Burlingame, CA) containing 4,6-diamino-2-phenylindoledihydrochloride (DAPI) (1 µg/ml).

Colocalization of *BCR* and *ABL* signals was analyzed in at least 10 to 15 nuclei per colony by fluorescence microscopy (Leica, Dialux). Only colonies with at least 90% evaluable nuclei were counted.

Results

Colonies formed by freshly isolated and long-term cultured PBMNC were studied by FISH. Cell donors were 18 patients with CML in chronic phase at initial diagnosis or under treatment with IFN-α and/or HU.

Clonogenic cells from nine patients at diagnosis of the disease showed *BCR/ABL* positivity in most colonies analyzed after LTC (median 100% [range 94–100%]). The colony assay before LTC revealed a high percentage of *BCR/ABL*-positive colonies in all patients (median 100% [range 86–100%]) (Table 1).

CFC of patient No. 3 were also tested after four and eight weeks of HU and repeatedly after IFN. Cells from those patients treated with HU (Nos. 3, 8–11) showed high numbers of *BCR/ABL*-positive colonies before and after LTC (median 100% [range 40–100] vs 90% [range 64–100]). In contrast, only one (No. 4) of the IFN-treated CML patients (Nos. 1–7) had a markedly increased number of *BCR/ABL*-positive colonies after LTC of PBMNC. A reduction of *BCR/ABL*-positive colonies after LTC was seen in four cases (Nos. 2, 3, 5, 6). There were only two *BCR/ABL*-positive colonies among 88 tested in the PBMNC of patient No. 1 when tested before LTC. After LTC we could not detect more than two positive colonies among 47 colonies tested. Over all, IFN-treated patients still showed high numbers of *BCR/ABL*-positive colonies as well before and after LTC (median 72% [range 2–100] vs 48% [range 4–100]) (Table 2).

In summary, even in IFN-treated patients high numbers of *BCR/ABL*-positive early progenitors could be

Table 1. BCR/ABL-positive colonies before and after LTC of PBMNC from CML patients at initial diagnosis

Pat. No.	No. of *BCR/ABL*-positive colonies over total number tested on day 0 (%)		No. of *BCR/ABL*-positive colonies over total number tested on day 35 of LTC (%)	
3	18/19	(95)	5/5	(100)
4	30/30	(100)	9/9	(100)
12	14/14	(100)	33/33	(100)
13	6/7	(86)	7/7	(100)
14	8/8	(100)	16/17	(94)
15	25/27	(93)	4/4	(100)
16	9/9	(100)	19/19	(100)
17	22/22	(100)	45/45	(100)
18	16/18	(89)	8/8	(100)

Table 2. BCR/ABL-positive colonies before and after LTC of PBMNC from IFN- or HU-treated CML patients.

Pat. No.	Treatment	Time in months after Dx[1)]	No. of *BCR/ABL*-positive colonies over total number tested on day 0 (%)		No. of *BCR/ABL*-positive colonies over total number tested on day 35 of LTC (%)	
1	IFN	18–26	2/88	(2)	2/47	(4)
2	IFN	2–7	13/34	(38)	7/34	(2)
3	IFN	3–7	33/46	(72)	12/25	(48)
4	IFN	25–30	5/66	(8)	20/43	(47)
5	IFN + HU	19–29	28/32	(88)	39/51	(76)
6	IFN	31	11/11	(100)	27/37	(73)
7	IFN	110	13/13	(100)	20/20	(100)
8	HU	1–4	8/20	(40)	26/37	(70)
9	HU	13–14	29/49	(59)	28/31	(90)
3	HU	1–2	10/10	(100)	58/62	(64)
10	HU	20	33/33	(100)	30/30	(100)
11	HU	46	12/12	(100)	23/23	(100)

1) Except for patients Nos. 6, 7, 10, 11, the tests were made at different time intervals after diagnosis (Dx).

detected. Obviously, LTC is not sufficient to achieve complete elimination of a leukemic clone *in vitro.*

Discussion

In our experiments, we show that IFN-treated patients still have *BCR/ABL*-positive progenitor cells in the peripheral blood, which are in late as well as in early stages of differentiation. This is concluded from the observation that colonies analyzed by FISH before and after LTC still showed *BCR/ABL* positivity.

FISH has been proven as a reliable method for the detection of *BCR/ABL* positivity in patients with CML. As it can be applied to interphase cells (interphase cytogenetics), especially analysis of patient probes after treatment with IFN is simplified when sufficient numbers of metaphases cannot be achieved. A good correlation with conventional cytogenetic data has been shown previously (Cox et al., 1988; Amiel et al., 1994; Cox et al., 1996). FISH might be more sensitive than conventional cytogenetic studies (García-Isidoro, 1997) and it allows detection of the *BCR/ABL* translocation at the single-cell level as well as correlations to cellular morphology. As a colony is assumed to be derived from a single hematopoietic cell, all nuclei are expected to show identical characteristics, so the evaluation of 10 to 15 nuclei is sufficient for determination of the Ph status of the whole colony.

Nevertheless, as colony formation by CML PBMNC before LTC is due to progenitors at a later stage of differentiation, the numbers of *BCR/ABL*-positive and negative CFC cannot be directly compared to those after LTC.

The reappearance in the blood of *BCR/ABL*-negative progenitor cells, particularly in IFN-treated patients, is a well-established phenomenon. It is also evident that there are *BCR/ABL*-positive CFC in the blood of patients showing complete cytogenetic remission. This was shown by RT-PCR analyses of CFU-GM (Reiter et al., 1998) colonies and by FISH experiments of our group (Heissig et al., 1997; Schultheis et al., 1997).

In IFN-treated patients showing a low proportion of *BCR/ABL*-positive colonies before LTC, a decreased number or even elimination of CML cells after LTC had been expected but, obviously, this was not the case. CFC after LTC even showed a higher percentage of *BCR/ABL* positivity than before LTC, which contradicts expected results.

None of the patients tested showed complete absence of leukemic progenitor cells after LTC in repeated experiments, even those with a low percentage of *BCR/ABL*-positive progenitors before LTC.

Therefore, we conclude that under LTC conditions the malignant cell clone cannot be completely eradicated from CML blood. Treatment with IFN-α may lead to a suppression of the *BCR/ABL*-positive cell clone for a certain time, but it still remains detectable even in patients with a good therapeutic response.

Acknowledgements

We gratefully acknowledge Susanne Brendel for excellent technical assistance, and Dr. Thomas Cremer and Dr. Nora Heisterkamp for providing the YAC and cosmid clones, respectively.

References

Amiel, A., Yarkoni, S., Slavin, S., Or, R., Lorberboum-Galski, H., Fejgin, M., Nagler, A. (1994) Detection of minimal residual disease state in chronic myelogenous leukemia patients using fluorescence in situ hybridization. *Cancer Genet. Cytogenet.* **76,** 59-64.

Arnoldus, E., Wiegant, J., Noordermeer, I., Wessels, J., Bevertock, G., Grosveld, G., van der Ploeg, M., Raap, A. (1990) Detection of the Philadelphia chromosome in interphase nuclei (with 2 color plates). *Cytogenet. Cell. Genet.* **54,** 108-111.

Barnett, M., Eaves, C., Phillips, G., Gascoyne, R., Hogge, D., Horsman, D., Humphries, R., Klingemann, H., Lansdorp, P., Nantel, S. (1994) Autografting with cultured marrow in chronic myeloid leukemia: results of a pilot study. *Blood* **84,** 724-732.

Bentz, M., Cabot, G., Moos, M., Speicher, M., Ganser, A., Lichter, P., Döhner, H. (1994) Detection of chimeric BCR-ABL genes on bone marrow samples and blood smears in chronic myeloid and acute lymphoblastic leukemia by in situ hybridization. *Blood* **83,** 1922-1928.

Boyum, A. (1976) Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* **5,** 9-15.

Brito-Babapulle, F., Bowcock, S., Marcus, R., Apperley, J., Th'ng, K., Dowding, C., Rassool, F., Guo, A., Catovsky, D., Galton, D., McCarthy, D., Goldman, J. (1989) Autografting for patients with chronic myeloid leukaemia in chronic phase: peripheral blood stem cells may have a finite capacity for maintaining haemopoiesis. *Br. J. Haematol.* **73,** 76-81.

Carella, A., Frassoni, F., Negrin, R. (1995) Autografting in chronic myelogenous leukemia: new questions. *Leukemia* **9,** 365-367.

Coulombel, L., Kalousek, D., Eaves, C., Gupta, C., Eaves, A. (1983) Long-term marrow culture reveals chromosomally normal hematopoietic progenitor cells in patients with Philadelphia chromosome-positive chronic myelogenous leukemia. *N. Engl. J. Med.* **308,** 1493-1498.

Cox, M. C., Maffei, L., Buffolino, S., Del Poeta, G., Venditti, A., Cantonetti, M., Aronica, G., Aquilina, P., Masi, M., Amadori, S. (1988) A comparative analysis of FISH, RT-PCR, and cytogenetics for the diagnosis of bcr-abl-positive leukemias. *Am. J. Clin. Pathol.* **109,** 24-31.

Cox Froncillo, M., Maffei, L., Cantonetti, M., Del Poeta, G., Lentini, R., Bruno, A., Masi, M., Tribalto, M., Amadori, S. (1996) FISH analysis for CML monitoring? *Ann. Hematol.* **73,** 113.

Daley, G., Goldman, J. (1993) Autologous transplant for CML revisited. *Exp. Hematol.* **21,** 734-737.

Dexter, T. (1989) Regulation of hemopoietic cell growth and development: experimental and clinical studies. *Leukemia* **3,** 469-474.

García-Isidoro, M., Tabernero, M., García, J., Najera, M., Hernandez, J., Wiegant, J., Raap, A., San Miguel, J., Orfao, A. (1997) Detection of the Mbcr/abl translocation in chronic