Immunogenicity of a p210$^{\text{BCR-ABL}}$ Fusion Domain Candidate DNA Vaccine Targeted to Dendritic Cells by a Recombinant Adeno-associated Virus Vector *in Vitro*[1]

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ABSTRACT

Chronic myelogenous leukemia (CML) is characterized by a t(9;22) translocation, which results in the expression of chimeric BCR-ABL fusion oncoproteins that are necessary for oncogenesis, unique to the leukemic clones, and represent enticing targets for immunotherapy. As a strategy for the immunotherapy of CML, we constructed a recombinant adeno-associated virus vector encoding the p210$^{\text{BCR-ABL}}$ b3a2 variant fusion region with flanking sequences (CWRBA) and used it to express the BCR-ABL fusion region within primary human dendritic cells (DCs), the most potent antigen-presenting cells currently known. Peripheral blood mononuclear cells from healthy donors were primed and restimulated *in vitro* with autologous DCs transduced with purified CWRBA, CWRAP (negative control), or pulsed with a peptide corresponding to the fusion domain (positive control). No specific responses were generated using DCs transduced with CWRAP. In contrast, CWRBA-transduced DCs primed autologous T cells in an antigen-specific, MHC-restricted fashion to levels comparable with the positive control. CWRBA-transduced DCs elicited both cytotoxic CD4+/Th1 and CD8+ responses, although the former were more readily detected in this system. Cytotoxicity against a tumor cell line endogenously expressing the p210$^{\text{BCR-ABL}}$ b3a2 variant fusion region was also demonstrable. In addition, HLA-DRB5*0101*+DRA (DR2a) was identified as a new restriction element capable of presenting the b3a2 BCR-ABL fusion region epitope. Thus, the construct developed herein may serve as a candidate vaccine for gene-based antigen-specific immunotherapy of CML and may serve as a paradigm for the use of DCs transduced with recombinant adeno-associated virus vectors encoding multiepitope immunogens for vaccine development.

INTRODUCTION

CML[3] is characterized by a t(9;22) chromosomal translocation that results in the expression of BCR-ABL oncoproteins. These fusion proteins are both critical to leukemogenesis and generate unique epitopes that represent appealing targets for immunotherapy (1–3). Host immune responses are important in disease control and influence rates of relapse after hematopoietic stem cell transplantation. In the allogeneic setting, relapsed CML has been successfully treated by: (*a*) stopping immunosuppressive therapy; (*b*) infusion of donor immune cells (4, 5); and (*c*) treatment with immunostimulatory agents, such as

IFN (6). Laboratory studies revealed that MHC alleles HLA-B8, A3, DR4, and DR2 avidly bind BCR-ABL peptides *in vitro* and, thus, are most likely to generate anti-BCR-ABL immune responses (7–9). In addition, specific immune responses directed against cells expressing the common p210$^{\text{BCR-ABL}}$ fusion domain could be generated both with human cells (1, 2, 10–12) *in vitro* and murine models (13) *in vivo* using cognate fusion domain peptides. In a recent clinical trial, a mixture of five BCR-ABL peptides containing HLA class I and II binding motifs was administered in conjunction with a QS-21 immune adjuvant to CML patients. The vaccination was shown to be nontoxic, and elicited antigen-specific proliferative and delayed type hypersensitivity responses (14). However, cytotoxic responses against leukemic cells expressing BCR-ABL were not demonstrable, and proliferative responses occurred in a low percentage (3 of 6 receiving the highest dose of a total of 12 patients), supporting the need for additional studies and strategies.

DC, the most potent APC known, are actively being studied for vaccine development because of their critical role in the generation of immune responses against tumor cells and infectious agents (15, 16). DCs from both healthy individuals and those with CML are capable of eliciting antigen-specific MHC class I- and II-restricted immune responses after pulsing with BCR-ABL peptides, demonstrating that BCR-ABL is a reasonable target for immunotherapy (1, 10, 11). Recombinant viral vectors are also currently being studied as a means to introduce specific immunogens into APC for processing and presentation to effector lymphocytes. In contrast to the use of peptides, this strategy has the advantage of introducing proteins comprised of multiple epitopes into APC and permitting the APC to present only relevant epitopes (17). Because immune responses are restricted by the necessity of MHC-epitope binding, the introduction of multiple epitopes increases the probability that a particular MHC molecule will bind with a particular antigen and elicit an immune response. rAAV vectors have emerged as highly promising for use in gene transfer because they are not pathogenic, have a wide host range, do not express virus encoded genes, and, thus, have a comparatively low immunogenicity and have the ability to transduce nonproliferating cells. Furthermore, rAAV vectors encoding potential immunogens have been successfully used to generate immune responses in animal models (18–20). In this report we demonstrate that rAAV vectors are capable of transducing primary human DCs. To explore the potentials for development of rAAV vector-based DNA vaccines targeting the BCR-ABL fusion region, we constructed an rAAV vector, CWRBA, containing an 835-bp cDNA fragment encoding the p210$^{\text{b3a2}}$ fusion domain and flanking sequences. DCs transduced with purified CWRBA were capable of priming autologous T cells *in vitro*, and generated Th1 helper T cells and CTLs that arose from CD4+ and CD8+ precursors. A new restriction element, HLA-DRB5*0101*+DRA (DR2a), was identified, which presented the b3a2 BCR-ABL fusion region epitope to an HLA-matched CD4+ CTL clone. The construct developed herein may serve as a candidate vaccine for gene-based antigen-specific immunotherapy of CML.

MATERIALS AND METHODS

Cell Lines and Plasmids. An adenovirus-transformed human embryonic kidney cell line, 293, was propagated in DMEM; other cells were maintained in RPMI 1640. Cells were maintained in medium containing 10% FCS and 2 mM glutamine at 37°C in humidified 5% CO_2. K562, a p210^{b3a2}-positive CML cell line, was obtained from American Type Culture Collection. The homozygous HLA reference cell line MGAR was obtained from the International Histocompatibility Workshop (Seattle, WA). EBV-treated PBMCs from healthy donors were used to derive transformed B-cell lines, EBL. All of the cell lines were monitored and proven to be *Mycoplasma* free. Plasmids pRSVgptDRB1∗*1501* and pRSVgptDRB5∗*0101* were kindly provided by Eric Long (NIH, Bethesda, MD).

Oligonucleotides and Peptides. The following oligonucleotide primers were synthesized using a 394 B DNA Synthesizer (Applied Biosystems, Foster City, CA). AS [5′-TAT AGC TCT AGA CCC GGA GCT TTT CAC C-3′] corresponding to nucleotides 547–565 of the p210^{b3a2} sequence (GenBank AJ131466); SP1 [5′-TAT GAG TCG ACG CTG CTG CTT ATG TCT C-3′] corresponding to nucleotides 3060–3084 of the BCR sequence (Y00661); and SP2 [5′-TTA AGT CGA CAT GGT GGA GCT GGT GGA G-3′] corresponding to nucleotides 2738–2756 of the BCR sequence (Y00661). Another pair of primers was designed based on sequence information from GenBank (J00194) to subclone HLA-DRA: DRAUP [5′-TAT ATA AGC TTC AAA CGA GCG CCC AAG-3′] and DRADOWN [5′-CTT CTC TCG AGG AAA CAT CAT CAC-3′]. A 25-residue peptide b3a2 (IVHSA TGFKQ SS-K-AL QRPVA SDFEP, where -K- represents the new amino acid at the fusion point of BCR-ABL), a negative control peptide b3b4 (IVHSA TGFKQ SSNLY CTLEV DSFGY) from BCR and an irrelevant peptide Flu-M$_{58-66}$ (GILGF VFTL) were synthesized by the Peptide Core Facility at the City of Hope.

Generation of the rAAV-based p210^{b3a2} Fusion Domain Construct. To generate the vector CWRBA plasmid, primer AS and SP2 were used to amplify an 835 bp cDNA fragment encoding the fusion domain and flanking sequences of p210^{b3a2} from mRNA isolated from K562 cells by RT-PCR. Primers SP2 and AS were designed to incorporate a 5′-Sal I and a 3′-Xba I restriction site into the amplified product so that it could be directionally inserted into the rAAV vector pCWRSP (21) downstream of the RSV promoter. The new open reading frame encodes a 276 AA truncated protein (from the 715th AA through the 991st AA of the p210^{b3a2}, Y00661 and AJ131466). Inserted sequences were verified as correct by double strand sequencing using the City of Hope Sequencing Core Facility.

Construction of a Plasmid Encoding DRA cDNA. mRNA isolated from the homozygous cell line MGAR was amplified by RT-PCR with the primers DRAUP and DRADOWN. The amplified fragment was inserted into vector pCWRSPN (22), and the DNA sequence of six clones was determined as described above. The coding sequence of DRA from MGAR was identical to that in GenBank (XM004209) except for a G to A substitution at position 354. This is a conserved mutation that does not affect the amino acid sequence of DRA. The DRA of MGAR belongs to the HLA-DRA∗*0102* group.

DNA Transfection. Cells (293) were transfected using calcium phosphate coprecipitation per the manufacturer's protocol (CellPhect Transfection kit; Amersham Pharmacia, Piscataway, NJ). Three days after transfection, the cells were either harvested for RNA extraction or metabolically labeled for immunoprecipitation assays. K562 cells were transfected with HLA-DR plasmids using Lipofectin (Life Technologies, Inc., Grand Island, NY) as per manufacturer's instructions, then selected with G418 at 400 mg/ml for 2 weeks. G418 resistant K562 cells were monitored for DR expression by FACS, and HLA-DR bright cells were sorted for experiments.

Encapsidation of rAAV Vectors. The recombinant viral vectors and CWRAP (an equivalent control vector containing the human PLAP gene; Ref. 21), were encapsidated as described previously (23). Vectors were purified by two rounds of isopycnic cesium chloride (density, 1.41 g/ml) gradient centrifugation. Particle titers were determined by dot blot hybridization with an insert-specific probe (522 bp) with densitometric comparison to known standards. A particle titer of 10^9/ml was obtained for CWRAP and 10^{10}/ml for CWRBA. Wild-type AAV was not detectable in the resulting vector stocks as assessed by hybridization of duplicate dot blots with a wild-type AAV-specific 1.7-kb Sac II fragment probe.

Immunoprecipitation Assays. The immunoprecipitation protocol has been described in detail elsewhere (24, 25). Briefly, both 2×10^7 transfected 293 cells (60–65 h after transfection) and 2×10^7 K562 cells were metabolically labeled with 0.5 mCi of an [^{35}S]methionine and [^{35}S]cysteine mixture (Amersham Pharmacia) for 4 h. Cells were then lysed with extraction buffer [50 mM Tris hydrochloride (pH 8.0), 5 mM EDTA, 150 mM sodium chloride, 0.5% NP40, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride solution] at 4°C. The supernatants of cell lysates were precleared with normal rabbit IgG-agarose beads (Sigma, St. Louis, MO), then incubated with Ab-1, a purified rabbit polyclonal IgG specific for the BCR protein (Oncogene Sciences, Cambridge, MD) and protein-A agarose beads (Sigma) at 4°C for 16 h. The final pellets were suspended in 40 μL electrophoresis sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE in 12% polyacrylamide gels and analyzed by autoradiography and phosphorimaging.

Culture and rAAV Transduction of Human Peripheral Blood-derived DCs. Blood was obtained from healthy volunteers according to a City of Hope Medical Center Institutional Review Board-approved protocol. PBMCs were separated by centrifugation on a Ficoll-Hypaque (Amersham Pharmacia) density gradient followed by plastic adherence. Adherent cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated autologous plasma, 1000 units/ml granulocyte macrophage colony-stimulating factor (Immunex, Seattle, WA) and 500 units/ml IL-4 (R&D, Minneapolis, MN) for 7–10 days and matured in 20 ng/ml tumor necrosis factor α (R&D Systems) for 3 days as described by Romani *et al.* (26). FACS analysis was performed on the derived DCs with antibodies directed against HLA-DR, CD14, CD80, CD83, and CD86 (BD PharMingen, San Diego, CA). DCs were transduced before maturation with CWRAP or CWRBA at a MOI of 200–300 particles/cell or pulsed with peptide b3a2 at 50 μM. CWRAP transduction efficiency was determined by *in situ* human PLAP expression as described previously (23).

Generation of T-Lymphocyte Lines and Clones. Nonadherent PBMCs ($20–30 \times 10^6$) were cocultured with 1×10^6 irradiated (3000 Rads) autologous APCs in 12-well plates, in RPMI 1640 supplemented with 10% heat-inactivated autologous plasma and 2 mM glutamine. Cultures were restimulated every 7–10 days with the same APC, and 5 units/ml of recombinant human IL-2 (R&D Systems) were added from day 10 onward. After two or three stimulations, a modified split-well method (27) was used for assessing primary T-lymphocyte-specific responses. Briefly, 1×10^4 viable lymphocytes and 1×10^3 irradiated APCs were seeded per well in 96-well plates in supplemented RPMI 1640. Thirty wells were seeded for each group. After two stimulations in 96-well plates, each well was split into three equal aliquots; the contents of two aliquots were used for proliferative assays, and the third was restimulated and propagated to generate cell lines. One specific T-cell line was cloned after the sixth stimulation by the limiting dilution method. Briefly, T cells were seeded at 0.5 cells/well with irradiated allogeneic PBMCs at 5×10^4/well as feeder cells in the presence of phytohemagglutinin at 2 μg/ml. Viable clones were expanded and tested for specificity by lymphocyte proliferation and CRA.

Lymphocyte Proliferation Assay. Lymphocytes stimulated with APCs (described above) were used as responders, and different APCs were matched as either stimulators or controls. Indicated numbers of responders and stimulators, or responders and controls in 100 μL supplemented RPMI 1640 were seeded into 96-well round-bottomed plates in triplicate and cultured for 3–4 days. Then 0.5 μCi/well of [^{3}H]thymidine was added into each well and incubated for 16 h. [^{3}H]Thymidine incorporation was measured using a scintillation counter (Beckman Coulter, Fullerton, CA). The stimulation index was calculated as $(\text{cpm}_{\text{T cells + stimulators}} - \text{cpm}_{\text{T cells alone}})/(\text{cpm}_{\text{T cells + controls}} - \text{cpm}_{\text{T cells alone}})$.

CRA and Antibody Blocking Analysis. This assay was performed as described previously (28). Briefly, autologous EBL targets were pulsed with 50 μM peptide (specific epitope or control) and labeled with 100 μCi ^{51}Cr (Amersham Pharmacia) per 10^6 cells for 1.5 h. The labeled targets were seeded in triplicate into 96-well V-bottomed plates in the presence of lymphocytes at indicated E:T ratios. After incubating at 37°C for 4 h, the release of radioactive ^{51}Cr in the supernatant was counted as experimental release (R_e) in a gamma counter (Packard Instrument, Meriden, CT). Spontaneous ^{51}Cr release (R_s) was determined by incubating targets alone with medium, and maximal ^{51}Cr release (R_m) was obtained by incubating targets with medium containing 1% SDS. Specific lysis was calculated as $100\% \times (R_e - R_s)/(R_m - R_s)$. Blocking experiments were performed with or without addition of monoclonal antibody at 10 mg/ml to the target preparation and to the CRA cultures. Monoclonal

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anti-HLA-A, B, DR, DQ, or DP antibody recognizes an intralocus determinant on HLA molecules (Lab Vision, Fremont CA).

RESULTS

The rAAV-based p210^{b3a2} Fusion Domain Construct, pCWRBA, Expresses the Appropriate Transcript and Truncated Protein. To confirm expression of the appropriate transgene product from the vector plasmid, we assessed the mRNA and protein expression from pCWRBA-transfected 293 cells. Fig. 1*A* shows that the expected sized (522 bp) band generated by RT-PCR with primers AS and SP1 was present only in 293 cells transfected with pCWRBA (Fig. 1*A, Lane 5*) and K562 cells (Fig. 1*A, Lane 7*), a positive control. This band was not demonstrable in control amplifications performed without addition of reverse transcriptase (Fig. 1*A, Lanes 4* and *6*), excluding the possibility of DNA contamination. This supports expression of the inserted BCR-ABL fragment. Finally, to confirm the identity of the product, the 522-bp DNA fragment was extracted, sequenced, and found to correspond to the BCR-ABL insert in pCWRBA.

Expression of the truncated p210^{b3a2} protein was examined by radioimmunoprecipitation with the Ab-1 antibody (Fig. 1*B*), using K562 cells (positive control) and 293 cells transfected with pCWRAP (negative control). The predicted M_r 25,000 band was identified only in lysates of 293 cells transfected with pCWRBA (Fig. 1*B, Lane 4*). High molecular weight bands immunoprecipitated with Ab-1 from lysates of K562 cells (Fig. 1*B, Lane 6*) correspond to cellular BCR and BCR-ABL proteins. These results demonstrate that the correct RNA transcript, and Ab-1- (anti-BCR) specific, truncated protein were expressed in 293 cells transfected with pCWRBA.
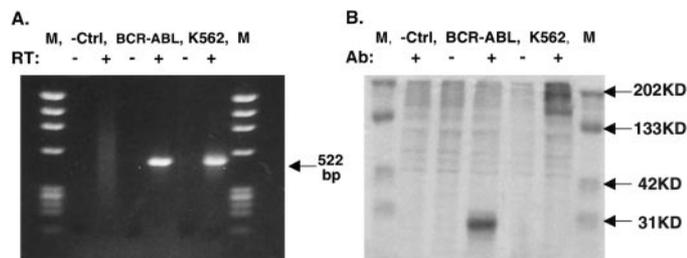

Fig. 1. Expression of the p210^{b3a2} transgene from pCWRBA. *A,* expression of the p210^{b3a2} fusion transcript: mRNA was extracted from 293 cells transfected with pCWRAP (*-Ctrl*) or pCWRBA (*BCR-ABL*), or from K562 cells and amplified by RT-PCR with primers AS and SP1. *Lanes 2, 4,* and *6* are negative controls without reverse transcriptase (*RT*). Marker (*M*) is the φX174 RF DNA digested with Hae III. *B,* expression of the truncated p210^{b3a2} fusion protein: 293 cells transfected with pCWRAP or pCWRBA, or K562 cells were metabolically labeled with a [^{35}S]methionine and [^{35}S]cysteine mixture, and immunoprecipitated using BCR-specific antibody. *Lanes 3* and *5* are negative controls without specific antibody (*Ab*). A predicted M_r 25,000 band in column 4 indicates a new Ab-1-specific protein is generated in 293 cells transfected with pCWRBA. The molecular weight maker is a Kaleidoscope Prestained Standard (BIO-RAD, Hercules, CA).

Fig. 3. Transduction of human DCs with rAAV. Human peripheral blood DCs were transduced with CWRAP, which encodes the human PLAP. Cells were stained *in situ* for human PLAP expression as described previously (23). *I. A,* untransduced controls. *B,* DCs transduced with CWRAP (MOI 200–300, particle titer). *II.* quantitative depiction of the percentage of CWRAP-transduced DCs (positive staining) isolated from 6 different healthy donors. *UTD,* untransduced; *TD1,* transduced at MOI 50–100; and *TD2,* transduced at MOI 200–300.

Isolation of Human Peripheral Blood-derived DCs and rAAV Vector Transduction. Primary human DCs generated herein were morphologically identical to those described in the literature. In addition, their strong expression of HLA-DR, CD80, CD83, and CD86, but not CD14 by flow cytometric analyses (Fig. 2) are consistent with mature DCs (26, 29). To assess rAAV transduction, DCs were transduced with CWRAP before tumor necrosis factor α maturation. Approximately 60–90% of primary human DCs isolated from multiple donors were transduced as determined by *in situ* human PLAP expression (Fig. 3).

CWRBA-transduced DCs Elicit Antigen-specific Lymphocytes in a MHC-restricted Fashion. To assess the capacity of CWRBA to induce anti-BCR-ABL immune responses, lymphocytes from healthy donors were primed and stimulated *in vitro* with CWRBA or CWRAP (as a negative control) transduced or peptide b3a2 (as a positive control of *in vitro* immunization) pulsed autologous DCs. Antigen-specific proliferative responses were analyzed by the split well
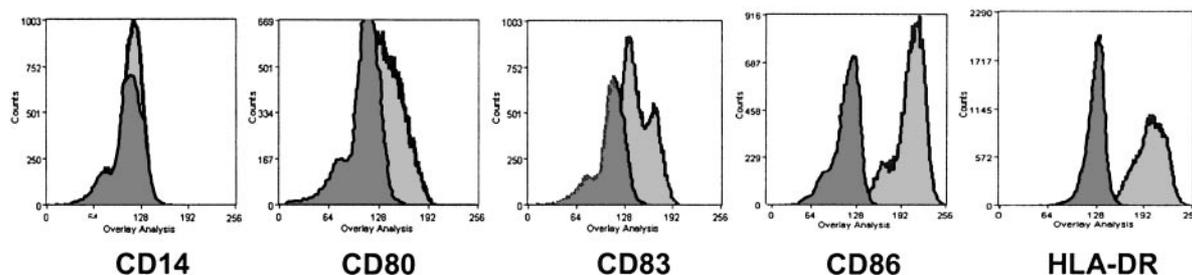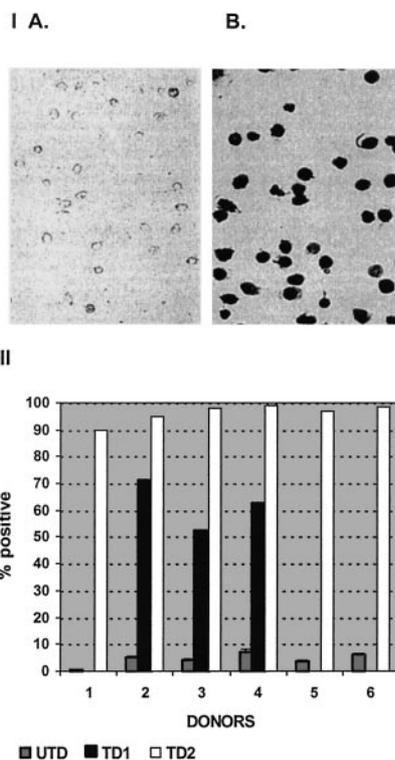

Fig. 2. Phenotypic analysis of DCs via FACS. DCs were derived from human PBMCs and analyzed by FACS with fluorescence-labeled antibodies directed against the indicated antigens. The *dark gray filled peaks* are isotype controls and the *light gray filled peaks* show cells labeled with indicated specific antibodies.
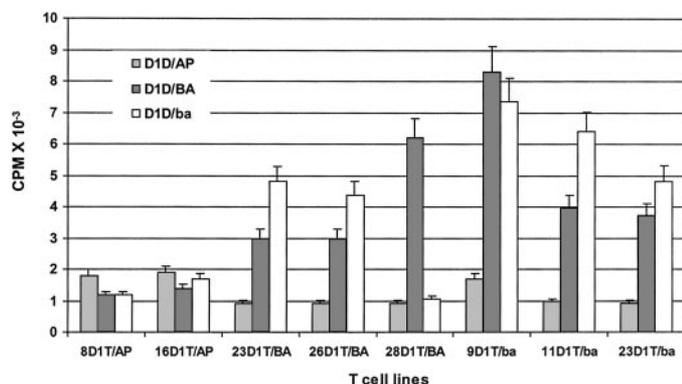
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Fig. 4. Proliferative responses of lymphocyte lines against autologous DCs antigen loaded by different techniques. Lymphocyte lines from donor D1 were generated by priming and restimulation with one of the following APCs: autologous DC (D1D) either transduced with CWRAP (*D1D/AP*), CWRBA (*D1D/BA*), or pulsed with the peptide b3a2 (*D1D/ba*), respectively. Proliferative responses were assessed in triplicate. Lymphocytes (1×10^4) as responders were cultured with 5×10^2 irradiated D1D/AP, D1D/BA, or D1D/ba as stimulators as the same as the regimen for priming and restimulation for 3 days. Data shown are representative of three experiments and expressed as the mean; *bars,* ±SD. SD varied from 1.7 to 14.1% of average, most of them <10%.

method after three to four stimulations. Cells from positive wells identified by the split method were additionally propagated as cell lines by repeated antigen stimulation. The results from one representative donor are depicted in Fig. 4. Two T-cell lines, (8D1T/AP and 16D1T/AP in Fig. 4) derived after stimulation with autologous DCs transduced with CWRAP did not show specific responses to DCs transduced with CWRBA or CWRAP, or pulsed with peptide b3a2. In contrast, five different cell lines derived after stimulation with either autologous DCs transduced with CWRBA or pulsed with peptide b3a2 displayed specific proliferative responses to both CWRBA-transduced or peptide-pulsed DCs. These data demonstrate that CWRBA-transduced DCs can present the BCR-ABL fusion domain epitope in an immunologically relevant fashion. Furthermore, one cell line, 28D1T/BA (Fig. 4), derived after stimulation with autologous DCs transduced with CWRBA, displayed specific proliferation to autologous DCs transduced with CWRBA, but not to autologous DCs transduced with CWRAP or pulsed with peptide b3a2. This cell line might recognize an immunogenic epitope existing within the flanking sequences of the BCR-ABL junction region, but additional studies are needed to map the exact epitope. A total of 5 donor results and their HLA genotypes are summarized in Table 1. Antigen-specific T-cell lines were readily generated from donors 1, 2, and 3 who possess HLA-DR alleles capable of presenting these epitopes on stimulation with CWRBA-transduced autologous DCs or peptide-pulsed DCs. In contrast, no p210^{b3a2}-specific T-cell lines were generated from donors 4 and 5, who lacked HLA class II alleles reported to be responsive to BCR-ABL epitopes (2, 11, 30, 31). Approximately 6–10% of wells analyzed showed antigen-specific proliferation in the first three donors. In contrast, no p210^{b3a2}-specific cell lines were obtained from

donors 1–5 primed with the negative transduction control. Importantly, CWRBA-transduced DCs were capable of priming MHC-matched lymphocytes *in vitro* to the equivalent extent as that of peptide-pulsed DCs, the positive control, as evidenced by the equivalent numbers of specific T-cell lines generated in both groups. This indicated that the transgene was efficiently presented by DCs transduced with CWRBA.

Phenotype of BCR-ABL Fusion Region-specific T Lymphocytes. The phenotype of BCR-ABL-specific cell lines was analyzed by flow cytometry. All of the cell lines tested were >99% CD3+ and CD56− indicating that the cells propagated in this culture system are T cells. BCR-ABL antigen-specific cell lines were primarily composed of CD4+ cells. Several nonspecific CD8+ cell lines were also obtained from the same priming events, suggesting that the culture system was permissive to the outgrowth of CD8+ T cells as well (data not shown). Although CD4+ cells were the major population in these specific cell lines, the possibility that a minor population of CD8+ cells or CD4+/CD8+ cells might be responsible for specific proliferative responses could not be excluded. To address this issue, two antigen-specific cell lines, 22D2T/BA and 23D1T/BA that specifically responded equally to both CWRBA transduced and peptide b3a2 pulsed DCs, were fractionated into CD4+ and CD8+ subpopulations using immunomagnetic beads. Fractionated cells were >99% CD4+ or CD8+, >95% viable, and were used to test specific proliferative responses to autologous peptide-pulsed EBL. Although the 25 residue fusion region peptide b3a2 is not exactly equivalent to the truncated BCR-ABL protein encoded from our rAAV vector, it is a reasonable reagent to define antigen specificity (see below). Purified CD4+ cells from these two cell lines demonstrated antigen-specific proliferation, whereas corresponding CD8+ cells did not (Fig. 5).
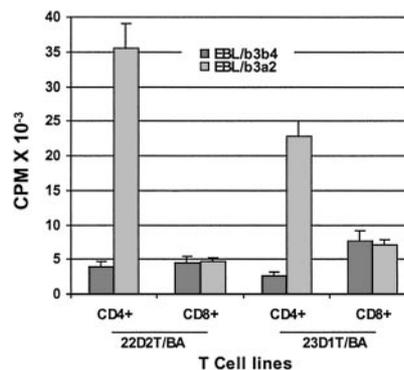

Fig. 5. Comparison of proliferative responses of CD4 and CD8 T cells from the same T-cell lines. CD4+ and CD8+ T cells were positively selected from each cell line using CD4 and CD8 isolation kits (DYNAL Inc., Lake Success, NY). Purified CD4 or CD8 cells were studied in proliferation assays using autologous EBL pulsed with the peptide b3a2 (*EBL/b3a2*) or b3b4 (*EBL/b3b4*) as stimulators. Depicted data represent the results from three experiments, and are expressed as the mean; *bars,* ±SD. This data demonstrates that CD4 but not CD8 T cells from two cell lines showed antigen-specific proliferative responses.

Table 1 *Specific T-cell lines identified by split well method*

Donor	HLA-				No. of specific lines[a]		
					DC/AP	DC/BA	DC/ba
1	A*0301;	B*0702, 3501.	DRB1***0101**, **1501**;[b]	DQB1*0501, 0602.	0	3	3
2	A*0301;	B*0702, 4001.	DRB1***1501**, 1602;	DQB1*0301, 0602.	0	3	2
3	A*0101;	B*0801.	DRB1***0301**, **1501**;	DQB1*0201, 0602.	0	2	2
4	A*1101;	B*4001.	DRB1*0801, 1201;	DQB1*0301, 0601.	0	0	0
5	A*0301, 3201;	B*0702, 1401.	DRB1*0701, 1401;	DQB1*0202, 0503.	0	0	0

[a] Split well method (see "Materials and Methods") criteria for possibly positive wells was a sample [³H]thymidine uptake after stimulation with antigen specific APC (see Fig. 4) greater than two times that of the control APC in two consecutive experiments. Specific T-cell lines from 30 wells of different groups (DC/AP, DC/BA, and DC/ba; see Fig. 4) listed in the table have been verified by triplicate proliferative assays.

[b] The bold numbers indicate alleles described in the literature that are involved in the restriction of immune responses directed against the p210^{b3a2} fusion domain.

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Table 2 *Cytokine profile of T cells responded to antigen*

T-cell cultures	Stimulation	Cytokine production (pg/ml)[a]			
		IL-2	IL-4	IL-5	IFN-γ
9D3T/BA	b3b4	20.48 ± 0.73[b]	UD[c]	UD	144.37 ± 6.50
+D3D	b3a2	97.84 ± 3.21	UD	UD	755.55 ± 26.79
29D2T/ba	b3b4	37.96 ± 1.21	UD	UD	233.58 ± 8.15
+D2D	b3a2	135.55 ± 5.57	UD	UD	915.67 ± 34.63
PBMC	PHA	ND	34.36 ± 0.99	32.08 ± 2.01	ND

[a] Cells (1×10^6) of the T-cell lines 9D3T/BA (from donor 3) or 29D2T/ba (from donor 2) were cultured in the presence of 5×10^4 irradiated autologous DC (D3D or D2D) with peptide b3a2 or b3b4 at 20 μM for 72 h. The concentration of each cytokine in the culture supernatants was determined by the Quantikine kit (R&D). PBMCs stimulated with phytohemagglutinin was included as positive control for IL-4 and IL-5.

[b] Data represent the mean ± SD from three experiments. The difference between peptide and no peptide stimulation was tested by the *t* test with the largest $P < 0.001$.

[c] UD, undetectable; ND, not determined.

We next analyzed whether CD4+ effector cells were of the Th1 or Th2 subtypes. 9D3T/BA and 29D2T/ba, as examples, were cultured for 3 days with irradiated autologous DCs in the presence of peptide b3a2 or b3b4, and culture supernatants were analyzed for the production of IL-2, IL-4, IL-5, and IFN-γ in triplicate. As shown in Table 2, the cell lines secreted IL-2 and IFN-γ in response to antigen-specific exposure. In contrast, the concentration of IL-4 and IL-5 in culture supernatants was below the level of detection. This data demonstrates that these cells were of the Th1 functional type.

rAAV-transduced DCs Generate Specific CTL Responses. Because CTLs have an important role in immune-mediated protection from tumors, we next determined whether or not priming and stimulation with CWRBA-transduced or peptide-pulsed DCs would lead to the generation of specific cytotoxic responses. All 14 of the T-cell lines derived in the first set of experiments that displayed antigen-specific proliferation against both CWRBA-transduced or BCR-ABL peptide-pulsed DCs were tested for cytotoxic activity against autologous EBL pulsed with the peptide b3a2. Specific cytotoxic responses to b3a2 were demonstrable in 4 of the 14 T-cell lines. Fig. 6*A* depicts the cytotoxic activity of 26D1T/BA, a representative cell line. Autologous EBL targets were lysed only when pulsed with b3a2 but not with the negative control peptide b3b4 or an irrelevant peptide Flu-M_{58-66}, demonstrating antigen specificity. The cytotoxic activity of 4 cell lines, which were predominantly (>90–95%) CD4+, is summarized in Fig. 6*B*. Innate cytolytic activity against K562 cells was not demonstrable supporting the lack of contamination with natural killer cells (data not shown).

Several reports have described *in vitro* generation of CD8+ CTL specific for the BCR-ABL fusion domain (10, 32, 33). To investigate whether or not the paucity of CD8+ CTL reflected low precursor frequencies, we empirically increased the scale of the split well experiment 4-fold (1.2×10^6 cells compared with 0.3×10^6 cells) using lymphocytes primed with CWRBA-transduced DCs. One antigen-specific CD8+ T-cell line (CTL-A1/CD8+ in Fig. 7) was identified among several antigen-specific CD4+ T-cell lines. Antibody blocking experiments demonstrated that this clone was HLA-B restricted (Fig. 7). These results demonstrate that CWRBA-transduced DCs were eliciting antigen-specific CD8+ CTL against the p210^{b3a2} fusion region epitope, although at a lower frequency than CD4+ CTL.

Identification of the CTL MHC Restriction Element. Cell lines generated via the split well method are unlikely to be clonal because 1×10^4 cells were plated per well. Table 1 depicts the BCR-ABL-specific cell lines representing 6–10% of seeded wells suggesting that BCR-ABL-specific cells could potentially have arisen from a single precursor. To determine the MHC restriction pattern, single cell clones were derived from the cell line 26D1T/BA by limiting dilution. Six BCR-ABL-specific clones were obtained from 120 wells. Flow cytometric studies demonstrated that 5 of these clones were CD4+ and 1 was CD8+. Antigen-specific cytotoxicity was demonstrable in 3 of the 5 CD4+ clones (B9, C9, and E5) but not with the CD8+

clone (E10; Fig. 8). Proliferative responses to peptide b3a2-pulsed autologous EBL mirrored the cytotoxicity studies (data not shown). These results provide support that the CD4+ population in cell line 26D1T/BA is responsible for the antigen-specific responses and that specific T-cells arose from a single precursor.

One representative CD4+ CTL clone, B9, was assayed for antigen-specific responses directed against b3a2 peptide-pulsed EBL or PBMCs from 5 donors to additionally define the CTL MHC restriction element. Fig. 9*A* demonstrates that clone B9 lysed b3a2 peptide-pulsed autologous targets (D1E) or allogeneic targets (D2E) sharing some HLA alleles (Table 1) but not autologous cells pulsed with a different peptide or b3a2 peptide-pulsed heterologous targets (D4–6E). These results were confirmed by proliferation assays (Fig. 9*B*). Additional loci for these donors including HLA-C, -DRB3, -DRB4, -DRB5, -DQA, and -DPB1 were identified (data not shown). Only donors 1–3 could present the b3a2 epitope to clone B9, and these three
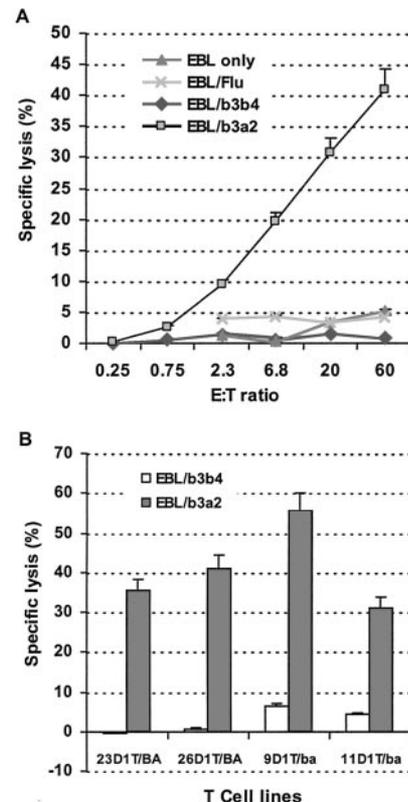

Fig. 6. Specific cytotoxicity of T-cell lines. ^{51}Cr-labeled autologous EBL were pulsed with the peptide b3a2, a BCR-derived peptide b3b4, or the Flu-M_{58-66} peptide (*EBL/Flu*) and incubated with serially diluted T cells at indicated E:T ratios in a standard 4-h CRA. *A*, cytotoxicity of a cell line, 26D1T/BA, to autologous EBL pulsed with peptide b3a2 or b3b4 at serial E:T ratios. *B*, cytotoxicity of four cell lines at E:T = 20. These data are derived from three experiments and expressed as mean; *bars*, ±SD.
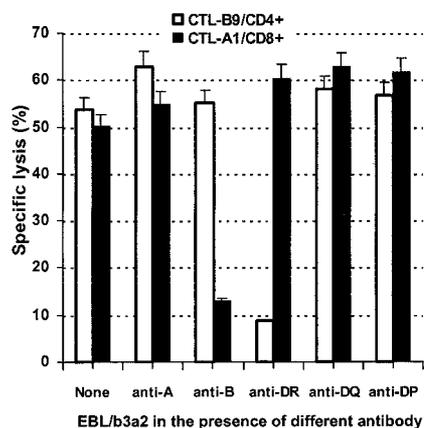
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Fig. 7. Antibody blocking analysis of CRA. Autologous EBL pulsed with peptide b3a2 were incubated with indicated anti-HLA monoclonal antibodies. The targets bound by different antibodies were cultured with CTL lines and the percentage of lysis measured. A CD8+ CTL line A1 (*CTL-A1/CD8+*) and the clone B9 (*CTL-B9/CD4+*) from cell line 26D1T/BA (see Fig. 8) were included in the blocking experiment for cross-reference to show antigen-presenting function of targets bound with an anti-HLA antibody. Data shown are at an E:T = 20 from three experiments and expressed as mean; *bars,* ±SD.
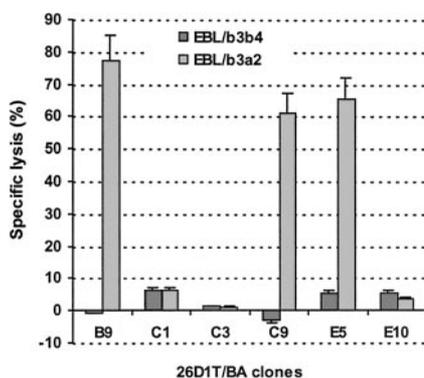

Fig. 8. Cytotoxicity of clones from cell line 26D1T/BA. ^{51}Cr-labeled autologous EBL were pulsed with the peptide b3a2 or a control peptide b3b4, and mixed with serially diluted T cells of different clones at different E:T ratios in a 4-h CRA. Data shown are at an E:T = 20 from three experiments, and are expressed as mean; *bars,* ±SD. Clones B9, C9, and E5 show antigen-specific recognition.

donors share the DRB1∗*1501*-DRB5∗*0101*-DQB1∗*0602* haplotype. To identify whether the response is DR or DQ restricted, antibody blocking experiments were performed, and the results shown in Fig. 7. B9 cytotoxicity was blocked only by an anti-DR antibody, suggesting that HLA-DR not DQ or DP was involved in B9 antigen recognition. Two variants of the DR2 complexes have been identified, DR2a and DR2b. To additionally address whether DR2a or DR2b is the antigen-presenting molecule, 293 cells were transfected with plasmids encoding DR2a or DR2b. Only 293 cells expressing DR2a were lysed by B9 after peptide pulsing with b3a2 (Fig. 9*A*). Thus, we have identified HLA-DR2a as a new HLA restriction element for p210^{b3a2} fusion region epitopes, a finding that might widen the application of immunotherapy for CML in human population.

Identification of the p210^{b3a2} Fusion Region MCE for DR2a. T lymphocytes recognize antigenic epitopes bound to MHC molecules. MHC class I molecules bind peptides of 8–11 amino acids with both amino and COOH termini tightly fixed in a structurally constrained groove. In contrast, the class II binding groove is open ended. MHC class II ligands, consisting of 9–25 residues, may extend out of the binding groove. The minimum peptide sequence that binds to an MHC molecule and elicits a CTL response is termed the MCE. To determine the MCE of BCR-ABL that was identified by HLA DR2a, a panel of

short peptides were synthesized corresponding to the p210^{b3a2} fusion region and tested as targets for the CTL clone B9 in CRA assays (Fig. 10). These studies demonstrated that CTL clone B9 recognized a MCE consisting of 9 residues (FKQSSKALQ, designated F9Q in Fig. 10, see arrow) that contained the p210^{b3a2} fusion region. Peptides that were truncated by one residue at either the COOH- (T10L) or NH$_2$-terminal (K9R) ends of F9Q were ineffective in this assay. Longer peptides that contained the full MCE sequence (F10R, F11P, G11R, and A17S) were also effective, whereas those with truncations (K11V and I15L) were not. The higher affinity of longer peptides may suggest that residues that extend out of the MHC-II binding groove can stabilize MHC peptide complexes.

The CTL Clone B9 Is Specifically Cytotoxic to the CML Cell Line K562 in an MHC-restricted Fashion. Because the B9-restricted MHC haplotype is comparatively rare, we have not yet obtained HLA-matched primary CML cells for cytotoxicity studies. Therefore, to address whether the T cells generated from priming and stimulation with CWRBA-transduced DCs could recognize leukemic cells, K562, a CML cell line encoding the full-length p210^{b3a2} BCR-ABL antigen, was transfected with DR2a or DR2b as described above. As depicted in Fig. 11, K562 cells expressing DR2a but not DR2b were recognized and lysed by clone B9, indicating that B9 recognizes an epitope presented by DR2a via an endogenous antigen presentation pathway involving native BCR-ABL protein. The absence of cytolysis of K562 expressing DR2b excludes contaminating natural killer activity.
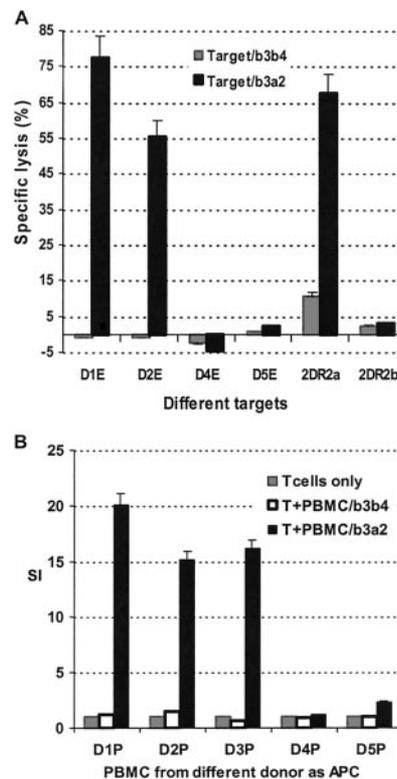

Fig. 9. Recognition profile of CTL clone B9 against a panel of APC. *A,* CRA. ^{51}Cr-labeled EBL from donors 1, 2, 4, and 5 (denoted as *D1E, D2E, D4E,* and *D5E*) or 293 transfected with HLA-DR2a or DR2b (denoted as *2DR2a* and *2DR2b*) were pulsed with the peptide b3a2 or b3b4, and mixed with serially diluted T cells. Data at a single E:T = 20 for all 6 targets were shown. *B,* proliferation assay. PBMC from donors 1–5 (denoted as *D1~5P*) were used to set up proliferation assays with clone B9 in the presence of different peptides (b3a2 or b3b4). Data are shown as stimulation indices (*SI*) derived from three experiments and expressed as mean; *bars,* ±SD.
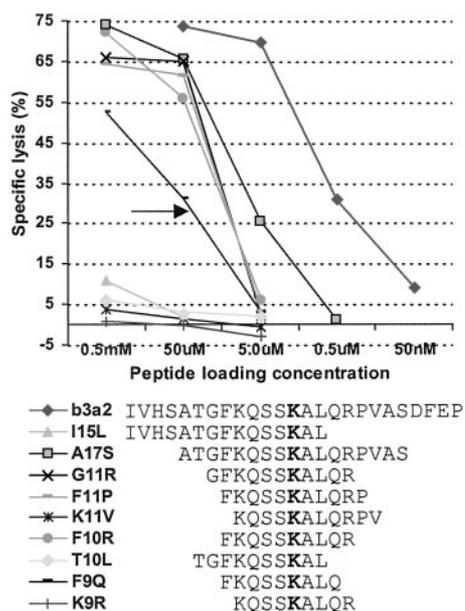
Fig. 10. Identification of the p210[b3a2] fusion region MCE for DR2a. Autologous EBLs were pulsed with various concentrations of depicted synthetic peptides corresponding to the p210[b3a2] fusion region (the *bolded K* indicates the new amino acid incorporated at the fusion point) and used as targets for CTL clone B9 at serial E:T ratios. Data shown are derived from a single E:T ratio of 20 and is representative of at least three experiments.

DISCUSSION

BCR-ABL fusion oncoproteins generated from the t(9;22) translocation are critical to the leukemogenic process, represent tumor specific antigens, and, thus, are appealing targets for possible immune intervention. In addition, immune mediated graft *versus* leukemia responses are known to influence rates of relapse after allogeneic hematopoietic stem cell transplantation for CML, additionally supporting an immune-based strategy for treatment of this disease. Therefore, it is not surprising that a variety of strategies have been developed to enhance host immune responses against BCR-ABL-expressing cells. Several reports support the potential immunogenicity of the BCR-ABL fusion region using human cells *in vitro* and in animal models *in vivo*. These encouraging results were followed by other reports, which described that human T cells derived from PBMCs stimulated with the BCR-ABL fusion region could recognize p210[b3a2]-positive marrow cells (10), p210[b3a2]-positive leukemic blasts (1, 2, 33), or APC-exposed to p210[b3a2]-containing cell lysates (6, 11). Furthermore, antigen-specific T cells were shown to inhibit CML clonogenic precursors in colony-forming assays *in vitro* (34) and demonstrated target specific cytotoxicity, although not all of the studies support this strategy (30, 35).

In these studies, we exploit the potential advantages of DCs as potent APC, rAAV vectors for expression of specific immunogens, and for the induction of an anti-BCR-ABL immune response. After first demonstrating that primary human DCs could be transduced with rAAV vectors (36–38), we constructed an rAAV encoding the p210[b3a2] fusion region and flanking sequences (CWRBA). We have shown that pCWRBA encodes an appropriate transcript and the predicted M_r 25,000 truncated protein, which was immunoprecipitable with a BCR-specific antibody. Furthermore, primary human DCs transduced with CWRBA were capable of priming autologous T cells *in vitro*. In our *in vitro* system all 15 of the antigen-specific lines generated from 3 donors were CD4+ T cells, including clones from a cytotoxic cell line 26D1T/F8. This CD4 predominance might be explained if the frequency of antigen-specific CD4+ is higher than that of CD8+ T cells and/or the BCR-ABL antigen itself promoted

CD4+ T-cell outgrowth. Indeed, the leukemia-reactive T-helper cells in CML patients have been reported to be present at a frequency of 1:4,000, whereas cytotoxic cells were present at a much lower frequency of 1:38,000 (39). Additional support was provided by Giralt *et al.* (40) who reported that CD8+ donor lymphocytes were not necessary to induce an anti-CML response in patients. It is possible that pulsing autologous EBL with the 25-mer b3a2 peptide to generate cytotoxicity targets could bias toward detection of CD4+ T cells, as MHC II molecules are less size constrained that MHC I for antigen binding. However, in these experiments T cells were primed with CWRBA-transduced DCs, in which truncated BCR-ABL was processed endogenously so only immunodominant epitopes were presented. Furthermore, initial screens for responsive T cells were based on detection of specific proliferative responses against CWRBA-transduced DCs as well as peptide-pulsed DCs. Finally, we demonstrated that our approach can generate antigen-specific CD8+ CTL as well.

Although the BCR-ABL fusion region is leukemia-specific, only targeting this region could be problematic. Because of its short length, it is possible that only limited MHC alleles are capable of binding to the BCR-ABL fusion region and inducing an immune response (2, 11, 30, 31, 32). Therefore, the binding motifs derived from the whole sequence of BCR-ABL were screened for immunogenic or immunodominant epitopes and several potential candidates were identified (41, 42). Because both the natural Bcr and Abl gene products are normal cellular components, it might not be possible to generate a vigorous immune response because of self-tolerance. However, self-tolerance may be quantitative rather than absolute (43) so that theoretically any antigen overexpressed on tumor cells might be recognized by the immune system when presented in the appropriate context, specifically in conjunction with potent APCs, MHC, and costimulatory molecules. For example, cytotoxic immune responses have been detected against epitopes of endogenous Her2-neu, a normal cellular protein, which is overexpressed in certain malignancies, particularly breast and ovarian cancer (44). BCR-ABL oncoproteins are both over- and aberrantly expressed in CML cells. In normal cells, only one allele of c-Abl or normal endogenous Bcr is expressed, whereas in CML cells, the BCR-ABL protein is produced in addition to the c-Abl and Bcr products (41). Also, c-Abl is normally found in both the nucleus and the cytoplasm, whereas BCR-ABL is exclusively cytoplasmic (45, 46). Thus, the structural alteration and delocalization of the hybrid BCR-ABL protein may modify processing and presentation of epitopes at the cell surface. Evidence for specific immune responses against p210[BCR-ABL] but not fusion domain peptides in IFN-treated CML patients in long-term has been reported (6). In
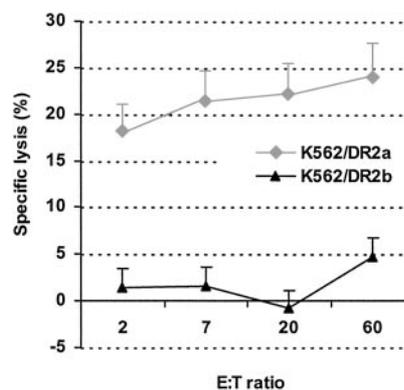

Fig. 11. CTL clone B9 specifically recognizes K562 cells expressing HLA-DR2a. K562 cells expressing HLA-DR2a or DR2b (*K562/DR2a* or *K562/DR2b*) were prepared as CRA targets for CTL clone B9. The data shown are representative of three 4-h CRA and expressed as mean values; *bars,* ±SD.

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addition to the p210[b3a2] fusion domain, our construct CWRBA included flanking sequences that contain most of the HLA-A2.1 binding motifs as well as two A2.1-restricted immunogenic epitopes defined by Buzyn *et al.* (41), and studies to evaluate this construct in DCs from HLA-A2.1 individuals are under way. In our first experiment, one cell line (28D1T/BA in Fig. 4) recognized a BCR-ABL epitope not contained in the 25 residue fusion region peptide, possibly within the flanking sequences. Additional experiments to define the epitope for this cell line are also underway. We hypothesize that vector-mediated expression of the larger BCR-ABL fusion region protein within DCs will generate multiple epitopes with increased opportunity for interaction with diverse MHC haplotypes and an augmented immune response.

T cells that exhibit antigen-specific recognition of peptide-pulsed APCs recognize tumor cells via an allogeneic HLA response rather than through an MHC-tumor associated antigen complex have been described (47). To address this issue we cloned BCR-ABL responsive T cells and carefully determined their restriction elements. One representative clone, B9, derived from the cell line 26D1T/BA was intensively studied, and a new restriction element, HLA-DRB5∗*0101*, was identified. ten Bosch *et al.* (31) previously described a BCR-ABL responsive CD4+ T cell line whose proliferative response was restricted by DRB1∗*1501*, and one which demonstrated b2a2 specific cytotoxic and proliferative responses in an HLA-DR2a (DRB5∗*0101*) restricted fashion (12). To confirm that the CTL response was BCR-ABL fusion specific, we identified the DR2a MCE for b3a2 as FKQSSKALQ, which includes the fusion region. In most cases, DRB5∗*0101* associates with DRB1∗*1501*, but it also can associate with DRB1∗*0101, 1502,* and *1601* (48). The antigen-specific T-cell lines generated after priming and stimulation with transduced mature DCs should be more likely to recognize p210[b3a2]-positive leukemic cells provided that the cells are HLA matched. Because of the relative rarity of the HLA-DRB5∗*0101* haplotype, we are still searching for HLA-matched primary CML cells for study. In the interim, we assessed the ability of the B9 clone to recognize and lyse BCR-ABL-containing cells using two strategies. In the first strategy, 293 cells were transfected with an expression plasmid encoding DR2a, the B9 restriction element, and then pulsed with the b3a2 peptide. In the second strategy, DR2a was introduced into K562 cells, a leukemic cell line, which constitutively expresses the b3a2 subtype of BCR-ABL. In both instances, B9-mediated cytotoxicity was shown to be both antigen-specific and MHC restricted (Figs. 9 and 11). Thus, we have identified a new p210[b3a2] fusion region restriction element, which could widen the application of immunotherapy for CML.

Under what clinical setting would we envision use of this construct? Although it has been reported that DCs from individuals with CML possess the t(9;22) translocation, data supporting antigen presentation of the BCR-ABL fusion region is sparse. Furthermore, it is possible that individuals with CML have an intrinsically muted response to BCR-ABL. This might be inferred by the higher relapse rates after transplantation with syngeneic as compared with allogeneic stem cells. Thus, although we are interested in examining the potential for stimulation of anti-BCR-ABL immune responses in individuals with CML, immune stimulation of the allogeneic donor before hematopoietic stem cell transplantation for CML might be a more viable strategy to prevent disease relapse. It is also clear that although drugs such as STI571 represent a major advance in tumor-specific therapy, only 40–50% of treated, IFN-resistant individuals achieve a cytogenetic response, and resistance to this agent has been reported, supporting additional investigations into other strategies for management of this disease (49). Because an immunotherapeutic strategy would be most effective in the context of "minimal residual disease," one could

easily envision combining this approach with STI571 to prevent disease recurrence.

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Immunogenicity of a p210^{BCR-ABL} Fusion Domain Candidate DNA Vaccine Targeted to Dendritic Cells by a Recombinant Adeno-associated Virus Vector *in Vitro*

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