Characteristic of Two Mouse *bcr-abl*-Transformed Cell Lines: I. General Properties of the Cells

(CML / Bcr-Abl / mouse cell lines)

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Abstract. In an effort to develop an experimental system suitable for immunological studies in which Bcr-Abl-positive cells are to be used as antigens, we examined the properties of two mouse (Balb/c) established cell lines that express the Bcr-Abl protein and are oncogenic for syngeneic animals. Under standard conditions the two cell lines, viz. Ba-p210 (B210) and 12B1, expressed comparable amounts of the Bcr-Abl protein. However, they differed in a number of characteristics. From the morphological point of view, B210 cells were the more homogeneous, being mainly represented by leukaemic blastic cells with a large number of AgNORs as markers indicating a high proliferative activity. 12B1 cells were more polymorphic and giant cells were detected within their populations. Many 12B1 cells exhibited nuclear segmentation and "band-like" structures. Markers of proliferation were less frequent in 12B1 and the tendency for aging was more pronounced in these cells. The 12B1 cells were slightly more sensitive to imatinib mesylate than B210 cells. In B210 cells, the expression of MHC class I was down-regulated, which was not the case with 12B1 cells. Both cell lines induced leukaemia-like disease in mice after intravenous application but, as compared with B210, 12B1 cells were about 100 times more oncogenic and the disease they induced was more aggressive. Moreover, 12B1, but not B210, induced tumours after subcutaneous or intraperitoneal inoculation.

Chronic myeloid leukaemia (CML) is associated with a chromosomal abnormality known as the Philadelphia chromosome (Ph+), which is a consequence of a reciprocal translocation between chromosomes 9 and 22. In it, a portion of the *abl* protooncogene is fused with a portion of the *bcr (breakpoint cluster region)* protooncogene, this resulting in the formation of a chimeric *bcr-abl* gene located on chromosome 22. The tyrosine-kinase activity (TKA) of its product is markedly increased as compared with the product of the *abl* protooncogene (Konopka and Witte, 1985). It is generally accepted that the formation of the chimeric gene and the activity of its product are the key events in the pathogenesis of CML. The nature of the *abl* and *bcr* genes and their products together with the biological consequences of their fusion have been described in several recent reviews (Holyoake, 2001; Melo and Deninger, 2004).

Depending on the site of the break in the *bcr* gene, the product of the fusion gene exists in three forms designated $p210^{bcr\text{-}abl}$, $p190^{bcr\text{-}abl}$ and $p230^{bcr\text{-}abl}$. Of these, the most frequent (in more than 90% of CML cases) is the fusion between the portion of the *bcr* gene ending with either the b2 or b3 exon and the portion of *abl* starting with the a2 exon. These connections have been denoted b2a2 and b3a2, respectively. In either instance the product of the chimeric gene is represented by the $p210^{bcr\text{-}abl}$ protein.

CML is a challenging target for immunotherapy. As a consequence of the fusion of the *bcr* and *abl* genes, the chimeric $p210^{bcr\text{-}abl}$ (both in its b2a2 and b3a2 form) contains sequences which are unique. The fusion results in a codon split that leads to the formation of a new amino acid: glutamic acid in the case of the b2a2 connection and lysine in the case of b3a2. In the recent past many attempts have been made to utilize the presence of these sequences, which represent true tumour antigens, for the immunotherapy of CML (Ten Bosch et al.,1995; Bocchia et al., 1996; Yotnda et al., 1998; Osman et al, 1999; Leeksma et al., 2000; Norbury et al., 2000; Pinilla-Ibarz et al., 2000; Cathcart et al., 2004). Although these studies have shown that it is possible to elicit specific immune responses against CML cells, no really significant clinical improvements have been

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Abbreviations: AgNORs – silver-stained nucleolus organizer regions, ATCC – American Tissue Culture Collection, CML – chronic myelogenous leukaemia; ECL – enhanced chemiluminescence, IM – inanitib mesylate, SDS – sodium dodecylsuphate, SDS–PAGE – SDS polyacrylamide gel electrophoresis.

reported as yet. Similarly as some other investigators, we strongly feel that efforts aiming at the development of more potent therapeutic vaccines should be based on the recent progress made in the fields of vaccinology, tumour immunonology and gene therapy. The verification of preparations based on the new biotechnologies has to be done in laboratory animals. In the work we started some time ago we have been using two murine (Balb/c) cell lines, viz. B210 and 12B1, which were isolated after transduction with retroviral vectors carrying the *bcr-abl* (b3a2) fusion gene. Both cell lines express $p210^{bcr\text{-}abl}$ and are oncogenic for syngeneic animals. The present and the next paper we submit (Jelínek et al. 2005, manuscript in preparation) describe the results obtained in investigating the properties of the two cell lines and the nature of the disease induced by them in syngeneic animals. We considered it appropriate to get the basic data on the disease we plan to prevent and treat.

Material and Methods

Cell lines

Two Balb/c mouse cells lines transformed by the *bcr-abl* fusion gene (b3a2) and expressing $p210^{bcr\text{-}abl}$ were used. Ba-p210 (B210) cells were kindly provided by G.Q. Daley (Whitehead Institute of Biomedical Research, Cambridge, MA). They had been derived from interleukin-3 (IL-3)-dependent BaF/3 cells (Palacios and Steinman, 1985). Their transformation by a retrovirus carrying the *bcr-abl* gene made them IL-3-independent (Daley and Baltimore, 1988). 12B1 cells (McLauglin et al., 1987) were obtained by courtesy of E. Katsanis (University of Arizona, Tuscon, AZ). They had been derived by the transformation of primary bone-marrow cells with a retrovirus-derived vector carrying the *bcr-abl* fusion gene. In some experiments K562 cells, derived from a CML patient, were used as a Bcr-Abl-positive control cell line and HL60 cells, isolated from the peripheral blood of a patient with acute promyelitic leukaemia (Ph-), were used as a Bcr-Abl-negative control. Both cell lines were obtained from ATCC. WEHI-3 cells, a Bcr-Abl-negative cell line derived from the peripheral blood of a Balb/c mouse with myelomonocytic leukaemia, were kindly provided by P. Dráber (Institute of Molecular Genetics, Prague). The cell lines were expanded in RPMI-1640 medium (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 2 mmol/l glutamine and antibiotics. In the case of 12B1 cells the medium was enriched with 1 mmol/l sodium pyruvate and 50 μmol/l 2-mercaptoethanol.

Cytological examination

Cells were examined in cytospins, being spun in a Shandon II cytocentrifuge (Shandon Southern Products, UK) at 6000 rpm for 10 min. The nuclear shape and chromatin structure were visualized by a simple cytochemical procedure for the demonstration of DNA (Smetana et al., 1967). Nucleoli were visualized with acidified methylene blue for the demonstration of RNA and nucleolar organizer regions by a silver reaction (silver-stained nucleolus organizer regions (AgNORs)) in unfixed smears or in cytospins under defined conditions as described previously (Ochs, 1998; Smetana et al., 1999). The cytoplasm of cells was easily seen in specimens stained for RNA or for DNA, with phase contrast microscopy being used. Micrographs were taken with a Camedia digital photocamera V-4040 ZOOM (Olympus, Tokyo, Japan) fixed on a Jenalumar microscope (Zeiss, Jena, Germany).

Sensitivity to imatinib mesylate (IM)

The IM used was a generous gift from Novartis (Basel, Switzerland). It was dissolved in distilled water (1 mg/ml), sterilized by filtration through a Millipore filter, distributed into vials and kept at –20°C until use. Third passages of freshly rethawed B210 and 12B1 cells, cultivated in parallel under standard conditions, were used. Counts of 5×10^3 cells/well were seeded in 100 μl culture medium in 96-well plates (TPP, Trasadingen, Switzerland). Then 100 μl volumes of IM solution diluted in twofold steps in complete medium were added. The final concentration of IM ranged from 0.01 to 5.0 μg/ml. In the first well the same amount of IM-free complete medium was added. Each sample was tested in four parallels. As a control, Bcr-Abl-negative WEHI-3 cells were employed. Cultures were incubated for 5 days and the proportions of surviving cells were determined by the Cell Titre AQ^{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) following the manufacturer's instructions. Background absorbance levels as determined in wells containing no cells were subtracted. The 50% inhibition concentration (IC_{50}) was calculated by non-linear regression analysis of the sigmoid curves fitted with variable slopes to normalized data, using the Graphpad PRISM 3.0 Software (San Diego, CA).

Flow cytometry

Third passages of freshly rethawed B210 and 12B1cells, cultivated in parallel under standard conditions, were used**.** Cells were washed twice with PBS. For the detection of MHC class I molecules, cells were incubated with FITC-conjugated anti-mouse $H\text{-}2K^dD^d$ monoclonal antibody (Cedarlane, Hornby, Ontario, Canada) or with isotype control antibody (Sigma) at 4°C for 30 min. In the second experiment the cells were incubated with phytoerythrin (PE)-conjugated anti-mouse $H\text{-}2L^d$ monoclonal antibody (Cedarlane) and with isotype control antibody (BD Biosciences, Pharmingen, San Diego, CA).

Animals and tumour induction

In all experiments, 5 to 8 week-old Balb/c mice were used. They were obtained from Charles Rivers, Sulzfeld, Germany. The mice were inoculated intravenously (i.v.) or subcutaneously (s.c.) with varying amounts of cells (see the Results section) in 0.2 ml PBS. Prior to inoculation, the cells were washed three times with PBS. The size of subcutaneously induced tumours was expressed as the area index (AI) in mm^2, calculated by multiplying tumour length by the longest tumour diameter perpendicular to tumour length. All work in animals was done in accordance with the Guidelines for Animal Experimentation valid in the Czech Republic.

Western blotting

Cells were lysed in a lysis buffer composed of 4% SDS, 20% glycerol, 10% mercaptoethanol, 2 mM/l EDTA, 100 mM/l Tris-HCl (pH 8.0) and bromphenol blue and boiled for 3 minutes. Lysates of about 10^5 cells were separated by 7% SDS-PAGE electrophoresis. The proteins separated were electroblotted onto nitrocellulose membranes. Remaining binding sites were blocked in 10% skimmed milk for 2 h at room temperature. Filters were incubated with mouse monoclonal anti-c-abl antibody (Oncogene Research Products, Boston, MA, AB-3, 50 ng/ml). Incubation proceeded at room temperature under constant agitation for 2 h and then at 4°C overnight. Membranes were then washed with 0.1% Tween 20 in PBS for 5 × 5 min and treated with peroxidase-labelled secondary anti-mouse antibody (Amersham Biosciences, Little Chalfont, UK). The blots were again washed in 0.1% Tween 20 in PBS for 7 × 5 min. Immunocomplexes were visualized using the ECL plus system (Amersham Biosciences).

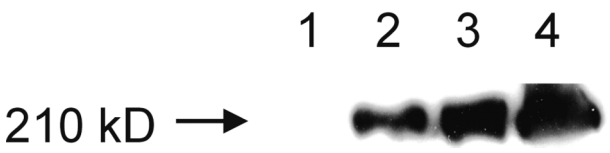

Fig. 1. Western blotting with lysates of different cells. Anti-c-abl monoclonal antibody (50 ng/ml) was used. Lane 1: HL60 (Bcr-Abl-negative control cells); lane 2: B210 cells; lane 3: 12B1 cells; lane 4: K562 cells. Each sample was a lysate from 2 × 105 cells.

Fig. 2. Western blotting with different doses of lysed Bcr-Abl-positive cells. Anti-abl monoclonal antibody (50 ng/ml) was used. B210 and 12B1 cells: 1, 2×10^5 cells; 2, 10^5 cells; 3, 5×10^4 cells; 4, 2.5×10^4 cells; 5, 1.25×10^4 cells; 6, 6×10^3 cells. K562 cells: 1, 10^5 cells; 2, 5×10^4 cells; 3, 2.5×10^4 cells; 4, 1.25×10^4 cells; 5, 6.125×10^3 cells; 6, 3×10^3 cells.

Results

Western blotting (WB)

The results of WB tests with lysates from 12B1, B210, K562 and HL60 cells are shown in Fig. 1. It may be seen that both of the mouse cell lines expressed $p210^{bcr\text{-}abl}$ similarly as K562 cells did. The results of a test aimed at determining the amount of $p210^{bcr\text{-}abl}$ produced by the different lines are shown in Fig. 2. For this experiment the third passages of freshly rethawed cells, grown in parallel under standard conditions, were used. As evident, B210 and 12B1 contained approximately the same amount of the Bcr-Abl protein, less than did the K562 cells tested in parallel.

Cytological characterization

Most of the B210 cells were mononuclear and resembled leukaemic blast cells, both lymhoblasts and myeloblasts. Their size was variable, ranging from 13 to 22 μm. The nuclei of these cells were large, with a fine chromatin structure (Fig. 3a), and mostly contained large nucleoli (Fig. 3b). The nuclei were characterized by a relatively uniform distribution of RNA and multiple small AgNORs corresponding to fibrillar centres (Fig. 3c). Such nucleoli are characteristic of proliferating cells (Smetana, 2002, 2003). Large, „giant" mononuclear, bi- or multinuclear cells (larger than 25 μm) (Fig. 3d) were very rare. Mitotic cells were also present (not shown).

In cytospins, 12B1 cells were widely polymorphous because of a large variability of nuclear shapes (Fig. 4). Numerous cells exhibited very unusual nuclear segmentation (Fig. 4b) and large cells with ''band-like" nuclei were also observed (Fig. 4a). The ''band-like" nuclei resembled such as occur during leukaemic granulocytic variation in chronic myeloid leukaemia. However, despite the variability in nuclear shape, the chromatin structure in the cells was fine, without any distinct chromatin condensation. On the other hand, some cells of small size (smaller than 15 μm) were characterized by round and almost pycnotic nuclei in

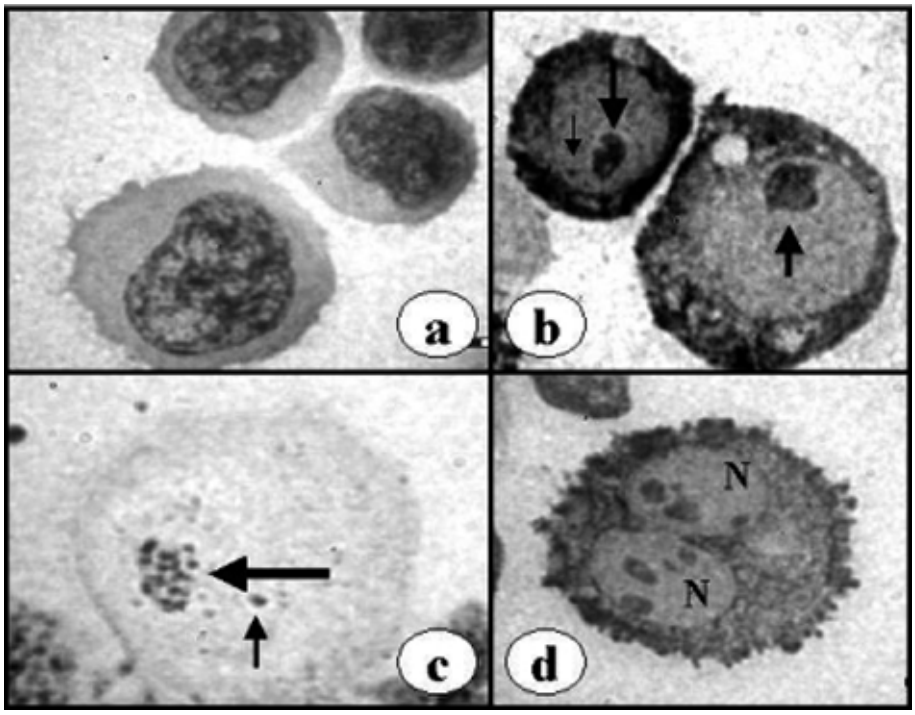

Fig. 3. B210 cells. (a) DNA staining and phase contrast microscopy. The cell size was highly variable. However, the chromatin structure was fine, similarly as in leukaemic lympho- or myeloblasts. (b) RNA staining. Nucleoli were usually large (large arrow), but micronucleoli representing satellite nucleoli (small arrow) could also be present in blastic cells. (c) Silver staining for AgNOR proteins. A large nucleolus containing multiple small AgNORs (large arrow) and a single AgNOR corresponding to the micronucleolus (small arrow). (d) RNA staining. Nuclei (A) in a large binuclear cell with multiple nucleoli. Magnification (approx.) 1300× (a), 2000× (b), 2400× (c), 1800× (d).

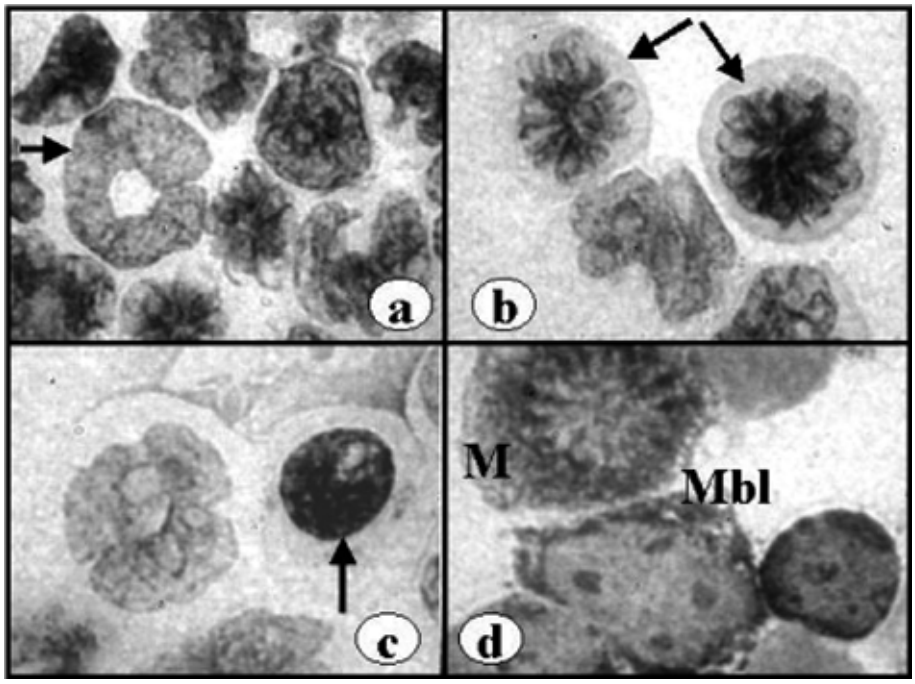

Fig. 4. 12B1 cells. The nuclear shape was highly variable (a-c). (a) Nuclei were rounded, sometimes band-like (arrow), with indentations of varying size (c) or (b) they were irregularly segmented (arrows), but the chromatin structure was fine, without distinct condensed chromocentres. However, in some cells resembling late erythroblasts (arrow) the nuclei were round, frequently pycnotic, and exhibited heavy chromatin condensation (c). In numerous cells the nuclei displayed very unusual nuclear segmentation around the central region (b). Figures a–c, DNA staining. In specimens stained for RNA (d), some cells resembling myeloblasts (Mbl) possessed several nucleoli. The chromosomes in mitotic cells (M) were unstained. Magnification (approx.) 1300× (a), 1700× (b), 1900× (c), 2000× (d).

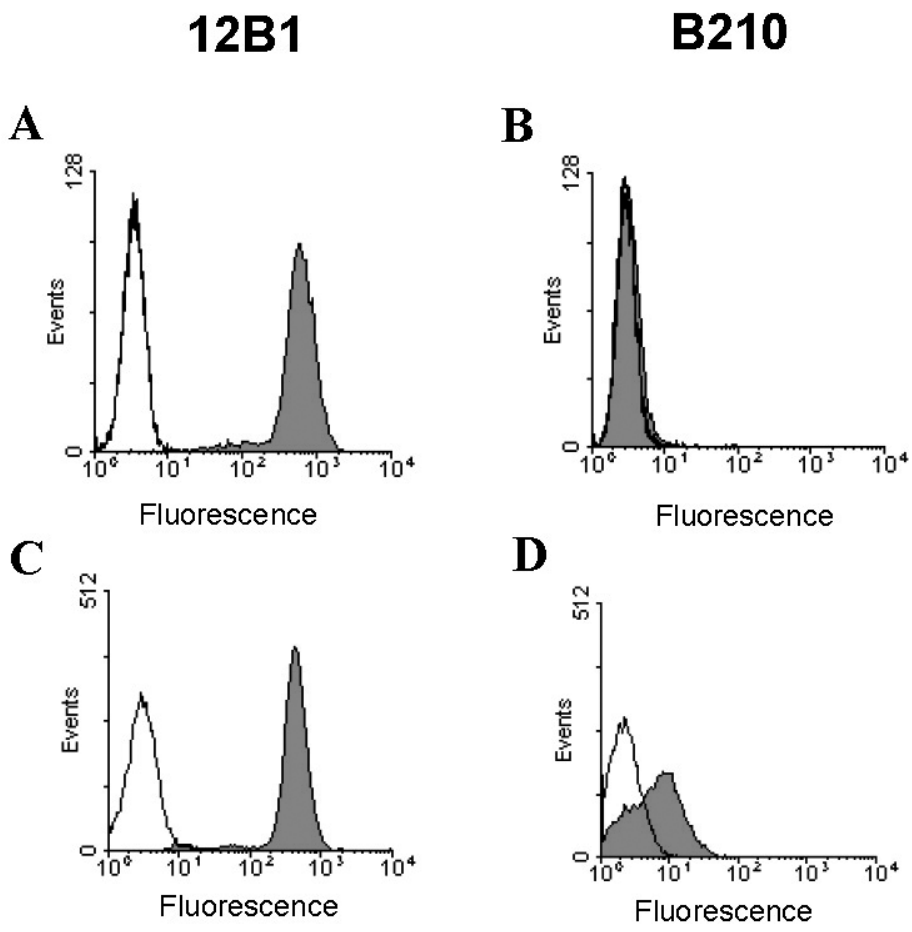

Fig. 5. MHC class I H-2Kd/H-2Dd (A, B) and H2-Ld (C, D) molecule expression on B210 and 12B1 cells. Empty histograms represent cells incubated with isotype control antibodies, black histograms represent cells incubated with specific antibodies.

which the chromatin structure was highly condensed. Such cells resembled late erythroblasts (Fig. 4c). It should also be noted that in cytospins a few cells with large nucleoli were similar to myeloblasts (Smetana, 1980). Rare mitotic cells were also observed in the specimens tested (Fig. 4d).

MHC class I expression

The results of flow cytometry are shown in Fig. 5. They indicate that MHC class I production was strongly downregulated in B210 as compared with 12B1 cells.

Sensitivity to imatinib mesylate

In repeated *in vitro* experiments, 12B1 cells were found slightly more sensitive to IM than B210 cells. The results of a representative experiment are presented in Fig. 6. WEHI-3 cells were not inhibited even in the presence of the highest concentration of IM used (results not shown).

Oncogenicity tests

Both cell lines induced a leukaemia-like disease in mice. After i.v. inoculation of B210 cells the animals remained in good health for about 16–17 days, while those inoculated with 12B1 cells for about 11–12 days. In subsequent days the animals developed symptoms of an acute disease, characterized by limited motility, bristling, hepatosplenomegaly, rapidly deteriorating malnutrition and apathy. Paralytic disease developed in nearly all animals inoculated with 12B1 cells, but only exceptionally in those injected with B210 cells. The animals died 3–5 days after the appearance of the first symptoms. After s.c. inoculation, only 12B1 cells formed rapidly growing solid tumours. These cells also induced diffuse intraabdominal tumours after intraperitoneal inoculation However, a few mice i.v. injected with B210 cells and surviving without any leukaemia-like disease for more than 2 months developed solid intraperitoneal tumours. From these tumours, cell lines resembling the original B210 cells were easily established. These cells were again capable of inducing a leukaemia-like disease and were positive for $p210^{bcr\text{-}abl}$ in WB (results not shown).

The oncogenic potential of the two cell lines was tested in three repeated experiments using different cell

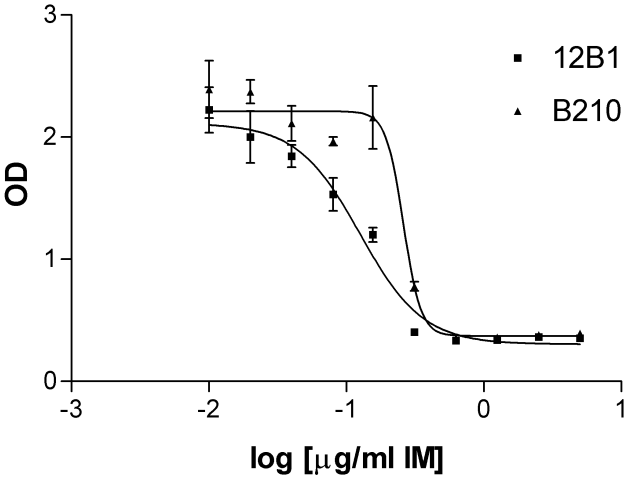

Fig. 6. In vitro sensitivity of B210 and 12B1 to imatinib mesylate. IC_{50} values calculated for the B210 and12B1 cells were 0.2601 and 0.1258, respectively.

Table 1. Oncogenicity of B210 and 12B1 cells in Balb/c mice

Cells	Route of inoculation[1)]	Cell dose inoculated											
		10^7	5×10^6	10^6	5×10^5	10^5	5×10^4	10^4	5×10^3	10^3	5×10^2	10^2	5×10^1
B210	i.v.		4/4[2)]	17/18	8/8	19/20	2/4	4/16	NT[3]	0/8			
			(100)	(94)	(100)	(95)	(50)	(25)					
	s.c.	0/3	0/3	0/3	0/3	0/3	0/3						
12B1	i.v							23/23	15/15	6/8	2/7	0/5	1/3
								(100)	(100)	(75)	(29)		(33)
	s.c.							9/9	6/6	6/9	0/3	0/6	
								(100)	(100)	(67)			

i.v. – intravenous, s.c. – subcutaneous
No. of mice with tumours/No. of mice inoculated. In parentheses – percentage
NT – not tested

doses. The results did not differ significantly from experiment to experiment and are summarized in Table 1. It is evident that the oncogenicity of the two cell lines differed markedly. 12B1 cells were approximately 100 times more oncogenic than B210 cells. Moreover, relatively low doses of 12B1 cells were capable of inducing solid tumours after s.c. inoculation.

Discussion

The recently increased interest in the immunotherapy of CML is paralleled by an increased interest in suitable animal models, in which specific immune responses to Bcr-Abl-positive cells can be studied much more easily than in human patients. Furthermore, vaccines resulting from the recent progress in medical biotechnologies, which may prove much more efficient than the peptide-based vaccines used in several clinical studies (see above), can be tested in mice without the restrictions necessarily imposed on the treatment of human patients.

We have recently initiated a study aimed at developing several types of genetic vaccines against *bcr-abl*-transformed cells in mice. At the first stage we plan to test their capability of inducing immunity against challenge with syngeneic Bcr-Abl-positive cells and at the second to use them therapeutically. For this purpose we decided to use two cell lines, viz. B210 and 12B1 cells, both transduced by retroviral vectors carrying the *bcr-abl* gene.

Before starting systematic work on the development of these vaccines we tried to define the basic characteristics of these cells and to characterize the disease they induced in syngeneic animals. As expected, this disease did not reproduce human CML either clinically or histologically, as will be shown in a subsequent paper (Jelínek et al., 2005, manuscript in preparation). Still, the presence of $p210^{bcr\text{-}abl}$ makes these cells a legitimate model system for studying specific immune responses to this protein. Quantitatively, the expression of the $p210^{bcr\text{-}abl}$ protein appeared to be comparable in both cell lines. The cell lines differed only slightly in their sensitivity to imatinib mesylate (IM), although some of the previous experiments with cells from high passage levels indicated an up to 10 times higher IM sensitivity in 12B1 cells (results not shown). This suggests that the degree of sensitivity of Bcr-Abl-expressing cells to IM may vary in the course of prolonged *in vitro* cultivation. Otherwise the two cell lines differed in a number of characteristics. These included differences in morphology and in the expression of MHC class I molecules, as well as, most importantly, a very marked difference in oncogenicity. The 12B1 cells were approximately 100 times more virulent than B210 cells and were capable of inducing tumours after subcutaneous and intraperitoneal inoculation. This is in line with earlier observations (He et al., 2001; Graner et al., 2003; Zeng et al., 2004). The high oncogenicity of 12B1 cells provides a number of advantages for the immunological studies we are planning. In addition, it is much easier to monitor the development of subcutaneous tumours than leukaemia-like disease. On the other hand, B210 cells provide us with an opportunity to study, in the present system, the influence of downregulation of MHC class I molecules, a phenomenon so frequently seen in cancer (Bubeník and Vonka, 2003; Algarra et al., 2004). Interestingly, it has been reported that both BaF3 cells and their *bcr-abl*-transformed derivatives do express MHC class I molecules (Deng and Daley, 2001). The reason for the discrepancy between these and our results is not clear. It might be a consequence of unknown selective pressures operative in the course of cultivating the cells *in vitro.* Furthermore, the capability of B210 cells to induce long-delayed solid tumours may provide a possibility of monitoring and analyzing processes not visible in the course of the acute leukaemia-like disease (to be published).

To summarize, the two mouse cell lines we chose for our immunological studies with $p210^{bcr\text{-}abl}$-positive cells differed in a number of important characteristics. The existence of this discrepancy may provide some

advantages in the ongoing immunological studies in mice.

Acknowledgement

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