DNA vaccination against *bcr-abl*-positive cells in mice

VINCENT LUCANSKY, EVA SOBOTKOVA, RUTH TACHEZY, MARTINA DUSKOVA and VLADIMIR VONKA

Department of Experimental Virology, Institute of Hematology and Blood Transfusion, Prague, Czech Republic

Received May 15, 2009; Accepted July 10, 2009

DOI: 10.3892/ijo_00000408

Abstract. A series of DNA vaccines based on the *bcr-abl* fusion gene were developed and tested in mice. Two mouse (BALB/c) *bcr-abl*-transformed cell lines, B210 and 12B1, which both expressed $p210^{bcr\text{-}abl}$ and were oncogenic for syngeneic animals but differed in some other respects, were used as a model system. In the first series of experiments, plasmids carrying either the complete *bcr-abl* fusion gene or a fragment thereof coding for a 25-amino acid-long junction zone (bcr-abl25aa) linked with genes coding for a variety of immunostimulatory factors were used as the DNA vaccines. A plasmid carrying the complete *bcr-abl* gene was capable of inducing protection against challenge with either B210 or 12B1 cells. However, the DNA vaccines based on the gene fragment coding for $p25aa^{bcr\text{-}abl}$ did not induce significant protection. To localize the immunizing epitopes on the $p210^{bcr\text{-}abl}$ protein, the whole fusion gene was split into nine overlapping fragments and these, individually or in various combinations, were used for immunization. Although none of the vaccines based on any single fragment provided potent protection, some combinations of these fragment-based vaccines were capable of eliciting protection comparable to that seen after immunization with the whole-gene vaccine. Surprisingly, a mixture of six fragment-vaccines was more immunogenic than the complete set of fragment DNA vaccines. To analyze this phenomenon, the three fragments missing from the hexavaccine were either individually or in various combinations mixed with the hexavaccine. The results obtained suggested that the product of the fragment coding for 197 amino acids forming the N-terminal of the BCR protein was involved in the decreased immunogenicity. However, further experiments are needed to clarify the point. Additional experiments revealed that all the important epitopes were located in the ABL portion of the $p210^{bcr\text{-}abl}$ protein. The livers, spleens and bone marrows of the successfully immunized animals were tested for the presence of *bcr-abl*-positive cells by RT-PCR. The results were negative, this suggesting that these animals were free of any residual disease.

Correspondence to: Dr Vladímir Vonka, Institute of Hematology and Blood Transfusion, U nemocnice 1, 128 20 Prague 2, Czech Republic
E-mail: vonka@uhkt.cz

Key words: CML, bcr-abl-transformed mouse cells, DNA vaccines

Introduction

Chronic myeloid leukaemia (CML) is a malignant disease of the haematological system and is characterized by clonal expansion of haemopoietic progenitor cells (1). Cytogenetically, it is characterized by the so-called Philadelphia chromosome (Ph+), which develops as a consequence of the reciprocal t(9;22)-(q34;q11) translocation. As a result, the fusion gene *bcr-abl*, consisting of only portions of the *bcr* and *abl* genes, is generated on chromosome 22 (1,2). Chromosomal breaks, conditioning the translocation, may occur at different sites of the *bcr* gene. In all instances the tyrosine-kinase activity of the fusion BCR-ABL protein is markedly increased over that of the product of the untruncated *abl* gene (2). Of the different junctions formed, the most common are those designated b3a2 and b2a2, which result in the formation of the $p210^{bcr\text{-}abl}$ fusion protein. They occur in more than 90% of CML cases. It is generally accepted that the deregulated tyrosine-kinase activity of the chimeric protein plays a key role in the cell transformation and in the maintenance of the transformed state.

During the recent past the therapy of CML has changed dramatically thanks to the introduction of inhibitors of bcr-abl-coded tyrosine-kinase activity. The first to be introduced and the one most widely used is imatinib mesylate (IM). It is relatively well-tolerated and is much more effective in inducing haematological and cytogenetic remissions than the drugs used previously. It prolongs the mean survival time considerably. Still, it cannot cure the disease. Two mechanisms are mainly responsible for this failure, viz. inability of the drug to kill tumour stem cells (3) and point mutations that make the tumour cell resistant to the drug (4,5). Novel tyrosine-kinase inhibitors have been developed, which can deal with the mutation problem (4,5); however, it is not expected that their use will result in the cure of CML. Thus, allogenic stem cell transplantation remains the only potentially curative approach. Unfortunately, it is limited by the rare availability of suitable donors and is followed by relatively high morbidity and mortality (2,6). These are the main reasons for a growing interest in immunotherapy of the disease. This approach is strongly supported by what seems to be substantiated hope that immunotherapy might result in cure of the disease.

Imunotherapy may be either non-specific or specific. Both alternatives have already been tested (7-18). The specific approach has usually been directed against the zone generated

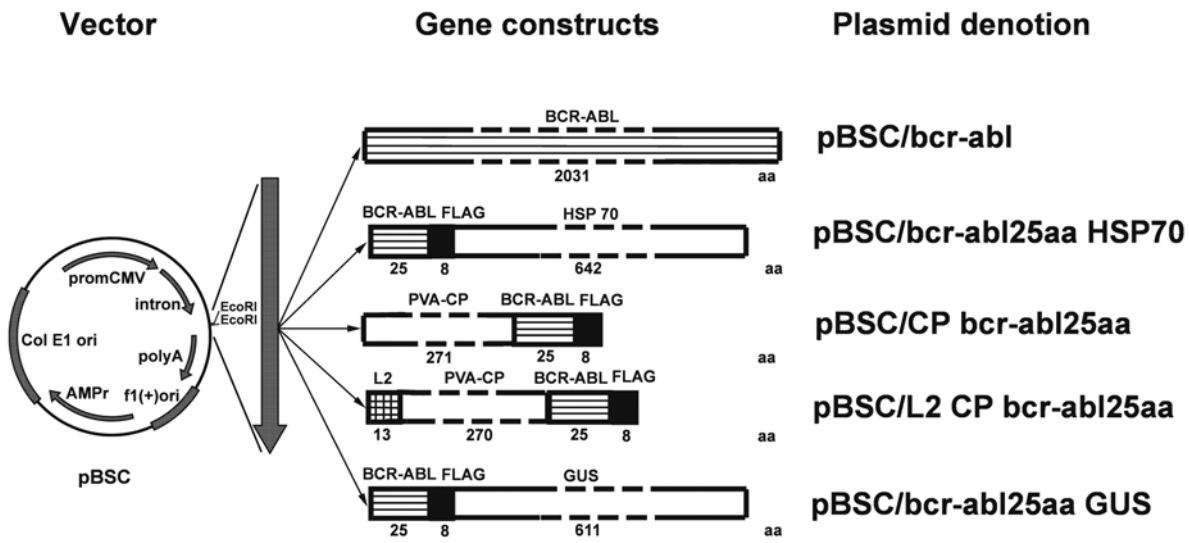

Figure 1. Plasmids carrying either the complete *bcr-abl* gene, or its fragment coding for the junctional zone, fused with adjuvant genes. The figures indicate the numbers of amino acids forming the respective gene products.

by the fusion of the two genes. The fusion is associated with a codon split and the appearance of a new amino acid, lysine in the case of b3a2. Thus, a new amino acid sequence is formed which is unique among all the proteins known and can serve as a target for immune reactions. It has recently been shown in mouse-model systems, using a cell line transformed by the human *bcr-abl* fusion gene, that it is possible to induce protection against challenge with these cells by peptide-based vaccines, by dendritic cells stimulated by synthetic peptides covering the junction site, or by tumour-cell lysates enriched with chaperons or with chaperon-enriched lysates embedded with peptide derived from the junctional zone (8,19-22). It has also been demonstrated in CML patients that a mixture of peptides spanning the junction sequence, or dendritic cells pulsed with b3a2 peptide (12) or infected with recombinant adeno-associated virus expressing the fusion zone and its flanking sequences (17) are capable of inducing specific immune reactions against leukaemia cells (13,14). In other studies, the presence of $p210^{bcr\text{-}abl}$ junction-specific $CD8^+$ cells has been demonstrated in the majority of CML patients (23,24). However, it is not yet known to what extent these specific immune reactions could contribute to the control of the disease. In this respect the recent report indicating that the antigens carried by the fusion protein may not be the dominant tumour antigens in bcr-abl-transformed human cells is of high interest (25).

We are convinced that more information on the immunology of *bcr-abl*-transformed cells obtained in experimental systems might be helpful towards future immunotherapy of CML. Some time ago we started a series of experiments designed to develop a variety of experimental vaccines against *bcr-abl*-transformed cells. BALB/c mice and two syngeneic *bcr-abl*-transformed cell lines have been used in these experiments. The disease induced by these cells is different from human CML (26); however, it provides a suitable system for studying immune reactions to *bcr-abl*-positive cells. The vaccines under development in our laboratory include DNA vaccines. In the present report we summarize the results obtained with DNA vaccines that carry either the complete *bcr-abl* fusion gene, or the gene fragment coding for the eicosapentapeptide that spans the fusion zone and is linked with some other genes known to stimulate anti-tumour immunity (27,28 and unpublished data), or fragments of the *bcr-abl* gene that code for 122 up to 315 amino acid-long sequences that represent various domains of the $p210^{bcr\text{-}abl}$ protein. To the best of our knowledge, this is the first report on the use of DNA vaccines for immunization against *bcr-abl*-transformed cells.

Materials and methods

Cell lines. Two mouse (BALB/c) cell lines transformed by human *bcr-abl* (b3a2 configuration), viz. B210 and 12B1, were used. The B210 cells were developed (29) and kindly provided by G.Q. Daley (Whitehead Institute of Biochemical Research, Cambridge, MA); 12B1 cells, originally isolated by J. McLaughlin *et al* (30), were obtained through the courtesy of E. Katsanis (University of Arizona, Tuscon, AZ). We recently compared the two lines (26,31) and the findings can be summarized as follows. Although both cell lines produced comparable amounts of $p210^{bcr\text{-}abl}$ protein and, in syngeneic mice, both induced a disease resembling acute myeloid leukaemia or CML in its blastic phase, they differed in several respects. In brief, B210 cells induced leukaemia-like disease only after intravenous (i.v.) injection and 1 TID_{50} corresponded to approximately $5x10^4$ cells. 12B1 cells, when the same route of administration was used, were approximately 100 times more oncogenic. The latter cells also induced tumours after subcutaneous (s.c.) injection, with approximately $10^{2.5}$ cells corresponding to 1 TID_{50}. In the past three years nearly 500 animals were inoculated s.c. with 12B1 cells. Of these, less than 1% of the s.c. inoculated animals developed leukaemia without any appearance of palpable tumours, about 3% of them died of systemic disease with relatively small s.c. tumours (<15 mm in their longest diameter) and another 14% died before the tumour size exceeded the critical diameter of 20 mm (unpublished data). The expression of MHC class I molecules on the surface of

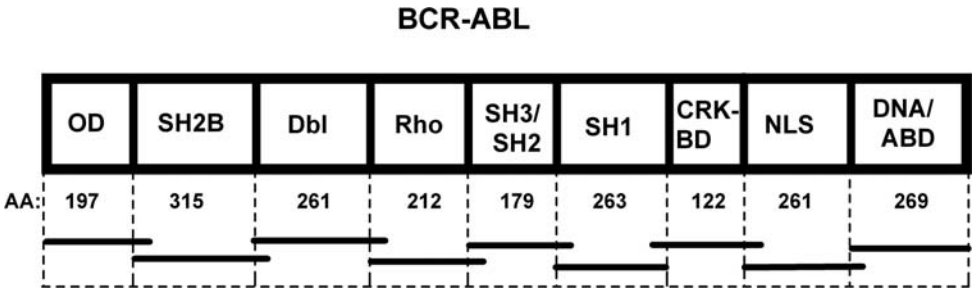

Figure 2. Portions of the *bcr-abl* gene inserted into the pBSC expression vector. The figure indicates the overlap of the amino acid sequences and the numbers of amino acids forming the products of the respective fragments. OD, oligomerization domain; SH2B, SH2 binding; Dbl, Dbl homology domain; Rho, Rho GTP-ase; SH3/SH2, Src homology 3/Src homology 2; SH1, Src homology 1; CRKBD, CRK binding domain; NLS, nuclear localization signal; DNA/ABD, DNA/actin binding domains.

B210 cells is downregulated, while 12B1 cells express high levels of these molecules. Furthermore, the cell lines differ morphologically somewhat: 12B1 cells are more polymorphic and exhibit morphological *in vitro* markers of proliferation less frequently than B210 cells. Finally, 12B1 are slightly more susceptible to IM than B210 cells (26,32).

In some experiments, *bcr-abl*-negative HL60 cells (23), obtained from American TCC, were used. For transfection experiments, 293T cells, already used in previous experiments (33) were employed.

All cell lines were maintained in RPMI media supplemented with 10% FCS (PAA Labs., Linz, Austria), 2 mM of glutamine and antibiotics.

Construction of plasmids. Plasmid p210GD (obtained through the courtesy of G. Q. Daley), which carries the complete *bcr-abl* gene (with b3a2 configuration), served as the source of the sequence coding for $p210^{bcr\text{-}abl}$. The fragment for ligation was obtained by cleavage with EcoRI enzyme and was inserted into the same restriction site of the mammalian expression plasmid pBSC, downstream of the immediately early human cytomegalovirus promoter (33). This construct, designated pBSC/*bcr-abl*, was used as a template DNA for PCR production of all other constructs as well as a positive control in all vaccination experiments (see below).

The construction of most of the plasmids containing the junction region of *bcr-abl* (listed in Fig. 1) was described in a previous report (33). In brief, the pBSC/*bcr-abl* plasmid served as the source of the sequence coding for the junction region of $p210^{bcr\text{-}abl}$. The junction region selected contained 25 amino acids (IVHSATGFKQSSKALQRPVASDFEP), with lysine in the centre (the fusion point), and 12 adjacent amino acids (aa) from the C-terminal of the BCR protein fragment and 12 adjacent aa from the N-terminal of the ABL protein fragment. The sequence coding for the bcr-abl 25-aa peptide was amplified by PCR together with the sequence coding for the FLAG epitope (DYKDDDDK). This hybrid sequence was linked to various genes known from earlier experiments to support the development of anti-tumour immunity (27,28 and unpublished data). These included the genes for mouse heat-shock protein 70 (HSP 70) and for the capsid protein of potato virus A (CP), either alone or linked with a sequence coding for that portion of the L2 protein of human papillomavirus 16 which carries the neutralization epitope (L2 CP), and for glucuronidase A of *E. coli* (GUS). A complete list of the primers used for the preparation of these and other constructs (see below) is in Table I.

The sequence of the whole *bcr-abl* fusion gene was divided into nine fragments (using PCR), which were inserted into pBSC plasmids. These fragments, each designed to code for a portion of the BCR-ABL protein corresponding to one of its various function domains, were designated OD, SH2B, Dbl, Rho, SH3/SH2, SH1, CRKBD, NLS and DNA/ABD (Fig. 2). Their products overlapped by seven amino acids to avoid the possibility that some important epitopes might be lost as a consequence of the splitting. The fragments were further modified by adjunction of the shorter version of the flag sequence (DYKDDDD) and appropriate restriction sites. All of these DNA fragments were prepared in our laboratory, except OD and SH2D, which was requested from, and was prepared by, GenScript (Piscataway, NJ, USA).

For the amplifications, the following PCR conditions were employed: denaturation at 95˚C for 5 min followed by 40x denaturation at 94˚C for 1 min, primer annealing at 50˚C for 1 min, primer extension at 72˚C for 1 min, and final extension at 72˚C for 4 min. In the case of the SH3/SH2 and CRKBD fragments, the primer-annealing temperature was 55˚C. Taq or DeepVent polymerase (New England BioLabs, Ipswich, MA, USA) was used for the amplification. DNA constructs were separated by elecrophoresis, isolated using DNA Lego Kit and were inserted in the pBSC vector.

All new constructs were sequenced using the Big Dye Terminator 1.1 Cycle Sequencing Kit (Applied Biosystems, USA), except the two (OD and SH2B) prepared by GenScipt. In these cases the sequencing was included into the service provided.

To monitor the functionality of the constructs, 293T cells were transfected, as described previously (33), with all the newly constructed plasmids and, in parallel, with the original pBSC as a control. Cell lysates were tested by Western blotting as described previously (33). Lysates from cells transfected with a plasmid containing the fragment coding for the bcr-abl25aa sequence (see Fig. 1) were tested with rabbit polyclonal antibody reactive with the fusion zone (33) and with peroxidase-labelled secondary goat anti-rabbit antibody (Amersham, Buckingshire, UK). To monitor the expression of the other fragments of the *bcr-abl* gene, anti-FLAG monoclonal antibody (Amersham) and peroxidase-labelled secondary sheep anti-mouse antibody (Amersham) were used. The results are shown in Figs. 3 and 4. Cells

Table I. List of the used primers.

Primer denotion	Primer sequence
CML-3	5'CCCCAAGCTTGCCGCCATGATCGTCCACTCAGCCACT 3'
CML-4a	5'CATGCCATGGCCTTGTCGTCATCGTCTTT 3'
CML-CP-F	5'GGGATGCATCACCTTGTTATCGTCCACTCAGCCACT 3'
CML-CP-R	5'GGGAAGCTTACTTGTCGTCATCGTCT 3'
CML-GUS-F	5'CTAGCCCGGGGCCGCCATGATCGTCGTCCACTCAGCCACT3'
CML-GUS-R	5'CTAGCCCGGGCCCTTGTCGTCATCGTCTTTG 3'
CML-Dbl-F	5'CGGAATTCGCCGCCATGTGGGTCCTGTCGGGAATC 3'
CML-Dbl-R	5'GCGAATTCTTAGTCGACGAATGCGTCGTCGTCGTCTTTGTAGTCATCCGTGA GCGGAATGTA 3'
CML-Rho-F	5'CGGAATTCGCCGCCATGTACATTCCGCTCACGGAT 3'
CML-Rho-R	5'GCGAATTCTTAGTCGACGAATGCGTCGTCGTCGTCTTTGTAGTCGCGTTCCA TCTCCCACTT 3'
CML-SH3/SH2-F	5'CGGAATTCGCCGCCATGAATGACCCCAACCTTTTC 3'
CML-SH3/SH2-R	5'GCGAATTCTTAGTCGACGAATGCGTCGTCGTCGTCTTTGTAGTCGCGTTCCA TCTCCCACTT 3'
CML-SH1-F	5'CGGAATTCGCCGCCATGGACTACAAAGACGACGACGACAAGTGGGAGATG GAACGC 3'
CML-SH1-R	5'GCGAATTCTTAGTCGACGAATGCCATTGTTTCAAAGGGTTG 3'
CML-CRKBD-F	5'CGGAATTCGCCGCCATGCAAGCCTTTGAAACAATG 3'
CML-CRKBD-R	5'GCGAATTCTTAGTCGACGAATGCGTCGTCGTCGTCTTTGTAGTCTGGGGCT GTCTTCTTCTT 3'
CML-NLS-F	5'CGGAATTCGCCGCCATGAAGAAGAAGACAGCCCCA 3'
CML-NLS-R	5'GCGAATTCTTAGTCGACGAATGCGTCGTCGTCGTCTTTGTAGTCCTTGCTGG TGCCCCCTGG 3'
CML-DNA/ABD-F	5'CGGAATTCGCCGCCATGCCAGGGGGCACCAGCAAG 3'
CML-DNA/ABD-R	5'GCGAATTCTTAGTCGACGAATGCGTCGTCGTCGTCTTTGTAGTCCCTCTGCA CTATGTCACT 3'

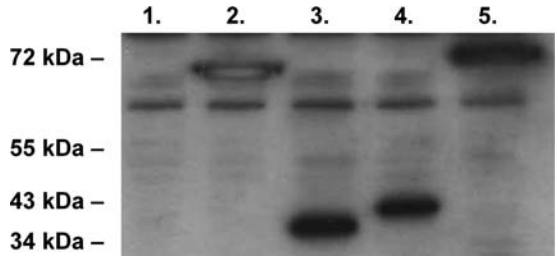

Figure 3. Western blotting with lysates of 293T cells transfected with the following plasmids: Lane 1, pBSC; lane 2, pBSC/*bcr-abl*25aa HSP70; lane 3, pBSC/CP *bcr-abl*25aa; lane 4, pBSC/L2 CP *bcr-abl*25aa; lane 5, pBSC/*bcr-abl*25aa GUS. Polyclonal rabbit antibody reactive with the b3a2 fusion zone, diluted 1:500, and peroxidase-labelled goat anti-rabbit antibody, diluted 1:2000, were used.

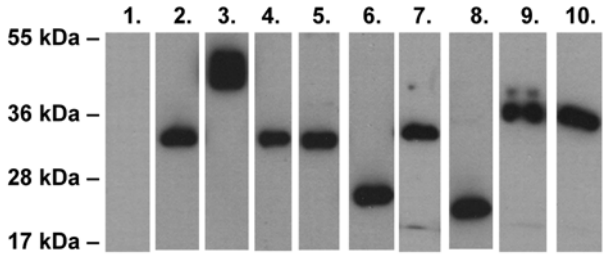

Figure 4. Western blotting with lysates of 293T cells transfected with the following plasmids: Lane 1, pBSC; lane 2, pBSC/OD; lane 3, pBSC/SH2B; lane 4, pBSC/Dbl; lane 5, pBSC/Rho; lane 6, pBSC/SH3/SH2; lane 7, pBSC/SH1; lane 8, pBSC/CRKBD; lane 9, pBSC/NLS; lane 10, pBSC/DNA/ABD. Monoclonal mouse antibody reactive with the FLAG sequence, diluted 1:1000, and peroxidase-labelled goat anti-mouse antibody, diluted 1:2000, were used.

Table II. List and composition of plasmid mixtures used in experiments.

Mixture denotion	Presence of respective plasmid in mixture								
	pBSC/ OD	pBSC/ SH2B	pBSC/ Dbl	pBSC/ Rho	pBSC/ SH3/SH2	pBSC/ SH1	pBSC/ CRKBD	pBSC/ NLS	pBSC/ DNA/ABD
MIX - compl	●	●	●	●	●	●	●	●	●
MIX - orig			●	●	●		●	●	●
MIX - bcr	●	●	●	●					
MIX - abl					●	●	●	●	●
MIX - orig +OD	●		●	●	●		●	●	●
MIX - origl + SH2B		●	●	●	●		●	●	●
MIX - orig + SH1			●	●	●	●	●	●	●
MIX - orig + OD + SH2B	●	●	●	●	●		●	●	●
MIX - orig + OD + SH1	●		●	●	●	●	●	●	●
MIX - orig + SH2B + SH1		●	●	●	●	●	●	●	●

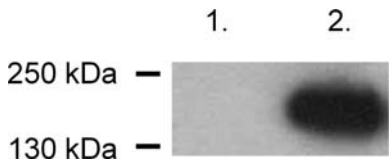

Figure 5. Western blotting with lysates of 293T cells transfected with the following plasmids: Lane 1: pBSC; lane 2: pBSC/*bcr-abl*. Monoclonal mouse antibody reactive with the C-terminus of *abl*, diluted 1:1000, and peroxidase-labelled goat anti-mouse antibody, diluted 1:2000, were used.

transfected with the plasmid carrying the complete *bcr-abl* gene (pBSC/*bcr-abl*) gave a positive reaction with monoclonal ABL-antibody reactive with an epitope located outside the 25aa fusion zone (33) (Fig. 5).

Preparation of plasmid mixtures. In several experiments, mixtures of plasmids were used. The composition of these mixtures is shown in Table II.

Several technical problems arose in the preparation of these mixtures. Owing to the different length of the constructs, different numbers of plasmid copies were present in 1 μg of DNA. The length of plasmids carrying the *bcr-abl* fragments varied over an interval of 5255-5849 bps. This was considered tolerable and we decided to use equal amounts of DNA, viz. 1 μg, for the immunization (see below). More annoying was the difference that occurred when the immunogenicity of the plasmid carrying the whole *bcr-abl* gene was tested in comparison with a mixture of plasmids that carried the different fragments of the fusion gene. Theoretically, to form an equivalent mass of BCR-ABL protein after transfection with a single copy of pBSC/*bcr-abl* (carrying the whole *bcr-abl* gene), a mixture of 9 fragments should contain 9 times more plasmid copies. Because of the immunization technique used (see below), this did not seem to be technically feasible, and, furthermore, we were aware that such a modification would introduce other variables into the system. To decrease the difference, we decided to apply 2 μg of DNA when mixtures of fragments were used for immunization and the same amount of empty plasmid DNA was administered as a negative control. Anyhow, less BCR-ABL product was formed than after administration of the plasmid carrying the whole *bcr-abl* gene, the amount depending on the number of fragment-based vaccines in the respective mixtures. We assumed that this shortcoming would not essentially influence the outcome of our experiments and the results obtained (see the 'Results' section) seemed to justify this expectation.

Animals and immunization procedure. BALB/c mice aged 5-6 weeks were used. The animals were purchased from Velaz, Prague, Czech Republic. All work with animals was carried out in accord with the regulations valid in the Czech Republic. For their immunization, plasmid DNAs were prepared using the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). The preparation of DNA cartridges for the gene gun followed the manufacturer's protocol (Helios gene gun system, Bio-Rad

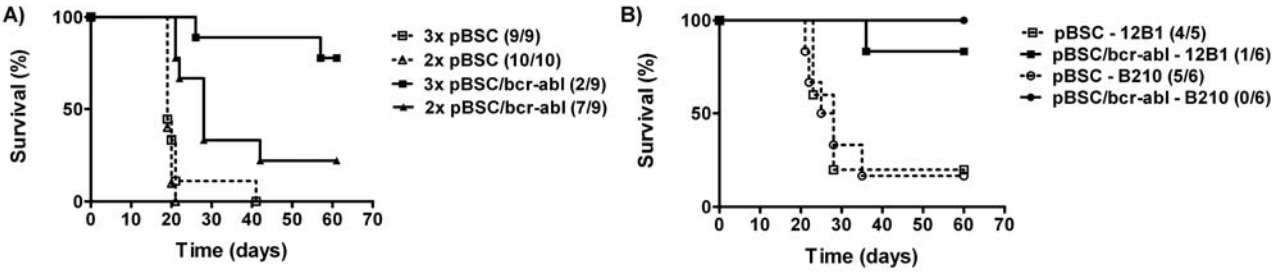

Figure 6. (A) Tumour development in mice immunized with either two or three doses of pBSC/*bcr-abl* vaccine and challenged with B210 cells. (B) Tumour development in mice immunized with three doses of pBSC/*bcr-abl* vaccine and challenged with either B210 cells administered intravenously or 12B1 cells administered subcutaneously.

USA). Each cartridge contained 1 μg of DNA on gold particles of 1 μm diameter. Using the gene gun, we applied either amount of DNA intradermally into the shaven abdominal area. If not indicated otherwise, three doses were given at two-week intervals. A group of mice immunized with an empty pBSC plasmid was included as a control in each experiment. A group of untreated mice was always included as well. The results of challenge experiments were always very similar in the groups of pBSC-inoculated and untreated mice. For the sake of simplicity, only the results obtained in mice treated with the empty plasmid were included in the figures (see the 'Results' section).

Two weeks after the last immunization dose the animals were challenged with either $5x10^5$ B210 cells given i.v. or with $5x10^3$ 12B1 cells injected s.c. In all experiments, third passage cells taken from a big frozen stock were used. If not stated otherwise, six animals per group were used. The animals were examined at least three times a week for up to 100 days after the challenge. However, if tumours did not appear before day 50, the animals remained tumour-free until the end of the observation period. When 12B1-induced tumours extended beyond 20 mm in their longest diameter, the animals were humanely sacrificed.

Detection of bcr-abl-positive cells in animal tissues. Sample collection, RNA extraction, RNA transcription and PCR performance were the same as described by Sobotkova *et al* (32).

Epitope prediction and sequence analyses. Human and mouse BCR and ABL protein sequences were obtained making use of NCBI Entrez database search tool. Comparison of the sequences was performed employing Clone Manager 9 software. Epitope prediction analysis was made using the on-line 'RANKPREP' (http://ww.bio.drgci.Harvard/RANKEP).

Statistical methods. The results were statistically analyzed using GraphPad Prism 4 software. For comparison of survival curves, the log-rank test was used. Tumour growth curves were analyzed by two-way analysis of variance (ANOVA).

Results

Immunization experiments with plasmids coding for whole BCR-ABL. The results of the experiment in which mice were immunized with either two or three doses of the pBSC/*bcr-abl* vaccine (carrying the whole *bcr-abl* gene) and challenged with B210 cells are shown in Fig. 6A. As shown, the immunization resulted in protection against the challenge, more so after three than after two doses of the vaccine. The differences both between 3x pBSC (control) and 3x pBSC/*bcr-abl*-vaccinated mice and between 2x pBSC (control) and 2x pBSC/*bcr-abl*-vaccinated mice were highly significant ($p<0.001$). Also the difference between 2x and 3x pBSC/*bcr-abl* was statistically significant ($p<0.02$). On the basis of these results, we used the three-dose regimen of immunization in all subsequent experiments.

As has been shown elsewhere (8,31), 12B1 cells, at variance with B210 cells, induce solid tumours after s.c. inoculation. To test the efficacy of the pBSC/bcr-abl vaccine against tumours induced by 12B1 cells, mice immunized with three doses of the vaccine were challenged, in parallel, with either $5x10^5$ B210 cells injected i.v. or with $5x10^3$ 12B1 cells administered s.c. Thus, 10 TID_{50} were employed in either case. The results are shown in Fig. 6B. They suggest that the immunization procedure was effective in either system, affording protection both against leukaemia-like disease induced by B210 and subcutaneous tumour formation by 12B1 cells ($p<0.005$ for B210 cells and $p<0.02$ for 12B1 cells). Since the 12B1 cells offer, in addition to survival, another parameter for measuring the immune reactivity, viz. tumour size, these cells were used in further experiments.

Immunization experiments with plasmids coding for the junction region of the $p210^{bcr-abl}$ (bcr-abl 25aa). Next, the immunogenicity of the products of plasmids carrying the information for the junction region of the $p210^{bcr-abl}$ protein (listed in Fig. 1) was tested. Fig. 7 presents data from an experiment in which mice were immunized with these vaccines. Again, the pBSC/bcr-abl vaccine protected a portion of the mice from tumour development and markedly post-poned tumour development in others. In this particular experiment, the immunization effect of this plasmid was less pronounced than in previous experiments, but it was still highly significant both in terms of survival ($p<0.005$) (Figs. 7A, B and C) and tumour size ($p<0.001$) (data not shown). On the other hand, none of the constructs coding exclusively for the bcr-abl25aa zone and fused with other genes elicited marked protection. Only in the case of the pBSC/bcr-abl25aa HSP70 vaccine was there a slight prolongation of animal survival

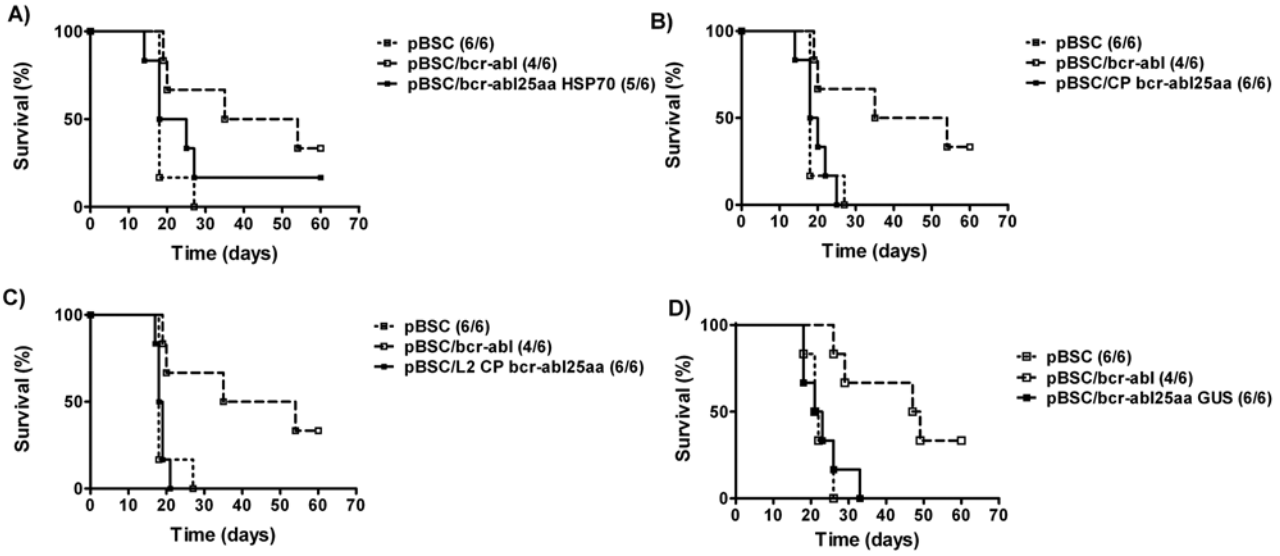

Figure 7. Tumour development in mice immunized with vaccines carrying either the whole pBSC/*bcr-abl* gene or its fragment coding for the 25 amino acid-long junction region (*bcr-abl* 25aa) fused with other genes and challenged with 12B1 cells administered subcutaneously. Plasmid pBSC was used as a negative and plasmid pBSC/*bcr-abl* as a positive control. The immunization effect of: (A) pBSC/*bcr-abl*25aa HSP70, (B) pBSC/CP *bcr-abl*25aa, (C) pBSC/L2 CP *bcr-abl*25aa, and (D) pBSC/*bcr-abl*25aa GUS.

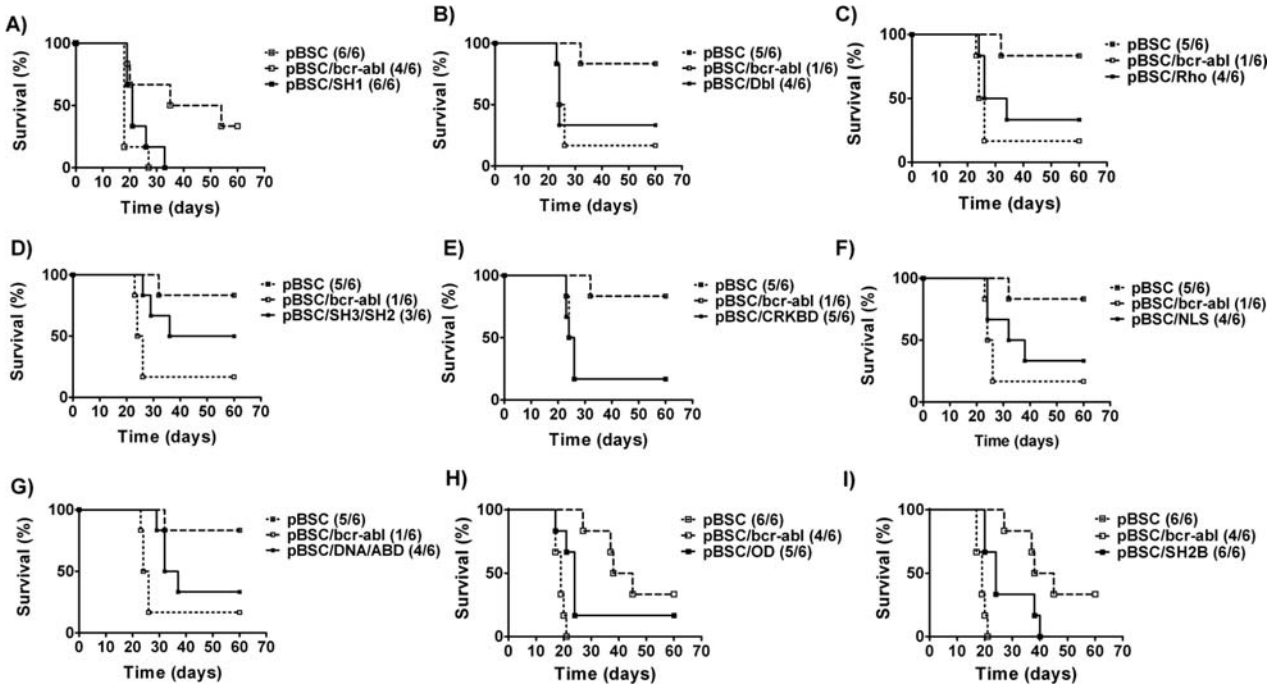

Figure 8. Tumour development in mice immunized with vaccines carrying different fragments of the *bcr-abl* gene and challenged with 12B1 cells administered subcutaneously. Plasmid pBSC was used as a negative and plasmid pBSC/*bcr-abl* as a positive control. The immunization effect of: (A) pBSC/SH1, (B) pBSC/Dbl, (C) pBSC/Rho, (D) pBSC/SH3/SH2, (E) pBSC/CRKBD, (F) pBSC/NLS, (G) pBSC/DNA/ABD, (H) pBSC/OD and (I) pBSC/SH2B.

and one of the six mice immunized remained tumour-free. However, this difference was not statistically significant. Also the differences in tumour size were small and did not reach statistical significance (results not shown).

In previous work with other systems, we found that the immunogenicity of DNA vaccines could be markedly increased by fusing the gene of interest with the gene coding for GUS of *E. coli*; this construct had immunized mice more effectively than the constructs described above (27 and unpublished data). Since our pBSC/bcr-abl25aa GUS plasmid was prepared later than the other plasmids carrying bcr-abl25aa, this particular DNA vaccine was tested separately. The results of the immunization experiment are shown in Fig. 7D. Again, the control pBSC/*bcr-abl* vaccine induced protection to challenge, but no significant difference between the control pBSC-inoculated and the pBSC/*bcr-abl*25aaGUS-immunized mice was observed.

Immunization experiments with plasmids carrying bcr-abl fragments. Plasmid pBSC/SH1 was prepared earlier than the

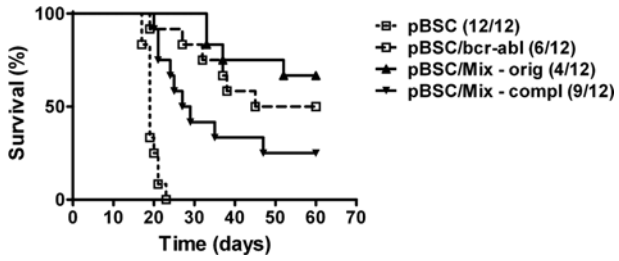

Figure 9. Tumour development in mice immunized with various mixtures (Mix) of vaccines carrying fragments of *bcr-abl*. Plasmid pBSC was used as a negative and pBSC/*bcr-abl* as a positive control.

other plasmids coding for bcr-abl fragments and was tested separately. Again, plasmids pBSC and pBSC/*bcr-abl* were used as negative and positive control, respectively. As is apparent from Fig. 8A, immunization with the SH1 domain failed to provide any significant protection against challenge with 12B1 cells. Moreover, its fusion with the gene for either HSP70 or GUS did not enhance the immunization potency (data not shown).

In a subsequent experiment, six vaccines containing either the pBSC/Dbl, pBSC/Rho, pBSC/SH3/SH2, pBSC/CRKBD, pBSC/NLS or pBSC/DNA/ABD fragment were tested separately. The results are shown in Figs. 8B-G. Some of these vaccines, viz. pBSC/SH3/SH2, pBSC/NLS and pBSC/DNA/ABD, did protect a portion of the mice immunized, and where tumours developed they tended to appear later and to grow at a somewhat slower rate (results not shown); however, none of these differences was statistically significant.

In the next immunization experiment, the vaccines based on the latter two fragments, viz. pBSC/OD and pBSC/SH2B, were tested. Both plasmids failed to induce significant protection against challenge (Figs. 8H and I), although, in the case of pBSC/SH2B, a certain delay in tumour development was apparent.

Immunization experiments with mixtures of the bcr-abl gene fragments. Thus, none of the constructs tested was capable of inducing protection comparable to the effect of the whole *bcr-abl* gene. This suggested that the immunizing epitopes were more widely distributed within the $p210^{bcr\text{-}abl}$ protein. To examine this possibility, various mixtures of the fragment-based vaccines were prepared and tested. A survey of the mixtures tested is presented in Table II.

In the next series of experiments we tested, in parallel, a mixture (Mix) consisting of the six plasmids enumerated in the above section, viz. pBSC/Dbl, pBSC/Rho, pBSC/SH3/SH2, pBSC/CRKBD, pBSC/NLS and pBSC/DNA/ABD, which mixture, as the first to be tested, is herein denoted Mix-orig, and a mixture of plasmids comprising all the nine fragments which together cover the whole *bcr-abl* gene; this preparation has been denoted Mix-compl. The results of the two repeated experiments are summarized in Fig. 9. It can be seen that Mix-orig induced an immunization effect that was comparable with that of immunization with the whole fusion gene vaccine administered in parallel. Paradoxically, when the complete set of fragments was used (Mix-compl) the immunization effect was weaker.

To analyze further this apparent discrepancy between the two vaccine mixtures, we tested the immunogenicity of Mix-orig and Mix-orig supplemented with fragment vaccines missing in the M-orig, either individually or in all three possible combinations. The results are shown in Fig. 10A and further summarized in Fig. 10B. It can be seen that the majority of the mice immunized with vaccines free of the OD fragment were protected against challenge with 12B1 cells. On the other hand, nearly all animals immunized with plasmid mixtures carrying the OD fragment developed tumours. The difference between these two groups of animals was significant ($p<0.02$). These results strongly suggested that the plasmid carrying the OD fragment was involved in the decreased immunogenicity of the vaccines. We repeated the tests with and without OD vaccines twice. In the second of these experiments the OD fragment was administered either in a mixture with the other fragments or applied separately at

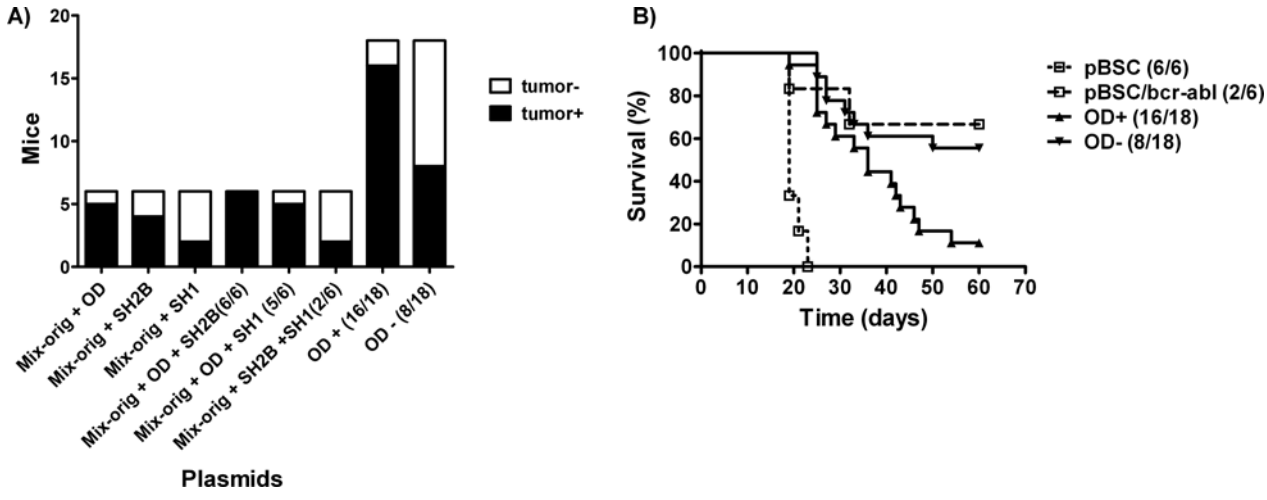

Figure 10. Tumour development in mice immunized with various mixtures of vaccines either including or not including pBSC/OD. Plasmid pBSC was used as a negative and plasmid pBSC/*bcr-abl* as a positive control.

Table III. Presence of *bcr-abl*-positive cells as determined by RT-PCR in successfully immunized and non-immunized animals inoculated with either B210 or 12B1 cells.

Cells	Immunization	No.	With tumours	With *bcr-abl* transcripts		
				L	S	BM
B210	yes	8	0	0	0	NT
	no	1	1	1	1	NT
12B1	yes	5	0	0	0	0
	no	5	5	5	5	5

Explanation of abbreviations: NT, not tested; L, liver; S, spleen; BM, bone marrow.

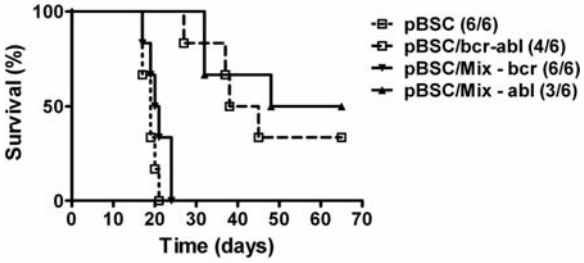

Figure 11. Tumour development in mice immunized with mixtures composed of either *bcr*- or *abl*-derived fragments. Mix *abl* vs. Mix *bcr*.

a different site. In both of these tests more mice survived after immunization with the OD fragment-free vaccines, and there was no additional protective effect of separate OD fragment administration. However, none of the differences were statistically significant (results not shown).

In the last experiments of this series, which aimed at localization of the immunizing epitopes, vaccines based on either *abl*- or *bcr*-derived fragments were compared. The results are shown in Fig. 11. They indicate that the *abl*-derived vaccines, but not the *bcr*-derived vaccines, were capable of inducing protection comparable to the effect of the whole fusion-gene-based vaccine.

Presence of bcr-abl-positive cells in tissues of animals inoculated with bcr-abl-transformed cells. In order to ascertain whether the animals successfully immunized with the DNA vaccines were free of *bcr-abl*-positive cells, we tested tissue lysates from different organs of these animals for the presence of *bcr-abl* transcripts by RT-PCR. The results from several experiments are summarized in Table III. It can be seen that *bcr-abl* transcripts were detected in all mice that had developed either leukaemia-like disease or s.c. tumours. All of the immunized mice that had survived the challenge with either B210 or 12B1 cells were free of *bcr-abl*-positive cells, this suggesting that these animals were free of residual disease.

Discussion

In the present study, a DNA vaccine based on the whole *bcr-abl* gene was shown to be able to protect mice against both types of the *bcr-abl*-transformed cells used, viz. B210 cells, which induce acute leukaemia-like disease after i.v. inoculation, and 12B1 cells, which, in addition to leukaemia, induce aggressively growing subcutaneous tumours. In spite of the difference in MHC class I expression between the two cell lines, the immunizing efficacy of the vaccine against both lines seemed to be similar. The ability of 12B1 cells to induce subcutaneously growing tumours made us to use the latter cells for measuring the efficacy of a variety of DNA vaccines based on the *bcr-abl* fusion gene. Some problems were encountered. Although we tried to standardize the conditions of the challenge by using cells derived from the same frozen stock, and all injections were administered by the same person, some variation was observed in different experiments. Nevertheless, in spite of this difficulty, statistically significant protection was achieved with the whole fusion-gene vaccine in all experiments.

The primary aim of the present undertaking was to ascertain whether it was possible to induce protection against challenge with syngeneic *bcr-abl*-positive cells by immunizing mice with a DNA vaccine that carried the *bcr-abl* gene sequence coding for the fusion zone (*bcr-abl*25aa). Quite clearly, the immunizing effect of the fusion zone was very low, if any, in the present system. Although the fusion zone sequence was combined with other genes, the products of which had, in previous experiments, been shown to be capable of stimulating anti-tumour immunogenicity, the results were rather disappointing: we failed to elicit any protection. To locate that portion of the BCR-ABL protein which carried the dominant protective epitopes, we gradually prepared and tested nine plasmid constructs coding for mutually overlapping peptides and together spanning the whole BCR-ABL protein. This was done to avoid losing any possibly important epitopes. Partial, though insignificant, protection was achieved with some of these vaccines. This suggested that several epitopes, probably widely distributed over the BCR-ABL protein, were involved in the induction of the protective immune response observed after immunization with the whole fusion-gene vaccine.

To examine this hypothesis, we used various mixtures of these single-fragment vaccines for immunization. Two results of these experiments appeared to be of major interest. First, it was apparent that all the important epitopes were located in the ABL portion of the $p210^{bcr\text{-}abl}$ fusion protein. This seemed

to be in consent with the partial protection provided by the single-fragment vaccines (see Fig. 8). The other observation that deserved a more detailed scrutiny concerned the immunization potency of the various mixtures of plasmids carrying the fragments of the *bcr-abl* fusion gene. If the transcription and translation of the particular genetic information from the whole gene and from its fragments were similarly efficient, one would have to find that even after the administration of 2 μg of the Mix-orig vaccine (consisting of six fragments) considerably less of the respective protein mass had been formed as compared with the product formed after vaccination with 1 μg of the whole *bcr-abl* gene. In spite of this, the protection induced by the Mix-orig vaccine was comparable with whole gene vaccine. This indicated that even small amounts of the immunogens were sufficient for eliciting the immunity. We were rather surprised to see that the immunizing potency of the M-compl vaccine, covering the whole gene, was lesser than that of Mix-orig. The first and most trivial idea was that the important epitopes might have been further diluted out beyond the critical level. However, further analysis carried out with new vaccine mixtures suggested that the pBSC/OD fragment, coding for the 197 amino acids forming the N-terminus of the BCR protein, played a negative role in the outcome of the immunization process. At this writing, it is not clear which mechanism might have been involved. One can speculate that the product of this gene fragment might have exhibited suppressive activity either on some component of the immune system or, alternatively, that it might have suppressed the expression of one or more products of some other fragment(s) coding for important immunizing epitopes. The preliminary data available thus far do not provide any support for the latter explanation and do not suggest that any type of interference occurred in the possibly co-transfected cells. Thus, the phenomenon deserves additional investigation and will be further analyzed.

When interpreting the present data on the efficacy of the *bcr-abl* DNA vaccines, one must take into consideration that while the junction region (outside the fusion point) of the human BCR-ABL has 100% homology with the corresponding mouse sections of the BCR and ABL proteins, there are differences in the amino acid composition in the other sections of the two proteins. Utilizing the data base and programs mentioned in 'Material and methods' we compared the sequences of human and mouse BCR and ABL proteins. We found a high but not complete homology between the respective human and mouse counterparts, 92% in the case of BCR and 87% in the case of ABL. It is likely, though not certain, that the differences in amino acid composition between the human and the mouse proteins, and their resulting heterogeneity, could primarily have been involved in the protection induced by the DNA vaccines used. The sequences of the human BCR-ABL fusion protein and its mouse homologue were further compared and an epitope prediction analysis was done using the above-mentioned tool (see 'Materials and methods'). No significant relations between the position of the predicted epitopes and the heterogeneity of the amino acid sequences were detected. Moreover, the negative results of immunization with Mix-*bcr* (see Fig. 11) suggested that the heterogeneity of the human and the mouse version of the protein did not necessarily lead to the induction of a protective immune response (at least in the model used). In spite of these findings, the results obtained are only valid for the present system and any attempts to extrapolate the present findings into human pathology should be extremely cautious. At the most, they might provide some leads for further research.

Using RT-PCR, we also tested whether *bcr-abl*-positive cells persisted in the successfully immunized mice. The data obtained indicated that these animals were free of such cells, yet could be considered healthy. This conclusion was further supported by the absence of the disease in the immunized animals when the observation period was prolonged by several additional months. It is noteworthy that in animals inoculated with 12B1 cells and possessing subcutaneous tumours, *bcr-abl*-positive cells were always observed in the liver, spleen and bone marrow. This indicates a strong propensity of the subcutaneous tumours to metastasize to distant organs. These results are in line with the data obtained after combined chemo- and immunotherapy with cell-based vaccines that we reported recently (32).

Acknowledgements

This work was supported by Grant No NR-9037/3 from the Internal Granting Agency of the Ministry of Health of the Czech Republic, and was co-financed as a Research Project of the Institute of Hematology and Blood Transfusion, No. MZ0UHKT2005. The authors thank E. Katsanis for critical reading of the first version of a portion of this manuscript.

References

1. Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R and Kantarjian HM: The biology of chronic myeloid leukemia. N Engl J Med 341: 164-172, 1999.
2. Mauro MJ and Druker BJ: Chronic myelogenous leukemia. Curr Opin Oncol 13: 3-7, 2001.
3. Barnes DJ and Melo JV: Primitive, quiescent and difficult to kill: the role of non-proliferating stem cells in chronic myeloid leukemia. Cell Cycle 5: 2862-2866, 2006.
4. Shah NP: Loss of response to imatinib: mechanisms and management. Hematology Am Soc Hematol Educ Program pp183-187, 2005.
5. Weisberg E, Manley PW, Cowan-Jacob SW, Hochhaus A and Griffin JD: Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia. Nat Rev Cancer 7: 345-356, 2007.
6. Goldman JM and Druker BJ: Chronic myeloid leukemia: current treatment options. Blood 98: 2039-2042, 2001.
7. Bocchia M, Korontsvit T, Xu Q, *et al*: Specific human cellular immunity to bcr-abl oncogene-derived peptides. Blood 87: 3587-3592, 1999.
8. He L, Feng H, Raymond A, *et al*: Dendritic-cell-peptide immunization provides immunoprotection against bcr-abl-positive leukemia in mice. Cancer Immunol Immunother 50: 31-40, 2001.
9. Leeksma OC, Kessler JH, Huijbers IJ, Ten Bosch GJ and Melief CJ: BCR-ABL directed immunotherapy: a virtual reality? Leuk Lymphoma 38: 175-181, 2000.
10. Li Z, Qiao Y, Liu B, *et al*: Combination of imatinib mesylate with autologous leukocyte-derived heat shock protein and chronic myelogenous leukemia. Clin Cancer Res 11: 4460-4468, 2005.
11. Norbury LC, Clark RE and Christmas SE: b3a2 BCR-ABL fusion peptides as targets for cytotoxic T cells in chronic myeloid leukaemia. Br J Haematol 109: 616-621, 2000.
12. Osman Y, Takahashi M, Zheng Z, *et al*: Generation of bcr-abl specific cytotoxic T-lymphocytes by using dendritic cells pulsed with bcr-abl (b3a2) peptide: its applicability for donor leukocyte transfusions in marrow grafted CML patients. Leukemia 13: 166-174, 1999.

13. Pinilla-Ibarz J, Cathcart K, Korontsvit T, *et al*: Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. Blood 95: 1781-1787, 2000.
14. Pinilla-Ibarz J, Cathcart K and Scheinberg DA: CML vaccines as a paradigm of the specific immunotherapy of cancer. Blood Rev 14: 111-120, 2000.
15. Reichardt VL and Brossart P: Current status of vaccination therapy for leukemias. Curr Hematol Rep 4: 73-76, 2005.
16. Ren R: Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. Nat Rev Cancer 5: 172-183, 2005.
17. Sun JY, Krouse RS, Forman SJ, *et al*: Immunogenicity of a p210 (BCR-ABL) vision domain candidate DNA vaccine targeted to dendritic cells by a recombinant adeno-associated virus vaccine in vitro. Cancer Res 62: 3175-3183, 2002.
18. Takahashi T, Tanaka Y, Nieda M, *et al*: Dendritic cell vaccination for patients with chronic myelogenous leukemia. Leuk Res 27: 795-802, 2003.
19. Deeb D, Gao X, Jiang H, Divine G, Dulchavsky SA and Gautam SC: Vaccination with leukemia-loaded dendritic cells eradicates residual disease and prevent relapse. J Exp Ther Oncol 5: 183-193, 2006.
20. Kislin KL, Marron MT, Li G, Graner MW and Katsanis E: Chaperone-rich cell lysate embedded with BCR-ABL peptide demonstrates enhanced anti-tumour activity against a murine BCR-ABL positive leukemia. FASEB J 21: 2173-2184, 2007.
21. Zeng Y, Feng H, Graner MW and Katsanis E: Tumor-derived, chaperone-rich cell lysate activates dendritic cells and elicits potent antitumor immunity. Blood 101: 4485-4491, 2003.
22. Zeng Y, Graner MW, Feng H, Li G and Katsanis E: Imatinib mesylate effectively combines with chaperone-rich cell lysate-loaded dendritic cells to treat bcr-abl+ murine leukemia. Int J Cancer 110: 251-259, 2004.
23. Butt NM, Rojas JM, Wang L, Christmas SE, Abu-Eisha HM and Clark RE: Circulating bcr-abl-specific CD8+ T cells in chronic myeloid leukemia patients and healthy subjects. Haematologica 90: 1315-1323, 2005.
24. Guilhot F, Roy L, Martineua G, Guilhot J and Millot F: Immunotherapy in chronic myelogenous leukemia. Clin Lymphoma Myeloma 2: 64-70, 2007.
25. Grünebach F, Mirakaj V, Mirakaj V, Müller MR, Brümmendorf T and Brossart P: BCR-ABL is not an immunodominant antigen in chronic myelogenous leukemia. Cancer Res 66: 5892-5900, 2006.
26. Jelinek F, Sobotkova E and Vonka V: Characteristics of two mouse bcr-abl-transformed cell lines. II. Pathological lesions induced in mice. Folia Biol (Praha) 51: 93-102, 2005.
27. Smahel M, Pokorna D, Mackova J and Vlasak J: Enhancement of immunogenicity of HPV16 E7 oncogene by fusion with E. coli beta-glucuronidase. J Gene Med 6: 1092-1101, 2004.
28. Smahel M, Sima P, Ludvikova V and Vonka V: Modified HPV16 E7 genes as DNA vaccine against E7-containing oncogenic cells. Virology 281: 231-238, 2001.
29. Daley G Q and Baltimore D: Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210 bcr-abl protein. Proc Natl Acad Sci USA 85: 9312-9316, 1988.
30. McLaughlin J, Chianese E and Witte ON: In vitro transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Philadelphia chromosome. Proc Natl Acad Sci USA 84: 6558-6562, 1987.
31. Sobotkova E, Ludvikova V, Petrackova M, *et al*: Characteristic of two mouse bcr-abl-transformed cell lines: I. General properties of the cells. Folia Biol (Praha) 51: 12-18, 2005. Erratum in: 158, 2005.
32. Sobotkova E, Duskova M, Tachezy R, Petrackova M and Vonka V: Combined chemo- and immunotherapy of tumours induced in mice by *bcr-abl*-transformed cells. Oncol Rep 21: 793-799, 2009.
33. Ludvikova V, Hamsikova E, Sobotkova E, Lucansky V, Smahel M and Vonka V: Use of polyclonal rabbit antibodies for detection of the bcr-abl fusion zone in cells transfected with experimental bcr-abl DNA vaccines. Int J Oncol 27: 265-274, 2005.