Abstract
In the present study, we analyzed the involvement of the BCR-ABL protein in the induction of antigen-specific CTLs in order to develop an immunotherapeutic approach in patients with chronic myelogenous leukemia (CML). To accomplish this, we generated dendritic cells (DC) in vitro and electroporated them with various sources of RNA harboring the chimeric bcr-abl transcript. These genetically engineered DCs were used as antigen-presenting cells for the induction of CTLs. By applying this approach, we found that the CTLs induced by DCs transfected with RNA extracted from bcr-abl-positive K-562 cells or CML blasts lysed DCs transfected with the corresponding RNA, but failed to recognize epitopes derived from the chimeric BCR-ABL fusion protein in ^51Cr-release assays. In contrast, they were able to lyse autologous DCs electroporated with RNA isolated from patients with acute myeloid leukemia, indicating that antigens shared among these malignant cells are involved and recognized by these CTLs. In patients with CML in complete cytogenetic remission during IFN-α treatment, we detected some reactivity of CD8^+ T cells against BCR-ABL in IFN-γ ELISPOT assays, which was weaker as compared with proteinase 3 (PR3)- or prame-}

Introduction
Chronic myelogenous leukemia (CML) is a myeloproliferative disorder, which originates from pluripotent hematopoietic bone marrow progenitor cells. It is characterized by excessive proliferation of the granulopoiesis in the bone marrow (1). In ~90% of the patients with CML, the Philadelphia chromosome (Ph) is the characteristic cytogenetic hallmark. Ph is a shortened chromosome 22 which arises from an acquired reciprocal translocation between the long arms of chromosomes 9 and 22 (t(9;22) (q34;q11); ref. (2)). This translocation fuses a portion of the bcr gene, with unknown function, with part of the c-abl gene, which encodes a protein-tyrosine kinase. The chimeric bcr-abl oncogene is translated into a 210-kDa chimeric protein (p210) which exhibits constitutive ABL kinase activity (3–6).

Hence, the tumor-specific BCR-ABL protein is a favored target for the development of new therapeutics for the treatment of CML including STI571 (Gleevec/Glivec, Novartis, Basel, Switzerland) which was designed as a selective competitive inhibitor of the ABL protein-tyrosine kinase (7).

Furthermore, several recent reports showed that the BCR-ABL protein may function as a tumor-specific antigen and T cells generated in vitro or in vivo specific for this neoantigen or peptides deduced from its sequence can recognize CML cells expressing the BCR-ABL protein (8–10). Based on these results, clinical vaccination trials were designed in an attempt to elicit CML-specific T cells capable of eliminating the malignant cells. In these studies, peptides plus adjuvants or dendritic cells (DC) generated from Ph-positive cells expressing the BCR-ABL product, and therefore, containing all the antigenic information of a malignant cell were used as vaccine. These vaccines rather induced delayed-type hypersensitivity and/or CD4 proliferative responses. However, BCR-ABL–specific cytotoxic T cell responses were rarely detected, indicating that this antigen might be less antigenic (11–14). In line with these observations, in patients with INF-γ-treated CML, antigen-specific CTLs recognizing overexpressed antigens like proteinase 3 (PR3), which is also involved in Wegener’s granulomatosis, were found to contribute to the elimination of malignant cells (15–18).

In the present study, we analyzed the involvement of the BCR-ABL fusion protein in the induction of antigen-specific CTLs. To accomplish this, we generated DCs in vitro and electroporated them with various sources of RNA harboring the chimeric bcr-abl transcript. These genetically engineered DCs were used as antigen-presenting cells (APC) for the induction of CTLs or CD4^+ T cells. The effectiveness of RNA-electroporation for CTL induction was shown in several previous studies (19–23). This technique was also adopted for the generation of target cells in ^51Cr-release assays, i.e., DCs electroporated with different species of RNA.

By applying this approach, we found that the CTLs induced by DCs transfected with whole tumor RNA extracted from Ph^+ cells, including CML blasts, did not recognize epitopes derived from the chimeric BCR-ABL fusion protein on target cells. In line with these results, we also show that BCR-ABL–derived epitopes did not substantially contribute to the stimulation of CD4^+ cells. However, we were successful in the induction of BCR-ABL–specific CTLs by DCs electroporated with an excess of pure in vitro–transcribed full-length p210 encoding bcr-abl-RNA (in vitro transcript, IVT).

Materials and Methods
Tumor cell line. The K-562 CML tumor cell line (CML in blast crisis, Ph^+; HLA class I–deficient; ATCC no. CCL-243) was grown in RPMI 1640 containing all the antigenic information of a malignant cell were used as vaccine. These vaccines rather induced delayed-type hypersensitivity and/or CD4 proliferative responses. However, BCR-ABL–specific cytotoxic T cell responses were rarely detected, indicating that this antigen might be less antigenic (11–14). In line with these observations, in patients with INF-γ-treated CML, antigen-specific CTLs recognizing overexpressed antigens like proteinase 3 (PR3), which is also involved in Wegener’s granulomatosis, were found to contribute to the elimination of malignant cells (15–18).

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Tumor cell line. The K-562 CML tumor cell line (CML in blast crisis, Ph^+, HLA class I–deficient; ATCC no. CCL-243) was grown in RPMI 1640 containing 10% heat-inactivated FCS and 100 U/mL penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C and 5% CO_2.

Generation of DCs from peripheral blood mononuclear cells. DCs were generated from peripheral blood mononuclear
previously (24, 25). In brief, peripheral blood mononuclear cells (PBMC) were isolated by ficoll/paque (Biochrom, Berlin, Germany) density gradient centrifugation of blood obtained from the buffy coats of healthy volunteers from the blood bank of the University of Tübingen. Cells were seeded (1 × 10^7 cells/ml per well) into six-well plates (Corning, Cambridge, MA) in X-VIVO 20 medium (Cambrex Bio Science, Verviers, Belgium). After 2 hours of incubation, at 37°C and 5% CO₂, nonadherent cells were removed. Immature DCs were generated by culturing the adherent blood mononcytes in RPMI 10 medium supplemented with human recombinant granulocyte-macrophage colony-stimulating factor (100 ng/ml). Leukine Liquid Sargramostim; Berlex Laboratories, Richmond, CA) and interleukin-4 (20 ng/ml R&D Systems, Wiesbaden, Germany) for 6 days. The medium was replenished with cytokines every 2 to 3 days. For maturation, DCs were cultured with tumor necrosis factor-α (10 ng/ml; R&D Systems) for an additional 24 hours. DCs were enumerated by flow cytometry as lineage (CD14, CD3, and CD19) negative and human leukocyte antigen DR (HLA-DR) bright. Furthermore, analysis of the expression level of the DC markers, CD1a and CD83, and costimulatory molecules, CD80 and CD86, was done.

Enrichment of CD34⁺ cells. After informed consent, peripheral blood cells were obtained from healthy donors or patients with nonhematologic malignancies during granulocyte colony-stimulating factor–induced stem cell mobilization according to the guidelines of the ethical committee of the University of Tübingen. Mononuclear cells were separated by ficoll/paque (Biochrom) density gradient centrifugation. Enrichment of CD34⁺ cells was done using immunomagnetic microbeads (magnetic-associated cell sorting (MACS) System; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. By passing the cells over two consecutive columns, all samples used throughout this study were purified to 96% to 99%, as determined by dual immunofluorescence staining with CD45-FTTC/CD43-phycocerythrin monoclonal antibodies and flow cytometry.

Isolation of CD4⁺ or CD8⁺ T cells from PBMCs. Isolation of T cells was done using MACS CD4 or CD8 Microbeads (Miltenyi Biotec) according to the protocol provided by the manufacturer. Cell populations were characterized by flow cytometry after isolation procedures. Purity was routinely found to be ~95%.

RNA isolation. Total RNA was isolated from tumor cell lysates using RNeasy Mini anion-exchange spin columns (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer for the isolation of total RNA from animal cells. The quantity and purity of RNA was determined by UV spectrophotometry. RNA samples were routinely checked by formaldehyde/agarose gel electrophoresis for integrity and stored at ~80°C in small aliquots.

Synthetic peptide. HLA-A3/2/HLA-A11 binding peptide BCR-ABL-A3 (amino acids KQSLSKQR; ref 26) was synthesized in an automated synthesizer (20 ng/ml; R&D Systems, Wiesbaden, Germany) for 6 days. The medium was replenished with cytokines every 2 to 3 days. For maturation, DCs were cultured with tumor necrosis factor-α (10 ng/ml; R&D Systems) for an additional 24 hours. DCs were enumerated by flow cytometry as lineage (CD14, CD3, and CD19) negative and human leukocyte antigen DR (HLA-DR) bright. Furthermore, analysis of the expression level of the DC markers, CD1a and CD83, and costimulatory molecules, CD80 and CD86, was done.

For the generation of bcr-abl-, MUC1-, and EGFP-IVTs, a bcr-abl-IVT, a bcr-abl RNA was synthesized in an automated synthesizer, by UV spectrophotometry. RNAs samples were routinely checked according to the protocol provided by the manufacturer for the isolation of mRNA. MUC1- and EGFP-IVTs were generated as described above (24). DCs electroporated with different species of RNA were incubated for 24 hours in RPMI medium supplemented with tumor necrosis factor-α (10 ng/ml) for maturation. For the induction of specific CTLs, 5 × 10⁶ electroporated DCs were washed, and incubated with 3 × 10⁴ PBMCs in RPMI 10 medium. After 7 days of culture, cells were restimulated with RNA-electroporated DCs, and 2 ng/ml of interleukin-2 (R&D Systems) were added on days 1, 3, and 5. The cytolytic activity of induced CTLs was analyzed on day 5 after the last restimulation in a standard ⁵¹Cr-release assay.

CTL induction using DCs transfected with RNA. DCs electroporated with different sources of RNA were incubated for 24 hours in RPMI medium supplemented with tumor necrosis factor-α (10 ng/ml) for maturation. For the induction of specific CTLs, 5 × 10⁶ electroporated DCs were washed, and incubated with 3 × 10⁴ PBMCs in RPMI 10 medium. After 7 days of culture, cells were restimulated with RNA-electroporated DCs, and 2 ng/ml of interleukin-2 (R&D Systems) were added on days 1, 3, and 5. The cytolytic activity of induced CTLs was analyzed on day 5 after the last restimulation in a standard ⁵¹Cr-release assay.

Standard ⁵¹Cr-release assay (CTL assay). CTL assays were done as described previously (24). DCs electroporated with different species of RNA or pulsed with defined peptides were used as targets. In brief, immature DCs were transfected with RNA as described above and pulsed with 50 μg/ml of synthetic peptide for 2 hours, washed, and labeled with ⁵¹Cr. DCs were incubated in RPMI 10 medium for 1 hour at 37°C/3% CO₂. Target cells (1 × 10⁵) were transferred to a well of a round-bottomed 96-well plate. Varying numbers of CTLs were added to a final volume of 200 μl and incubated for 4 hours at 37°C/3% CO₂. At the end of the assay, supernatants (50 μl/well) were harvested and counted in a beta-plate counter (MicroBeta, Perkin-Elmer Wallac, Freiburg, Germany). The percentage of specific lysis was calculated as: 100 × (experimental release – spontaneous release) / (maximal release – spontaneous release). Spontaneous and maximal releases were determined in the presence of either RPMI medium or 2% Triton X-100, respectively. Inhibition of HLA class I or class II molecules was achieved by incubating DC for 1 hour prior to the assay either with the monoclonal antibodies Pa2.1 (10 μg/ml) directed against HLA class I molecules or Tü39 (10 μg/ml) and L 243 (10 μg/ml) directed against HLA class II molecules (kindly provided by S. Stevanović, Institute for Cell Biology, Department of Immunology, Tübingen, Germany).

The antigen specificity of cell lysis was further determined in a cold target inhibition assay by analyzing the capacity of unlabeled DCs transfected with K-562-RNA (‘‘cold target’’) to block lysis of ⁵¹Cr-labeled DCs transfected with acute myeloid leukemia (AML)-RNA (‘‘hot target’’) at a ratio of 20:1 (inhibitor/target ratio).

IFN-γ ELISPOT assay. CTLs were induced from PBMCs in vitro using autologous DCs transfected with RNA purified from Pl CML blasts as described above. Enrichment of CD8⁺ T cells was done using immunomagnetic microbeads (MACS System; Miltenyi Biotec) according to the manufacturer’s instructions. These cells were incubated at a concentration of 1 × 10⁶ cells/ml with an antihuman IFN-γ antibody (10 μg/ml; Hölzel Diagnostika, Cologne, Germany)–coated, 96-well plate, and 1 × 10⁴ autologous PBMCs, electroporated with different species of RNA for 40 hours. For the detection of spots, a biotin-labeled antihuman IFN-γ antibody (2 μg/ml; Hölzel Diagnostika) was used. Spots were counted using an automated ELISPOT reader (Immunospot Analyzer, CTL Analyzers LLC, Cleveland, OH). As negative controls, we used DCs transfected with an irrelevant RNA such as MUC1- or EGFP-IVT.

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Proliferation assay. Purified CD4+ T cells (1 × 10^6, responding cells) were cultured in a 24-well flat-bottomed microplate (Nunc, Wiesbaden, Germany) together with 2 × 10^5 RNA-electroporated autologous DCs (stimulator cells) for 1 week. After one restimulation, 1 × 10^6 CD4+ cells were incubated with 1 × 10^5 DCs electroporated with various species of RNA and thymidine incorporation was measured on day 5 by a 16-hour pulse with [3H]-thymidine (1 μCi/well; Amersham Life Science, Braunschweig, Germany). Inhibition of HLA class I or class II molecules was achieved by incubating DC for 1 hour prior to the assay either with the monoclonal antibodies Pd2.1 (10 μg/mL) directed against HLA class I molecules or Ti39 (10 μg/mL), and I.243 (10 μg/mL) directed against HLA class II molecules (all antibodies were kindly provided by S. Stevanović).

Results

CTL induction by DCs electroporated with Ph+ K-562 total RNA. Initially, we analyzed the feasibility of the induction of antigen-specific CTLs by DCs electroporated with total RNA purified from Ph+ cells that represents the pool of all mRNA species. Because it was established from a patient with CML in terminal pluripotent stem cell, we next wanted to find out whether the antigens recognized by CTLs induced by Ph+ K-562 RNA–transfected DCs are expressed on hematopoietic progenitor cells. Therefore, we tested whether the CTLs generated as described above are able to lyse DCs electroporated with total RNA extracted from CD34+ bone marrow cells. As shown in Fig. 1C, CTLs efficiently lysed the K-562-RNA–transfected DCs but spared DCs electroporated with CD34+-RNA as well as DCs electroporated with total RNA from normal PBMCs (buffy coat cells) or electroporated with an irrelevant MUC1-IVT.

In cold target inhibition assays, DCs transfected with the K-562 RNA could efficiently inhibit the recognition of AML-derived antigens, thus confirming that the in vitro–induced CTLs are specific for epitopes expressed in both K-562 and AML cells (Fig. 1D). In contrast, DCs transfected with irrelevant EGFP-IVT could not inhibit the recognition of AML-derived antigens. In this assay, we also included DCs electroporated with PBMC-RNA as well as DCs electroporated with pure bcr-abl-IVT. Both targets were not recognized by the CTLs, thus confirming the previous results.

In summary, these experiments indicate that DCs transfected with Ph+ K-562 total RNA could induce CTLs that recognize antigens expressed or shared by Ph+ and Ph- myeloid leukemias but not by Ph+ ALL, CLL, CD34+ progenitor cells or normal PBMCs.

Induction of K-562-specific CTLs using DCs pulsed with cell lysates. The lack of recognition of BCR-ABL–derived epitopes by these in vitro induced CTLs might be due to the way how the antigens were delivered to DCs (whole-cell RNA electroporation). In the following experiments, we therefore used DCs pulsed with tumor cell lysates generated from K-562 cells as APCs for in vitro T cell priming.

As shown in Fig. 2, in line with previous results, CTLs obtained after several restimulations with K-562 lysates recognized and efficiently lysed DCs electroporated with the cognate RNA as well as DCs containing RNA isolated from CML or AML cells. The specific lysis of the K-562-RNA–transfected target cells could be blocked using a monoclonal antibody directed against HLA class I molecules indicating that the elicited T cell responses were HLA class I–restricted. Consequently, the antibody directed against HLA class II molecules did not inhibit the lysis of DCs transfected with K-562-RNA.

There was no recognition of targets transfected with an excess of pure in vitro transcribed bcr-abl RNA or with total RNA purified from normal PBMCs (Fig. 2B). Furthermore, in cold target inhibition assays, DCs transfected with the K-562 RNA could efficiently inhibit the recognition of AML-derived antigens, thus confirming that the in vitro induced CTLs are specific for epitopes expressed in both K-562 and AML cells (Fig. 2B).

CTL induction by DCs electroporated with Ph+ CML blasts’ total RNA. In the next experiments, we examined whether the findings obtained with RNA isolated from the Ph+ cell line K-562 could be reproduced with RNA purified from primary Ph+ blasts obtained from patients with CML. This experimental setting should mimic Ph+ blasts in patients with CML.

Therefore, we repeated the experiments using monocyte-derived DCs electroporated with total RNA now isolated from Ph+ CML blasts. The cytotolytic activities of the CTLs generated were determined in a standard 51Cr-release assay. As shown in Fig. 3A, these CTLs efficiently lysed DCs transfected with RNA isolated from Ph+ CML blasts or Ph+ K-562 cells. DCs transfected with Ph+ CLL-RNA as well as DCs transfected with irrelevant MUC1-IVT were spared. In line with previous experiments, these CTLs also recognized DCs transfected with RNA isolated from patients with Ph+ AML (Fig. 3A, patient 2; Fig. 3B, patient 3). Additionally, we again included DCs electroporated with an excess of pure bcr-abl-IVT as targets in this cytotoxicity assay. As shown in Fig. 3B, these target cells were ignored by the CTLs as well as by DCs electroporated with MUC1-IVT.

To further confirm and extend our findings, we included autologous DCs pulsed with the HLA-A3-binding BCR-ABL–derived peptide A3 and used them as targets in a standard 51Cr-release assay using the CTLs as above that were induced from an HLA-A3+ donor. As shown in Fig. 3C, these cells were not lysed by the CTLs as well as DCs loaded with an irrelevant HIV peptide and the cell line K-562.

The cytolytic activity of the CTLs against AML cells was inhibited in cold target inhibition assays using CML-RNA–transfected DCs, demonstrating that they recognized epitopes shared among these malignancies (Fig. 3D). In contrast, DCs transfected with irrelevant
EGFP-IVT could not inhibit the recognition of AML-derived antigens. DCs electroporated with PBMC-RNA were not recognized by the CTLs. These findings indicate that DCs transfected with Ph+ CML total RNA could induce CTLs that recognize antigens expressed or shared by Ph+ and Ph− myeloid leukemias.

Taken together, the results obtained by the transfection of DCs with Ph− whole cell RNA show that although bcr-abl transcripts, among others in the total RNA preparations, are delivered to the APCs, no BCR-ABL–specific CTLs are induced. Based on this experimental approach and results, BCR-ABL does not represent an immunodominant antigen when competing against all other proteins processed and presented by the DCs.

**Induction of BCR-ABL–specific CTLs by DCs transfected with pure bcr-abl-IVT.** In the next set of experiments, we sought to analyze the immunogenicity of BCR-ABL protein and the possibility to induce BCR-ABL–specific CTLs by DCs electroporated with an excess of pure full-length bcr-abl-IVT that codes for the entire BCR-ABL protein. Monocyte-derived DCs were generated from the PBMCs of an HLA-A3+ donor. As shown in the standard 51Cr-release assay (Fig. 4A), the generated CTLs did lyse DCs electroporated with bcr-abl-IVT as well as DCs electroporated with Ph− K-562-RNA or with Ph− CML-RNA. However, they did not recognize DCs electroporated with Ph− AML total RNA or with irrelevant EGFP-IVT as a control. D, for cold target inhibition experiments, unlabeled DCs electroporated with K-562 total RNA or with irrelevant EGFP-IVT were added to 51Cr-labeled DCs electroporated with Ph− AML total RNA at the cold/labeled target ratio of 20:1.

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These data indicate that DCs electroporated with an excess of pure full-length bcr-abl-IVT could result in the presentation of BCR-ABL–derived peptides on the cell surface that can induce BCR-ABL–specific CTLs that recognize Ph+ target cells in vitro.

**In vitro induction of IFN-γ-secreting CD8+ T lymphocytes by Ph+ CML-RNA–transfected DCs.** The long-term objective of our study is the development of a DC-based adoptive immunotherapy for patients with CML. As a result of our previous experiments,
it was apparent that BCR-ABL is not an immunodominant antigen when competing against other proteins to be processed and presented by Ph+ whole-cell RNA-transfected DCs in vitro. However, BCR-ABL could be immunogenic when it is used as a single antigen, thus, probably avoiding competition with other tumor antigens. Hence, the remaining questions were, whether functional DCs could be developed ex vivo from Ph+ CML patients under IFN-α treatment, corresponding to the clinical conditions and, which antigens are capable of stimulating a specific T cell response.

To address these questions, we generated monocyte-derived DCs under serum-free conditions from two CML patients who were in cytogenetic complete remission during IFN-α treatment. These DCs were then used as APCs for the induction of T lymphocytes after electroporation with RNA purified from the autologous Ph+ CML cells cryopreserved prior to therapy.

In order to analyze the induction and expansion of polyclonal T cell responses in vitro after two restimulations, CD8+ T lymphocytes were isolated using magnetic bead technology, and analyzed in an IFN-γ ELISPOT assay using PBMCs electroporated with various species of RNA as APCs. The RNAs coded for various proteins that were described to be recognized by CTLs in myeloid leukemias such as MUC1, survivin, PR3 or prame. As negative controls, we used PBMCs transfected with EGFP-IVT.

As shown in Fig. 5, transfection of DCs with Ph+ CML-RNA-stimulated CD8+ T cells that specifically recognized autologous PBMCs electroporated with CML-RNA, survivin-IVT, PR3-IVT, or prame-IVT. In contrast, CD8+ T lymphocytes showed lower IFN-γ response to PBMCs electroporated with bcr-abl-IVT as compared with PBMCs electroporated with whole CML-RNA, PR3, or prame-IVT, confirming the results obtained in previous experiments.

These data show that in patients with CML in complete cytogenetic remission during IFN-α treatment, there was some reactivity of CD8+ T lymphocytes against BCR-ABL in IFN-γ ELISPOT assays, which was weaker as compared with PR3- or prame-directed responses, supporting the observations obtained in healthy donors. Thus, DCs electroporated with Ph+ CML-RNA induce a strong T lymphocyte response specific for the PR3- and prame-derived epitopes suggesting the immunodominance of the presented epitopes. Interestingly, according to our results, survivin might represent a tumor-associated antigen in CML.

**In vitro induction of CD4+ T lymphocytes by Ph+ CML-RNA–transfected DCs.** As described previously, vaccination of CML patients with BCR-ABL–derived peptides is able to induce peptide-specific CD4 cell proliferation (14). To analyze the possible induction of BCR-ABL–specific CD4+ T helper cells in our experimental approach, we electroporated DCs generated from a healthy donor with total RNA isolated from Ph+ CML blasts and cultured them together with purified autologous CD4+ cells. After one restimulation, CD4+ T cells were incubated with DCs transfected with various species of RNA, and thymidine incorporation was measured on day 5 by a 16-hour pulse with 3H-thymidine. As shown in Fig. 6, in line with the results using CD8+ CTLs, CD4+ T cells stimulated with DCs pulsed with cell lysates from the Ph+ leukemia, K-562, and used as APCs for the induction of antigen-specific CTLs. The cytolysis activity of the generated CTLs was determined after two weekly restimulations in a standard 51Cr-release assay. A, DCs electroporated with K-562 total RNA or with EGFP-IVT as a control were used as target cells. Inhibition of HLA class I or class II was done by incubating DCs prior to the assay with anti-HLA class I or II antibodies. B, for cold target inhibition experiments, unlabeled DCs electroporated with Ph+/C0 AML total RNA or with irrelevant EGFP-IVT as a control were added to 51Cr-labeled DCs electroporated with Ph+ AML total RNA at the cold/labeled target ratio of 20:1.
recognized CML-RNA–transfected DCs, DCs transfected with K-562-RNA or with AML-RNA, but not DCs transfected with pure bcr-abl-IVT, CLL-RNA, or Ph+ ALL-RNA. The induced proliferative response could be completely blocked using antibodies directed against HLA class II molecules, thus confirming that the elicited CD4+ T cell response was HLA class II–restricted.

Discussion

Almost all CMLs are characterized cytogenetically by the translocation t(9;22) (q34;q11) which gives rise to one of two intracellular chimeric p210 BCR-ABL fusion proteins. These fusion proteins are unique to the CML blasts. Therefore, this leukemia offers the opportunity to be specifically targeted by the respective therapeutic approach. Accordingly, drug development and pharmaceutical research are focusing on this molecular abnormality. Imatinib mesylate (Glivec or Gleevec, Novartis), a potent inhibitor of the ABL tyrosine kinase, was recently developed. This compound has since been shown to produce impressive results in the treatment of patients with chronic phase CML (32–36).

Another strategy to make use of this tumor-specific determinant is to induce a T lymphocyte response against cells expressing the hybrid BCR-ABL protein. In 1995, it was shown for the first time that leukemia oncogene–derived breakpoint peptides are able to bind HLA class I molecules (26). Subsequently, four peptides, all derived from the bcr exon 3-abl exon 2 (b3a2) junction of the bcr-abl fusion gene, were found to be able to bind with either intermediate or high affinity to purified HLA-A3, -A11, -B8, or both -A3/-A11 molecules. In vitro, these peptides were able to induce specific class I–restricted cytotoxic T cells in the PBMCs of four out of seven HLA-matched healthy donors (10). Furthermore, it was shown that a 17-amino acid peptide corresponding to the b3a2 breakpoint region could also be presented by DRB1*0401 molecules and peptide-specific DR2- and DR4-restricted CD4+ T cell lines could be induced in vitro (37). However, it remained unclear whether the intracellular BCR-ABL protein is processed by the proteasome and derivative peptides are presented on the surface of fresh CML cells. Yotnda et al. identified a HLA-A2.1–restricted peptide from the b3a2BCR-ABL junctional region which bound to HLA-A2.1 molecules, stimulated in vitro–specific CTL responses using PBMCs from both healthy donors and patients with CML, and (c) was spontaneously processed and presented at the surface of CML cells in some patients. Such antigen presentation resulted, for particular patients, in sizeable

Figure 3. CTL induction by DCs electroporated with Ph+ CML blasts' total RNA. Immature monocyte-derived DCs generated from healthy donors (HLA-A2+/A3+) were electroporated with total RNA isolated from the Ph+ CML blasts and used as APCs for the induction of antigen-specific CTLs. The cytolytic activity of the generated CTLs was determined after two weekly restimulations in a standard 51Cr-release assay. A, DCs electroporated with K-562 total RNA or with total RNA isolated from Ph+ AML blasts or isolated from Ph+ CLL cells were used as target cells. DCs electroporated with pure bcr-abl-IVT (B) or K-562 cells (C) as well as DCs loaded with the BCR-ABL–derived HLA-A3-binding peptide A3 or with an irrelevant HIV peptide were included in the assay. D, for cold target inhibition experiments, unlabeled DCs electroporated with CML blasts' total RNA or with irrelevant EGFP-IVT as a control were added to 51Cr-labeled DCs electroporated with Ph+ AML total RNA at the cold/labeled target ratio of 20:1.
expansion of CML-specific CTLs. Certain b3a2 BCR-ABL–specific, in vitro–induced CD8+ CTL lines from different individuals were able to lyse either HLA-matched transfected K-562 (b3a2 BCR-ABL+) or autologous CML leukemic cells. This indicated that tumor cells could process the p210 b3a2 BCR-ABL fusion molecules with efficient surface antigenic peptide presentation by either HLA-A3, -A11, -A2.1, or -B8 class I molecules. However, in ~75% of cases, the investigators failed to elicit in vitro–sizable CTL responses in healthy donors as well as in patients with CML (9).

Scheinberg et al. used the four previously described peptides (10) for s.c. vaccination of patients with CML in phase 1 and phase 2 trials. These vaccines consistently generated peptide-specific CD4+ responses and only weak CD8+ responses (evidenced by ELISPOT IFN-γ assays) in patients with HLA-A*0301 or HLA-A*1101. In line with these studies, Bocchia et al. detected stable residual disease after Gleevec or INF-α treatment in CML patients vaccinated with a cocktail of five b3a2 breakpoint-derived peptides, delayed-type hypersensitivity responses that seemed to correlate with the degree of residual disease reduction (14). However, the authors did not definitively show that these immune responses were capable of recognizing native CML blasts or progenitors (11, 38). Accordingly, Moldrem et al. showed that in CML blasts, overexpressed antigens (proteinase 3) apparently contribute to the surveillance of disease in INF-α-treated patients (17). Interestingly, this antigen is obviously involved in the pathogenesis of CML.

Figure 4. Induction of BCR-ABL–specific CTLs by DCs transfected with pure bcr-abl-IVT. Immature monocyte-derived DCs generated from healthy donors (HLA-A2+/-A3+) were electroporated with pure bcr-abl-IVT and used as APCs for the induction of antigen-specific CTLs. The cytolytic activity of the generated CTLs was determined after several weekly restimulations in a standard 51Cr release assay. A, DCs electroporated with K-562 total RNA or with total RNA isolated from Ph+ CML blasts or isolated from Ph-/C0 AML cells as well as DCs electroporated with pure bcr-abl-IVT were used as target cells. DCs electroporated with MUC1-IVT were included as a control. B, DCs transfected with Ph+ ALL-RNA or Ph-/C0 CLL-RNA as well as DCs loaded with the BCR-ABL–derived HLA-A3 peptide were included in the assay. DCs loaded with irrelevant HIV-peptide or K-562 cells served as a control.

Figure 5. In vitro induction of IFN-γ-secreting CD8+ T lymphocytes by Ph+ CML-RNA–transfected DCs. Monocyte-derived DCs from two Ph+ CML patients (A and B) were generated under serum-free conditions and used as APCs after electroporation with RNA purified from the autologous CML blasts. After two restimulations, the CD8+ T lymphocytes generated were analyzed in an IFN-γ ELISPOT assay using PBMCs electroporated with different species of RNA as APCs. As controls, PBMCs transfected with MUC1- or an irrelevant EGFP-IVT were used. Columns, mean number of spots per 1 × 10^6 CD8+ T cells of duplicate wells.

Figure 6. In vitro induction of CD4+ T lymphocytes by Ph+ CML-RNA–transfected DCs. DCs generated from a healthy donor were electroporated with total RNA isolated from Ph+ CML blasts and cultured together with purified autologous CD4+ cells. After one restimulation, CD4+ T cells were incubated with DCs transfected with various species of RNA and proliferation was measured by 3H-thymidine incorporation on day 5. Inhibition of HLA class I or class II was done by incubating DCs for 1 hour prior to the assay with anti-HLA class I or II antibodies. DCs or CD4+ T cells alone were used as controls. Values represent counts per minute (cpm) after incubation of 1 × 10^6 CD4+ T cells with 1 × 10^5 RNA-electroporated DCs. Columns, mean of triplicate wells; bars, ±SE.
of Wegener’s granulomatosis as autoantibodies recognizing the PR3 protein can be found in the sera of these patients (18, 39).

Our study focused on the possible role of the BCR-ABL antigen in the induction of antigen-specific CTLs on the platform of RNA-transfected monocyte-derived human DCs in vitro.

It was recently shown that DCs transfected with RNA coding for a tumor-associated antigen or even whole tumor RNA are able to induce potent antigen and tumor-specific T cell responses directed against multiple epitopes. The latter technique does not require the definition of the tumor-associated antigen or HLA haplotype of the patients and has the potential for broad clinical application. Such a polyclonal vaccine may reduce the probability of a clonal tumor escape and elicit CTL responses directed against naturally processed and presented immunodominant tumor antigens. Additional targeting of HLA class II–restricted epitopes may further amplify and prolong the induced T cell responses (reviewed in ref. 40).

Sources of Ph+ total RNA, which were used in our study, were the cell line K-562 as well as primary Ph+ CML blasts. Additionally, pure full-length in vitro–transcribed RNA encoding p210 BCR-ABL was included in our assays.

In the first series of experiments, we electroporated DCs with Ph+ K-562 total RNA and used them as APCs for the induction of CTLs. These CTLs very efficiently lysed DCs electroporated with K-562 RNA as well as DCs transfected with Ph+ CML-RNA. The lysis was blocked by the addition of anti-HLA I monoclonal antibodies, indicating that recognition of the target cells was HLA class I–restricted. Remarkably, however, these CTLs also efficiently recognized DCs transfected with total RNA isolated from Ph+ AML blasts. Instead, DCs electroporated with Ph+ ALL-RNA as well as electroporated with Ph+ CLL-RNA or with pure bcr-abl-IVT were spared. DCs electroporated with RNA purified from normal CD34+ or PBMCs (buffy coat cells) were also not recognized by these CTLs. Hence, these experiments revealed that DCs electroporated with Ph+ total tumor RNA induce CTLs specific for antigens shared by Ph+ and Ph− myeloid leukemias but not by leukemias of the lymphoid lineage. The antigens recognized by these CTLs are not presented on normal CD34+ progenitor cells but are (over)expressed on neoplastic transformed myeloid leukemia cells. Thus, in these experiments, BCR-ABL is not an immunodominant antigen against which specific CTLs are induced.

In the second series of experiments, we tested whether BCR-ABL–specific CTLs could be induced by DCs electroporated with an excess of pure bcr-abl-IVT in vitro. In fact, we were successful in the generation of BCR-ABL–specific CTLs by the RNA-based technique. In 51Cr-release assays, these CTLs efficiently lysed all Ph+ target cells or DCs transfected with Ph+ RNA. In contrast, target cells electroporated either with Ph− total RNA purified from myeloid or lymphoid leukemias were not recognized by these CTLs. These results indicate that, with this experimental design, BCR-ABL is processed by APCs and target cells, and is presented in the context of HLA class I molecules. A possible explanation for this finding is that an excess of bcr-abl mRNA delivered to the cells and translated into an excess of BCR-ABL protein prevents other proteins, by competition, from being cleaved into peptides by the multicatalytic proteasome and from being transported via transporters associated with antigen presentation into the endoplasmatic reticulum cellular compartment. In the endoplasmatic reticulum, an excess of BCR-ABL peptides primarily assembles with MHC class I molecules. These complexes are then transported to the cell surface for a predominant presentation to CD8+ BCR-ABL–specific CTLs.

We next expanded our analysis by using PBMCs from patients with CML in complete cytogenetic remission during INF-α treatment. The outstanding question was, which antigens are expressed in common by Ph+ and Ph− myeloid leukemias, but not by Ph+ lymphatic leukemias, and elicit cytotoxic T cell responses. In these experiments, we included IVTs coding for PR3, prame, or survivin. Some of them were shown to be expressed in CML and acute leukemia (15, 16, 41, 42). We found that in IFN-γ ELISPOT assays, DCs electroporated with Ph+ CML-RNA induced CD8+ T lymphocytes specific for the CML-derived RNA as well as for PR3 and prame. Interestingly, in contrast with experiments in which PBMCs from healthy donors were used, in CML patients who were in cytogenetic complete remission during INF-α treatment, BCR-ABL–specific T cells were induced by CML-RNA electroporated DCs, although, at a lower number. One reason for this could be the fact that there is a difference in the T cell repertoire in the peripheral blood of healthy donors and patients with CML treated with INF-α that might result in the expansion of T cells recognizing a different set of epitopes. Nevertheless, in line with previous experiments, BCR-ABL–directed responses were lower as compared with PR3 or prame. Interestingly, there was some activity against survivin, indicating that this antigen might also be involved in the immune responses directed against CML.

In the next set of experiments, we examined the induction of CD8+ T cells by DC electroporated total RNA isolated from Ph+ CML blasts. In line with the results obtained with CD8-mediated immune responses, we found that BCR-ABL–derived HLA class II–restricted epitopes did not substantially contribute to the stimulation of CD4 cells.

In summary, BCR-ABL is processed and presented by Ph+ cells, but is not the immunodominant antigen that induces CD8+ T lymphocytes in competition with other tumor-associated antigens. However, APCs transfected with an excess of pure bcr-abl-IVT in vitro are capable of inducing BCR-ABL–specific CTLs that efficiently lyse Ph+ target cells. These findings could contribute to the development of antileukemia immunotherapies using DCs electroporated with whole tumor-RNA or IVTs coding for defined antigens like prame, PR3, or survivin.

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References


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