CD8+ T-Cell–Dependent Immunity Following Xenogeneic DNA Immunization against CD20 in a Tumor Challenge Model of B-Cell Lymphoma

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ABSTRACT

The CD20 B-cell differentiation antigen is an attractive target for immunotherapy of B-cell lymphomas. In an experimental lymphoma model, BALB/c mice were immunized with mouse or human CD20 cDNA (mCD20 and hCD20, respectively) or their extracellular domains (minigenes). IFNγ secretion by CD8+ T cells against CD20 was detected in mice vaccinated with hCD20 or human minigene, indicating that hCD20−primed CD8+ T cells recognize syngeneic CD20. Systemic challenge with syngeneic A20 cells, an aggressive lymphoma, resulted in long-term survival in a subset of immunized mice. Overall long-term survival was 14% in groups vaccinated with human minigene versus 4% in control groups (P < 0.001). CD8+ T-cell depletion during the effector phase completely abrogated this effect. Antibodies against a recombinant mouse CD20 protein produced in insect cells were detected in mice immunized with hCD20 DNA and human and mouse minigene, but not in mice receiving mCD20 DNA. These results show that active immunization with xenogeneic DNA vaccines can induce CD8+ T-cell–dependent immunity against CD20.

INTRODUCTION

Passive immune therapy for B-cell lymphomas has become a standard of care following the introduction of rituximab, a chimeric IgG1 monoclonal antibody (mAb) directed against the extracellular domain of CD20 (1–4). CD20 is a 33- to 37-kDa nonglycosylated B-cell–specific membrane phosphoprotein (2, 5, 6) predicted to traverse the plasma membrane four times, forming a single 44-amino-acid extracellular loop between the third and fourth transmembrane domains (7). A mouse CD20 homologue has also been characterized (8). There is no known definitive function or ligand for CD20. CD20-null mice generated independently by two groups had no defects in B-cell development and maturation (9, 10).

CD20 has been shown to be an excellent target for passive immune therapy. The chimeric mouse-human mAb rituximab and radiolabeled variants are being used to treat indolent and aggressive B-cell lymphomas and more recently autoimmune diseases (3, 11–14). Rituximab binds to malignant clonal B cells and also to normal CD20+ B cells. This activity is mediated largely through one or more immunologic mechanisms, including complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (15–17). Other mechanisms, including caspase and src family kinase–mediated activation of apoptosis, potentially contribute to the activity of rituximab (18–20); however, the function of anti-CD20 mAbs seems to be largely dependent on activation of innate immunologic mechanisms (21).

CD20 is expressed on normal B lymphocytes at most stages of their differentiation, and therefore there is a differentiation antigen. Among tumor antigens, differentiation antigens are frequently recognized by the immune system. Targeting this category of tumor antigens with vaccine strategies is theoretically difficult, because immune tolerance is presumably maintained.

We have previously shown that tolerance to a self-antigen can be overcome by immunizing with an altered form of the antigen, such as closely related gene products, including those derived from a different species (xenogeneic). In the B16 mouse model of melanoma, we have shown that xenogeneic DNA immunization with differentiation antigens such as tyrosinase, TYRP-1, TYRP-2, and gp100 breaks tolerance to the native corresponding self-antigen and that this results in enhanced survival from a lethal tumor cell challenge as well as autoimmune (22–25).

In the present study, we used naked DNA immunization to target the CD20 antigen in a BALB/c mouse lymphoma model. Mice were immunized with plasmids encoding mouse or human CD20. Based on the hypothesis that truncated peptides might be processed and presented more efficiently, we also tested plasmids expressing only the extracellular domains of mouse and human CD20 (minigenes). We show that mice immunized with DNA encoding the human extracellular domain of CD20 mount a T-cell–mediated immune response to mouse CD20. In addition, we identified an H2-Kd−restricted epitope in mouse CD20. Furthermore, we observed a small but significant survival advantage in mice challenged i.v. with a supralethal dose of A20 lymphoma cells.
compared with mice immunized with full-length mouse CD20 DNA or its extracellular portion, and this antitumor activity was CD8+ dependent.

MATERIALS AND METHODS

Mice. Female BALB/c mice were purchased from the National Cancer Institute (Frederick, MD) or from Taconic (Germantown, NY). All mice entered the studies between 7 and 12 weeks of age. Animals were cared for under institutional animal care protocols.

Cell Lines and Tissue Culture. A20 is a murine B-cell lymphoma cell line originally derived from a BALB/cAnN mouse (ref. 26; American Type Culture Collection, Manassas, VA). A20 cells were passaged in vivo, expanded, and frozen in aliquots. Mouse BALB/c-3T3 fibroblasts were obtained from American Type Culture Collection and cultured in DMEM medium (Mediatech, Herndon, VA) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L glutamine (all Life Technologies, Grand Island, NY), and 10% FCS (HyClone, Logan, UT). Cells were routinely tested for Mycoplasma.

Plasmids. The murine CD20 cDNA was cloned by reverse transcription-PCR from C57BL/6 mouse spleen and from A20 cells, and the human counterpart was cloned from PBMCs from a healthy donor using blood drawn under a protocol that was approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center (MSKCC). Briefly, total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) from 10^7 cells. The cDNA was synthesized using oligo dT primers and the DNAcycle kit (Invitrogen), as described in the kit instructions. Sequences of the CD20-specific primers modified with appropriate restriction sites at the 5' end (in both cases BamHI in the downstream primer and XbaI in the upstream primer) were for human cDNA, the 5' primer 5'-ATGGATCCCTGGAG-GAAATGCTGAGAG-3' and the 3' primer 5'-GTGCTA-GATGCTGCTATGTGCAAGAGA-3'; for mouse cDNA, the 5' primer 5'-AAGGATCCAGTACACTGTGAAGTCTCTT-CAAGTAC-3' and the 3' primer 5'-CCTCTAAGAGGTTAAGGAGCATACTT-3'. All primers were synthesized by Operon, Alameda, CA. The PCR products were digested with BamHI and XbaI and subcloned into the mammalian expression vector pCR3 (Invitrogen) to create the plasmids pCR3-hCD20 and pCR3-mCD20. CD20 identities were confirmed by DNA sequencing.

For the construction of the minigenes, the inserts coding for the extracellular domain of CD20 were cloned by PCR into the plasmid pNERIS (provided by M. Engelhorn, Department of Immunology, Sloan-Kettering Institute, New York, NY). The minigenes were placed downstream of the COOH terminus of an endoplasmic reticulum insertion sequence (ERIS) derived from the adenovirus E3/19K glycoprotein, driven by the cytomegalovirus promoter. The downstream primer 5'-CTAGCTGCACTGTCGAGGAG-3' and upstream primer 5'-TGCTCGAGTCAACAGTACCTGATAGGGAGGAG-3' (mCD20 sequences bold) were used to amplify the mCD20 extracellular domain, SHFLKRMRELLI QTSKPVVDYDCEPTNSSSEKNSSTQYC. These PCR products were subcloned into the pNERIS vector to create pNERIS-hmini and pNERIS-mmini, respectively. The plasmids were sequenced for accuracy.

Transfections. BALB/c-3T3 cells were transfected with 1 μg of each of the plasmids pCR3, pCR3-hCD20, pCR3-mCD20, pNERIS-hmini, and pNERIS-mmini using the LipofectAMINE reagent (Invitrogen), as per manufacturer’s instructions. Transfected cells were collected 48 hours after transfection in medium containing 1 mg/ml G418 (Life Technologies). Expression of the transfected DNA was confirmed by reverse transcription-PCR.

Gene Gun Immunization. DNA immunization was done as previously described (27). Briefly, 100 μg DNA (plasmids described above) were mixed with 50 μg of 0.95 to 2.6 μm diameter gold particles in the presence of 0.05 μmol/L spermidine. CaCl_2 (400 mmol/L final concentration) was added dropwise and the DNA was allowed to precipitate onto the gold particles. The beads were then washed and resuspended in 100% ethanol. This solution was instilled into plastic Tefzel tubing, the ethanol was gently removed by aspiration, and the gold particles were dried with a flow of nitrogen gas at 400 ml/min. The tube was then cut into 1.27 cm bullets. The gold-DNA complexes were injected into the shaved and depilated skin of anesthetized mice using a helium-driven gene gun (Powderject, Inc., Madison, WI). Four injections at 400 p.s.i. were delivered to each mouse, one per abdominal quadrant, for a total of 4 μg DNA per mouse. Mice were immunized in this fashion weekly, for a total of 5 weeks for tumor challenge experiments and for a total of 3 to 5 weeks for ELISPOT assays.

Tumor Challenge and Follow-up. Five days following the last of five immunizations, each mouse received 5 × 10^6 A20 cells resuspended in 200 μL PBS by tail vein injection. Three weeks after the tumor challenge, the baseline weight was measured for each mouse. Symptoms of tumor take included the development of ascites and consequently weight gain, and palpable abdominal masses arising from the liver and/or spleen. A few mice developed hind leg paralysis. Daily weights were recorded beginning the third week after tumor challenge. Mice were sacrificed when overtly sick or when body weight increased by ≥3 g within a 2-week period.

Generation of Heteroclitic Epitopes and Peptide Immunization. The murine CD20 amino acid sequence was analyzed for potential K4 epitopes using a computer program developed in our laboratory.2 A total of 21 octamers and nonamers were selected and optimized for enhanced HMC I binding using a computer algorithm that specifically selects single residue mutations able to enhance the predicted binding half life of the peptide-MHC complex (ref. 28; Table 1). Peptides were


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Table 1  Comparison of predicted binding half-life of optimized heteroclitic peptides and the respective wild-type counterparts for binding to K<sup>d</sup> MHC I molecules

<table>
<thead>
<tr>
<th>mCD20 Peptides</th>
<th>Wild-type sequence</th>
<th>Predicted half-life (s)</th>
<th>Optimized sequence</th>
<th>Predicted half-life (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C74Y</td>
<td>ICLSVWVYPL</td>
<td>115</td>
<td>YLYSWVYPL</td>
<td>5,760</td>
</tr>
<tr>
<td>I125Y</td>
<td>GILSISMDI</td>
<td>96</td>
<td>GYILSISMDI</td>
<td>4,800</td>
</tr>
<tr>
<td>G186Y</td>
<td>LGILSAML1</td>
<td>96</td>
<td>LYILSAML1</td>
<td>4,800</td>
</tr>
</tbody>
</table>

NOTE: Amino acid residues in bold represent the altered predicted MHC binding sites.

synthesized (Genemed Synthesis, Inc., San Francisco, CA), resuspended in 100% DMSO at a concentration of 40 mg/mL, and diluted with PBS (2 mg/mL, final concentration). For immunization, equal volumes of peptide stock and Titermax (Sigma, St. Louis, MO) were vortexed for 20 minutes to create an emulsion with a final peptide concentration of 1 mg/mL. This emulsion (20-30 μL) was injected in the foot pad of anesthetized mice. ELISPOT assays were done 1 week after immunization. Alternatively, a booster was given 3 weeks after the first immunization and the ELISPOT was done 5 to 7 days later.

ELISPOT Assays. Groups of five mice were immunized thrice at weekly intervals. Five to 7 days after the last immunization, CD8<sup>+</sup> T cells were isolated by positive selection by incubation with 20 μL anti-mouse CD8 immunomagnetic beads (Miltenyi Biotec, Inc., Auburn, CA) and passed through MACS columns (Miltenyi Biotec). Upon elution with 4 mL MACS buffer (PBS with 0.5% bovine serum albumin), cells were washed and resuspended in fresh medium. CD8<sup>+</sup> T cells (5 × 10<sup>5</sup> to 2 × 10<sup>6</sup>) were plated in each well in a 96-well plate coated with 5 μg/mL anti-IFNγ antibody (Mabtech, Cincinnati, OH) in PBS. Cells were incubated at 37°C for 1 hour before overlaying 5 × 10<sup>3</sup> 3T3 irradiated transfectants to each well. In some experiments, untreated or lipopolysaccharide (LPS)–activated naïve B lymphocytes were used as APCs. LPS activation was achieved by incubation of B cells in PBS for 1 hour at 37°C (4 μg/mL LPS). The ELISPOT assays were carried out at 37°C for 20 hours. Plates were washed extensively with PBS/0.05% Tween (PBST). Biotinylated anti-IFNγ antibody (Mabtech) diluted 1:1 μg/mL in PBS/0.5% bovine serum albumin was added to the wells and incubated at 37°C for 2 hours. Plates were washed six times with PBST. Spots were developed with avidin-biotin complex reagent (Vectors Laboratories, Burlington, CA) for 30 minutes at room temperature and with AEC in 0.5% dimethyl formamide (4-minute incubation). Plates were washed with tap water, air dried, and then read with a Zeiss ELISPOT reader by an independent investigator.

CD4<sup>+</sup> and CD8<sup>+</sup> T-Cell Depletion. Mice were immunized five times, as described. On days −1, 0, +7, +14, and +21 they were injected i.p. with 500 μg of monoclonal rat anti-mouse anti-CD4 or anti-CD8 antibodies (bioreactor supernatant of clones GK1.5 and 53-6.72, respectively, produced at the MSKCC Monoclonal Antibody Core Facility). Intravenous tumor challenge with 5 × 10<sup>5</sup> A20 cells was given on day 0, as described.

Production of Recombinant Mouse CD20 (recCD20) Fusion Protein. The baculovirus expression system was used to produce a mouse recCD20 protein. Mouse cDNA was amplified by PCR from pCR3-mCD20 using the same downstream primer described above, with a BamHI site at the 5' end, and the following upstream primer 5'-CGGAATTCAAGGAGTTAAG-

GAGCGATCTC-3' containing an EcoRI site (underlined; mCD20 sequence in bold characters). All reagents for the baculovirus expression system were purchased from Invitrogen. The PCR product was digested with BamHI and EcoRI and subcloned into pBlueBacHis2 (b), a vector that expresses CD20 with a 6xHis tag at the C terminus and a unique epitope tag (named Xpress) fused at the NH<sub>2</sub> terminus, under the control of the polyhedrin promoter. The plasmid was transfected into S9 cells using the CellFECTIN reagent together with linearized Bac-n-Blue viral DNA, following the manufacturer’s instructions. The final result is recombinant virus with a functional lacZ gene for plaque screening and purification by plaque assay. Two positive recombinant plaques were isolated and propagated. High-titer viral stock was prepared from one of the two clones (named B9). The infected cell lysate and the supernatant were tested at several time points by Western blot analysis, using the anti-Xpress mAb. The expected size of the fusion protein was ~40 kDa.

Immunoprecipitation/Western Blot and ELISA. After five immunizations, serum was collected from each mouse by eye bleed. Serum (5 μL) was incubated with 10 μL recCD20 overnight at 4°C. Anti-mouse IgG agarose beads (Sigma; 10 μL) were added to the mixture and incubated for 1 hour at 4°C. Beads were washed five times with 10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, and 1% NP40 (TNEN); five times with 1/10 strength TNEN; and finally, five times with water. Proteins were analyzed by 4% to 20% gradient SDS-PAGE under reducing conditions and then transferred to Immobilon membranes (Millipore, Bedford, MA). The membranes were blocked overnight with 5% nonfat dry milk in TBS-0.05% Tween (TBST), washed with TBST, and then probed with anti-Xpress antibody diluted 1:5,000 in TBST/3% bovine serum albumin. After additional washes with TBST, the membranes were incubated with horseradish peroxydase-labeled anti-mouse IgG (Sigma) diluted 1:10,000. After the final wash in TBST, membranes were incubated with the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ), according to the manufacturer’s instructions.

For ELISA assays, 96-well plates (Nunc, Naperville, IL) were coated with 1 μg recCD20 protein per well in PBS overnight at 4°C. The wells were blocked with 300 μL 2% bovine serum albumin in PBS for 2 hours at 37°C. The plates were washed in PBS, and 50 μL mouse serum diluted 1:20, 1:200, and 1:2,000 in 2% bovine serum albumin/PBS were added to duplicate wells. The plates were incubated at room temperature for 60 minutes and washed; 50 μL of a 1:5,000 dilution of goat anti-mouse immunoglobulin G and immunoglobulin M antibody conjugated to peroxide (Jackson ImmunoResearch, West Grove, PA) was added to each well. After 1-hour incubation at room temperature, plates were washed, and 50 μL of TMB peroxide substrate (BioFX,
RESULTS

Immunization with Xenogeneic hCD20 DNA Elicits T-Cell Responses against Cells Expressing mCD20. CD8+ T cells isolated from immunized mice were tested for response to wild-type mCD20 by IFNγ ELISPOT assays. Groups of three to five mice were immunized three to five times on a weekly schedule. CD8+ T cells isolated from mice immunized with the extracellular domain of mCD20 were able to recognize not only 3T3 cells expressing hCD20, but also 3T3 cells expressing wild-type mCD20 and its truncated form, as well as syngeneic A20 tumor cells. The response to wild-type mCD20 was detectable after five immunizations (Fig. 1B), but was minimal after three immunizations (Fig. 1A). The specificity of the assay was shown by the lack of IFNγ production in response to untransfected 3T3 cells or 3T3 cells transfected with control vector, as shown in Fig. 1B. These results were highly reproducible.

Antibody Responses against Recombinant mCD20 in Mice Immunized with Xenogeneic CD20 or with Truncated Forms of Human and Syngeneic CD20. Sera from mice immunized with full-length mCD20 or hCD20 or with mouse or human minigenes were tested for antibody responses to mCD20. For this purpose, the baculovirus system was used to produce a fusion protein containing mCD20 flanked by two tags, the HISx6 tag for easy purification and the Xpress tag for sensitive antibody recognition (see MATERIALS AND METHODS). By these means, precipitating antibodies recognizing recombinant mCD20 protein were identified in the sera of most, although not all, mice immunized with the mouse minigene, full-length hCD20, and human minigene. In contrast, no antibodies were detected in the sera of mice immunized with the control vector or the full-length mCD20 (Fig. 2). Interestingly, the presence and level of precipitating serum antibodies did not correlate with long-term survival (data not shown).

In addition, postimmune sera were diluted 1:20, 1:200, and 1:2,000 and tested in ELISA assays against the same recombinant protein (recmCD20) or against peptides spanning the extracellular domain of mCD20. No antibody responses were detected by this assay (data not shown). These results are consistent with low titer immunoglobulin G antibody responses against syngeneic mCD20.

Immunization with hCD20 minigene Rejects Lethal Tumor Challenge in a Small but Measurable Proportion of Mice. Plasmids expressing full-length mouse and human CD20 and truncated, extracellular domain of CD20 (minigenes) were used to immunize BALB/c mice followed by challenge with a lethal dose of A20 lymphoma cells. A20 is an aggressive lymphoma that is resistant to anti-CD20 mAb-based therapies in mice3 or anti-CD20 antibodies induced by active CD20 peptide immunization (29). Mice were immunized five times at weekly intervals followed by challenge with 5 × 104 A20 cells i.v. 5 days after the last immunization. Manifestations of disease included development of ascites, palpable abdominal masses corresponding to liver and spleen tumor nodules, and occasionally paralysis of the hind limbs. Mice were taken off the study at the first objective sign of lymphoma progression.

3 T.F. Tedder, personal communication.
In initial experiments, tumor-free survival of groups vaccinated with full-length mouse or human CD20, or empty vector pCR3 were compared. The group vaccinated with pCR3-hCD20 showed a minimal delay (mean, 4-day delay) in the onset of signs and symptoms of tumor and did not achieve a statistically significant survival advantage over mice treated with mouse CD20 or the control vector. To test the possibility that truncated forms of the protein would result in unstable protein products that enhanced antigen processing and presentation to T cells, in the subsequent experiments we included two additional groups of mice vaccinated with mouse or human CD20 minigenes.

Whereas mice vaccinated with hCD20 showed again a minimal delay in tumor onset, mice immunized with the human minigene showed a 10% to 30% long-term survival following the tumor challenge. This improvement in tumor-free survival was consistently reproducible. A representative experiment is shown in Fig. 3A. Pooled data from seven consecutive experiments are shown in Fig. 3B. The significant trend in delayed tumor onset and protection was confirmed, with a median event-free survival of 48 days for mice vaccinated with hCD20 minigene versus 38 days for mice that received only empty vector (P < 0.001). Furthermore, a tumor-free long-term survival of 14% for the

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**Fig. 2** Analysis of humoral autoimmune response upon vaccination with CD20, full-length, or minigenes. Sera from mice immunized five times were incubated with recombinant mouse CD20 fusion protein (recCD20). Immune complexes were bound to anti-mouse immunoglobulin G beads and analyzed by Western blot using an anti-Xpress epitope antibody to detect the precipitated protein. A, goat anti-mouse CD20 was used to immunoprecipitate recCD20 (arrow, first two lanes). Sera from mice immunized with control vector only (n4) and with mCD20 (sera from four individual mice, M1-M5) did not contain precipitating anti-CD20 antibodies. - negative control, sample incubated without recCD20; +, sample incubated with recCD20. B, three out of five mice immunized with mouse CD20 minigene (MM6-MM10) produced antibodies able to immunoprecipitate recCD20. C and D, sera from five mice immunized with the human CD20 (H1-H5) or the human minigene (HM1-HM5) were analyzed.
hCD20 group versus 4% in the control group was observed at day 90 following tumor challenge ($P = 0.029$). Five long-term survivors were boosted at day 100 post-tumor challenge and then rechallenged with $10^5$ A20 cells (a 2-fold higher tumor load). None of the rechallenged mice developed tumors after >100 days (data not shown).

**Tumor Rejection Requires CD8$^+$ T Cells during the Effector Phase.** The role of T-cell subsets in mediating tumor immunity was investigated. Following immunization, and just prior to a tumor challenge, CD4$^+$ and CD8$^+$ T cells were depleted by i.p. administration of anti-CD4 and anti-CD8 mAbs. Depletion of CD8$^+$ T cells induced a slight acceleration of tumor growth in nonimmunized mice, indicating that A20 lymphoma alone elicits a minimal, ineffective CD8$^+$ T-cell immune response (Fig. 4A). More notably, mice vaccinated with the human minigene died at a significantly faster rate if CD8$^+$ T cells were depleted compared with the immunized, nondepleted control (Fig. 4B). CD4$^+$ T-cell depletion did not have a significant effect, although a trend towards enhanced survival was observed in some experiments, suggesting a minimal role for CD4$^+$ T-cell regulation in moderating CD8$^+$ T-cell responses.

**Identification of a MHC Class I Epitope Capable of Eliciting T-Cell Responses In vitro.** We examined the amino acid sequence of mCD20 for potential peptide epitopes that might be presented by class I MHC K$^b$ molecules (28). All potential epitopes were predicted to have low affinity for MHC I. Heteroclitic peptides are variant antigens with enhanced binding to MHC molecules that elicit T-cell responses against the original nonmutated wild type peptide. We used a computer

**Fig. 3** Kaplan-Meier survival curves for mice immunized with full-length mCD20 or hCD20, mouse or human minigenes (mmCD20 or hmCD20) or pCR3 empty vector. After five weekly vaccinations, BALB/c mice received a lethal tumor challenge of $5 \times 10^5$ A20 cells, given i.v. (15 mice per group). A, most mice immunized with vector control and wild-type mCD20 and who received tumor challenges died from lymphoma by day 40 post-challenge. Of the mice immunized with human minigene, 20% survived beyond 90 days ($P = 0.002$; no mouse recurred after 60 days). B, disease-free survival data from seven consecutive experiments were pooled and analyzed. There were 78 mice in the control group (dashed line, pCR3null) and 81 mice in the experimental group (continuous line, hmCD20). Median disease-free survival of mice immunized with hmCD20 was significantly longer ($P < 0.001$). Overall survival was also significantly improved ($P = 0.029$).

**Fig. 4** Depletion of CD4$^+$ or CD8$^+$ T cells during the effector phase of the immune response. Mice ($n = 18-20$ per group) were depleted of CD4$^+$ or CD8$^+$ T cells via intraperitoneal injection of anti-CD4 and anti-CD8 monoclonal antibodies as described in MATERIALS AND METHODS. Depletion of CD4$^+$ or CD8$^+$ T cells was started 2 days before tumor challenge. A, mice were immunized five times weekly. At 50 days, 50% of the mice immunized with the human minigene (hmCD20) were alive and free of tumor, while only 5% of the CD8$^+$ depleted mice immunized with the minigene (hmCD20, CD8$^-$) were tumor-free. $P < 0.0005$ for CD8$^+$ depleted versus not depleted. B, nonimmunized mice were depleted of CD4$^+$ or CD8$^+$ cells. $P = NS$, for all groups.
program designed in our laboratory to select, from the mouse CD20 amino acid sequence, three heteroclitic peptides with predicted enhanced binding to MHC I alleles based on single amino acid substitutions at anchor residues. Mice were immunized with three heteroclitic peptides (C74Y, I125Y, and G186Y; Table 1) and IFNγ secretion by activated CD8+ T cells was measured by ELISPOT assay.

Freshly isolated splenocytes from syngeneic mice, either unstimulated or activated with bacterial LPS, were used as APCs for endogenous presentation of candidate CD20 antigenic peptides. Splenocytes pulsed with heteroclitic peptides were also assessed. Immunization with peptide I125Y plus TiterMax adjuvant elicited CD8+ T cell responses to the I125Y heteroclitic peptide presented on syngeneic splenocytes (with or without activation by LPS). CD8+ T cells also reacted to unpulsed, LPS-activated splenocytes, which are rich in CD20+ B cells, consistent with recognition of the corresponding native, endogenous CD20 peptide (Fig. 5A). Following a booster immunization, CD8+ T cell activation was also observed in response to unstimulated naïve B cells (Fig. 5B).

Furthermore, CD8+ T cells isolated from mice immunized once with the heteroclitic peptide I125Y reacted with B cells pulsed with the native CD20 peptide counterpart (Fig. 6). These results indicate that lack of T-cell reactivity to the CD20 self peptide, which suggested tolerance, was overcome by using an epitope with predicted higher affinity for MHC I molecules.

DISCUSSION

Active immune therapy for lymphoma is directed against the B-cell clone idiotype (30–33). Humoral and cellular responses have been observed in about half of the patients treated with this form of therapy, but its limitation is that the vaccines have to be custom-made for each patient. Therefore, we sought to investigate the approach of active DNA immunization for lymphoma against the shared B-cell differentiation antigen CD20.

Passive administration of mAbs against CD20 is an effective therapy in patients with B-cell lymphoma. Preclinical studies by Clynes et al. (34) showed that mice deficient in the inhibitory FcγRIIB molecule were more susceptible to antibody-dependent cellular cytotoxicity in response to rituximab treatment, whereas mice deficient in the activating receptor FcγRIII did not clinically respond to treatment with rituximab, indicating that Fc receptor–dependent mechanisms are fundamental for the activity of innate effectors in vivo. Roberts et al. (29) previously reported that immunization of mice with CD20 peptides corresponding to the human or mouse extracellular sequences conjugated with keyhole limpet hemocyanin induced specific antibodies against the native mouse CD20 protein, including antibody-mediated complement killing of CD20+ cell lines, and stimulated specific proliferation and interleukin-4 and IFNγ secretion by mouse splenocytes.

These studies show that CD20 can be recognized by the immune system and antibodies against CD20 can provide effective immunity against B-cell lymphoma. These observations in turn lead to questions about the possibility of cellular immune responses against CD20. One rationale for searching for T-cell reactivity to CD20 is based on experiments showing that the combination of passive mAb treatment or active induction of antibody in combination with active induction of T-cell responses can be more effective than either modality alone (35, 36). With regard to T-cell recognition, Grube et al. have shown that the immune repertoire of healthy individuals and patients with chronic lymphocytic leukemia contains T cells that can recognize and lyse cells presenting HLA-A*0201–restricted CD20 peptides. However, peptide-stimulated T cells did not lyse CD20+ B cells or lymphoma cells (37).

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In the present experiments, we sought to determine whether DNA immunization against CD20 induced immunity against a transplantable syngeneic lymphoma. We showed that mice immunized with the human extracellular domain had a significant delay in the development of signs and symptoms of lymphoma and about 20% of immunized mice survived beyond 90 days. Whereas resulting in only a small improvement in survival, this effect was highly reproducible and statistically significant. Mice immunized with full-length human CD20, although showing some delay in tumor appearance, had no significant protection against the tumor challenge. This observation shows discordance between the level of IFNγ production by antigen-specific CD8+ T cells and in vivo survival benefit. Because we observed this discrepancy between absolute number of responding T cells and tumor rejection in other models, we think the number of antigen-specific T cells in draining lymph nodes in response to vaccination may not be the best measure of efficacy. Activated T cells may undergo activation-induced cell death during response or may accumulate at the tumor sites. A detailed analysis of T cell responses over time and at sites of tumor might clarify this question.

The exact mechanism by which the minigenes are processed and presented to MHC molecules is not known. We have observed that truncations of some antigens are necessary and sufficient to protect against a tumor cell challenge and to confer autoimmunity. Therefore, we postulate that the DNA encoding CD20 fused to an endoplasmic reticulum insertion sequence produces a short-lived, unstable protein product in the endoplasmic reticulum that does not move to the Golgi complex, but is rather translocated to the cytosol for degradation by the proteasome complex, providing peptides for recognition by CD8+ T cells. In addition, the adenovirus-derived leader sequence could provide help for T-cell epitopes. The extracellular domain of CD20 contains several weak potential class I MHC epitopes and is the least conserved region between the mouse and human sequences. We have recently reported that a heteroclitic peptide of the melanosomal differentiation antigen gp100 is necessary and sufficient to induce protective tumor immunity in the B16 melanoma model (38). We hypothesize that the human CD20 minigene similarly results in the expression of one or more heteroclitic epitopes that are more efficiently processed and/or presented than the corresponding mouse peptides.

To elucidate the mechanisms responsible for tumor protection after xenogeneic immunization with the CD20 minigene, both cellular and humoral immune responses were studied. Depletion of CD8+ T cells efficiently abrogated the effect of the immunization, indicating an important role for this T-cell subset. Furthermore, IFNγ secretion by CD8+ T cells activated by exposure to human and mouse full-length CD20 and minigenes was detected after immunization with the human sequences, both full-length and extracellular domain. It is not clear at this time why the stronger CD8+ T-cell responses seen after immunization with full-length CD20 cDNA do not correspond to better tumor protection in vivo. It does suggest, however, that the mechanism implicated in the tumor protection effect of the human minigene is not purely CD8 dependent. One possibility is that T-cell help elicited by leader sequences during immunization enhances CD8+ T-cell responses against CD20. Although we did not observe a role for CD4+ T cells during the effector phase, ERIS sequences could be crucial for T-cell help during immunization. For CD8+ T-cell recognition, we identified the I125Y peptide epitope in CD20. However, the I125Y epitope lies outside the extracellular domain of the protein. Because little or no immunity was detected in vivo after immunization with full-length hCD20, but only after immunization with the hCD20 minigene, we think that immunization with this minigene may lead to intramolecular epitope spreading in recognition of the I125 native epitope. However, no T-cell responses against the native I125 peptide were detected following immunization with either full-length hCD20 or human minigene. We are presently searching for possible MHC I peptide ligands to Kd and Dd MHC I molecules within the extracellular domain of CD20, despite low predicted binding of potential peptides within this region.

Antibody responses were studied by assessing the serum of immunized mice for the presence of antibodies against recombinant mouse CD20. Our findings of antibodies in all groups except for that immunized with full-length mCD20 suggest that these antibodies might be directed against linearized epitopes that are not present on the native folded protein. We could not detect any reactivity against normal B cells in the sera of immunized mice, but the background was high and precluded a definitive answer. The number of circulating B cells, as

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5 M. Engelhorn, unpublished data.

6 Unpublished observations.
assessed by cytometry, was not affected in any of the groups tested, likely because native CD20 on normal B lymphocytes was not recognized (data not shown).

Clearly, the issue of actively immunizing against an antigen expressed on normal B cells presents the problem of long-term consequences triggered by an autoimmune response. In our model, the number of circulating B cells was found to be unaltered by the vaccine. In addition, there were no obvious signs of autoimmunity and the surviving mice did not show any signs of infection for up to 1 year after the tumor rejection.

These initial studies show that CD8+ T-cell responses can be elicited against CD20 and that CD8+ T-cell–dependent immunity elicited by DNA immunization can lead to tumor rejection in a limited number of hosts. We are currently exploring a series of strategies for augmenting this immune response, including the use of plasmid vectors that allow better antigen processing, incorporation of heteroclitic epitopes, and application of cytokine and chemokine genes as adjuvants.

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CD8+ T-Cell–Dependent Immunity Following Xenogeneic DNA Immunization against CD20 in a Tumor Challenge Model of B-Cell Lymphoma

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