demonstrate quantitative differences in gene expression between chronic and blastic phase CML cells which, at least in part, are a reflection of the differences in the level of maturity of the cell populations being studied. Additionally studies of poly A+ and poly A− c-*myc* RNA demonstrated differences between immature blastic and chronic phase cells.

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Intratumoral Injection of OK432 and Lymphokine-activated Killer Activity in Peripheral Blood of Patients with Hepatocellular Carcinoma

Mutsunori Shirai, Seishirou Watanabe and Mikio Nishioka

Lymphokine-activated killer (LAK) activity of peripheral blood mononuclear cells (PBMC) from 33 patients with hepatocellular carcinoma was significantly decreased compared with that of healthy volunteers. There was less LAK activity in PBMC from patients with larger tumours (5 cm or more in diameter) than in patients with smaller tumours (under 5 cm in diameter). In 8 out of 20 patients with larger tumours there was none or little LAK activity. Flow cytometry revealed that the percentage of Leu11b+ cells in PBMC was lower in patients than in normal volunteers, and was lowest in patients with larger tumours. 10 patients with hepatocellular carcinoma were treated with intratumoral injection of OK432. LAK activity was enhanced after treatment in 7 cases, and the percentage of Leu11b+ cells was increased. Enhancement of LAK activity in response to OK432 was more significant in patients with smaller rather than larger tumours. Of the 7 high LAK responders, 4 showed 50–100% tumour regression at 6–9 weeks after injection.
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INTRODUCTION

THE INCUBATION of lymphocytes with interleukin-2 (IL-2) generates cytotoxic cells that can lyse natural killer (NK) resistant tumour cells and a wide variety of other tumour cells without major histocompatibility complex restriction. This cytotoxicity is known as lymphokine-activated killing (LAK) [1–3], and occurs in the absence of any apparent antigenic stimulation. LAK is mediated mainly by IL-2 activated NK cells.

OK432 is a heat and penicillin treated lyophilized powder of the Su strain of *Streptococcus pyogenes* A3 and is a strong immunopotentiator and a useful immunotherapeutic agent for cancer. OK432 induces cytotoxic T lymphocytes against tumour cells [4], activates NK cells [5, 6] and reduces suppressor macrophages against NK cells in cancer patients [7]. Uchida and Micksche [8] demonstrated that autologous tumour killing activity can be induced in peripheral blood mononuclear cells (PBMC) by incubation with OK432. Grimm *et al.* [9] reported that LAK-like cells can be induced by treating PBMC with lower concentrations of OK432 and demonstrated that higher concentrations of OK432 significantly inhibited generation of LAK effectors to the NK resistant Daudi cell line, probably because of penicillin G potassium contained in OK432.

Our aim was to investigate LAK activity in patients with hepatocellular carcinoma and the *in vivo* effect of OK432 on LAK generation and tumour size.

Correspondence to M. Shirai.
The authors are at the Third Department of Internal Medicine, Kagawa Medical School, 1750–1, Ikenobe, Miki-cho, Kita-gun, Kagawa, 761–07, Japan.

SUBJECTS AND METHODS

Subjects

For studies of LAK activity in PMBC there were 33 patients with hepatocellular carcinoma (HCC) (27 M/6F, mean age 59.6 years). HCC was confirmed histopathologically and no patient had metastases. 32 of the 33 had liver cirrhosis. 2 were HBsAG and HBeAG positive. The others were not alcoholics and did not have hepatitis B but did have non-A, non-B hepatitis (NANBH). The liver cirrhosis was functionally well compensated. No patient in our study had received previous anticancer therapy or drugs known to cause immunological changes, or any such treatment during this study except for patients 1, 6 and 9 who had had transhepatic arterial embolization 2 months before OK432 injection. Tumour volume and diameter of the ideal tumour sphere were calculated by modelling the tumour portion of computed tomography (CT) scans ("IBAS", Zeiss). 18 patients with liver cirrhosis were also investigated (15M/3F, 58.2 years). These patients were not alcoholics but did have