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*J Immunol* 2002; 168:3983-3991; doi: 10.4049/jimmunol.168.8.3983
http://www.jimmunol.org/content/168/8/3983

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Immunization with a Recombinant Adenovirus Encoding a Lymphoma Idiotype: Induction of Tumor-Protective Immunity and Identification of an Idiotype-Specific T Cell Epitope

Anne C. Armstrong,* Said Dermime,* Christopher G. Allinson,* Tapan Bhattacharyya,* Kate Mulryan,† Karin R. Gonzalez,* Peter L. Stern,† and Robert E. Hawkins2*

The Ig Id of a B cell lymphoma is a tumor-specific Ag, although as a self-Ag it is likely to be a weak immunogen. Provision of a foreign gene may enhance the immunogenicity of the idiotype. Viral vectors allow highly efficient transfer of genetic material and are themselves innately immunogenic. We have investigated the ability of recombinant adenoviral vectors, encoding the idiotypic gene with or without fusion to the human Fc region, to produce anti-idiotypic Ab- and T cell-mediated responses in a syngeneic BALB/c A20 murine lymphoma model. The idiotypic \( \text{V}_H \) and \( \text{V}_L \) sequences were assembled as a single chain variable fragment (scFv) and adenoviral vectors encoding the A20 scFv (Ad.A20) and A20 scFv linked to the Fc fragment of human IgG1 (Ad.A20hFc) were constructed. A single immunization of BALB/c mice with Ad.A20hFc but not Ad.A20 induced a specific anti-idiotypic Ab response. T cell lines generated from mice vaccinated with either vector displayed specific cytotoxicity, proliferation, and IFN-\( \gamma \) release against a syngeneic dendritic cell line transduced using a retroviral vector to express the A20 scFv idiotype (XSS2.A1.A20). Importantly, both T cell lines lysed the A20 lymphoma cells. An immunodominant H-2K\( ^d \)-restricted CD8+ T cell peptide, DYWGQGTEL (A20[106–114]), was identified as a naturally occurring A20 scFv epitope. A single immunization with Ad.A20hFc but not Ad.A20 provided protection in >40% of animals challenged with a lethal dose of the A20 tumor line and was more effective, in this model, than a previously optimized plasmid vaccine. The Journal of Immunology, 2002, 168: 3983–3991.

The idiotypic Ig determinants of B cell lymphomas, formed by rearrangement of \( \text{V}_H \) and \( \text{V}_L \) genes, are tumor specific and therefore suitable targets against which to direct immunotherapy. Idiotype Ags are unique; therefore, any anti-idiotypic strategies must be tailored to individual patients. In a murine model, vaccination with idiotypic protein leads to Ab and T cell responses capable of protecting mice against tumor challenge (1–3). Clinical trials in patients with low-grade B cell lymphomas have demonstrated the ability of idiotypic protein mixed with an immunological adjuvant and GM-CSF to induce idiotype-specific immune responses (4), with some patients achieving molecular remissions.

Preparing individual protein vaccines for patients is time consuming and expensive. Advances in molecular biological techniques have allowed the development of rapid methods to isolate tumor-derived V genes (5) and assemble them as a single chain variable fragment (scFv), with the two chains separated by a linker peptide to allow the scFv to fold properly.

Initial attempts at using DNA vaccines encoding the scFv alone induced only low levels of anti-idiotypic immunity (6, 7), necessitating the use of methods to enhance the immunogenicity of the vaccines. Immunization with DNA encoding a fusion protein of the idiotype to a foreign Ig constant region and GM-CSF was more effective, with the vaccine efficacy comparable to an equivalent protein vaccine (8). Fusion of the idiotypic DNA to fragment C of tetanus toxoid to provide Th cell epitopes also appeared to improve the vaccine, inducing strong protection against tumor challenge (9).

Although anti-idiotypic cytotoxic T cells have been generated against human B cell idiotypes (10–12), in mice the main effector mechanism appears to be the induction of anti-idiotypic Abs (13). In murine models where protective immunity is dependent on the existence of CD8+ or CD4+ T cells (14–18), the existence of a cytotoxic anti-idiotypic T cell has yet to be demonstrated. T cell-mediated immunity may be particularly important against tumor cell variants that no longer express cell surface idiotype, and are therefore resistant to anti-idiotypic Abs, but are able to continue to produce idiotypic peptides to present to T cells. Until it is clear exactly which effector mechanisms are needed to provide therapeutic immunity, it is prudent to investigate the best methods of inducing both cell-mediated and humoral immunity.

Recombinant adenoviral vectors allow more efficient and reliable gene transfer than naked DNA vaccines. In a number of animal models adenoviral vectors have been shown to induce both humoral and cellular immune responses to a variety of Ags, including tumor Ags (19, 20). Adenoviral vectors owe part of their efficacy to their innate ability to infect eukaryotic cells. They are also highly immunogenic, and expression of the viral genes may act as an immunological adjuvant.
It has recently been demonstrated that vaccination of mice with idiotype-encoding adenoviruses leads to an anti-idiotype Ab response and protection from tumor challenge (21), both dependent on inclusion of a human Ig constant region. In this report we have investigated the ability of recombinant adenoviral vectors to induce both anti-idiotype humoral and cellular immunity. In an animal model we compare the efficacy of adenoviral vectors expressing the idiotype alone, or the idiotype fused to human Fc (hFc), at inducing anti-idiotype immunity. Both humoral responses and cytotoxic CD8+ T cells were generated and an immunogenic idiotypic T cell epitope was defined. The cytotoxic T cells were able to specifically lyse both target cells expressing the lymphoma idiotype and the B cell lymphoma line itself. Finally, we demonstrate that anti-idiotype immunity induced by the adenoviral vector expressing Id-hFc fusion is an efficient means of inducing tumor protection and is more effective, in this model, than a previously optimized plasmid vaccine (9).

Materials and Methods

Mice and cell lines

Six- to 8-wk-old female BALB/c mice were bred and housed under specific pathogen-free conditions. All procedures were conducted in accordance with British Home Office guidelines. A20, a BALB/c B cell lymphoma line originally derived from a spontaneous reticulum cell neoplasm (22), was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cell line was cultured in complete RPMI 1640 medium (10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 50 μM 2-ME). XS52, a dendritic cell (DC) line established from newborn BALB/c mouse epidermis, was grown in complete RPMI 1640 medium supplemented with recombinant murine GM-CSF at 20 U/ml (R&D Systems, Minneapolis, MN) and supernatants were collected from the syngeneic fibroblast cell line NS47 (5% v/v). Both cell lines were kindly provided by A. Takashima (University of Texas Southwestern Medical Center, Dallas, TX) (23). The 293T tumor cell line (ATCC), the HeLa cell line (ATCC), and the Cre8 cell line (kindly provided by S. Hardy, Somatix, Alameda, CA) were grown in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. All media were obtained from Sigma (Dorset, U.K.), and all supplements were from Life Technologies (Paisley, U.K.).

Identification of the idiotype from the A20 cell line and generation of A20 single chain (A20 scFv) constructs

H and L chain variable sequences from the A20 cell line were obtained by inverse PCR amplification from total A20-derived RNA using primers based on published H chain genes for VH (24), and with V gene family-specific primers from VL. Following cloning and sequencing, the idiotypic sequence were identified and assembled to encode a scFv with a flexible linker sequence, in a two-step procedure, as previously described (5). Appropriate cloning sites were added to the 5′ end of the A20 scFv to allow subcloning of the A20 scFv into eukaryotic and prokaryotic expression vectors. For expression from adenoviral, plasmid eukaryotic, and retroviral vectors, the leader sequence, the vector expression, and the leader sequence of the human costatin-M gene was used (25).

The assembled A20 scFv was cloned into the plasmid pVAC2.FrC as a NotI-XhoI fragment for expression of the A20 scFv as a fusion protein with the fragment C (FrC) of tetanus toxin (pVAC2.A20.FrC) (9).

The A20 scFv was also cloned into the adenoviral shuttle vector, pAdlox (kindly provided by Dr. S. Hardy) as a ClaI-NolI fragment for production of recombinant adenoviruses. Two A20-scFv-expressing vectors were generated. One contained only the A20 scFv (pAd.A20). The second vector (pAd.A20.hFc) contained the A20 scFv linked to the C fragment of human IgG1, which itself was generated as a NotI-XhoI fragment by RT-PCR from total human PBMC-derived RNA. These vectors allow the expression of the A20 scFv alone (pAd.A20) or as a fusion protein with hFc (pAd.A20.hFc). A third adenoviral vector, containing the scFv B1.8 (25), specific for the hapten 4-hydroxy-3-nitrophenyl acetyl/4-hydroxy-3-iodo-phenylacetyl, also as a fusion with hFc (pAd.B1.8.hFc), was generated for use as a control scFv.

For retroviral expression, the A20 scFv and the B1.8 scFv were also cloned as ClaI-NolI fragments into the vector rKat (26), into which an internal ribosome entry site (IRES) and the cDNA of enhanced green fluorescent protein (GFP) had previously been cloned (rKat.A20.IRES.GFP and rKat.B1.8.IRES.GFP) (33).

For bacterial expression, the A20 scFv was cloned into a pUC119-based vector (5) with the pel b leader sequence removed by restriction enzyme digestion (NcoI-HindIII) to allow intracellular localization of the A20 scFv. The expressed A20 scFv protein contained a c-myc and a hexahistidine tag to allow for detection of the scFv by Western blotting and purification using a metal affinity column.

Generation of recombinant adenovirus expressing the A20 scFv

Ad.A20 and Ad.A20hFc

Replication defective E1-E3-deleted Δ5 adenovirus was kindly provided by Dr. S. Hardy. Recombinant viruses were constructed as previously described (27) by cotransfection of Cre8 cells with Δ5 viral DNA and Δ5I-digested pAdlox.A20 or pAdlox.A20.hFc. Recombinant viruses were passaged twice in Cre8 cells to reduce contamination of residual Δ5 adenovirus. Cre8 cell lysates were prepared by freeze-thawing the cells three times, and recombinant adenoviral particles were purified on CsCl gradients and titrated in cytotoxic effect assays.

Confirmation of A20 scFv expression from the plasmid pVAC2.A20FrC and the adenovirus Ad.A20hFc

To confirm the expression of the A20 scFv from pVAC2.A20.FrC and Ad.A20hFc, plasmid DNA was purified from bacteria and isolated using a Gene Clean kit (BIO101, Venta, CA). The plasmid DNA was analyzed by agarose gel electrophoresis, and confirmed by sequencing. Specific primers were designed for the A20 scFv and the hFc, and used to detect the presence of the gene of interest on agarose gels. The A20 scFv and hFc proteins were expressed in 293T cells and purified using a metal affinity column. A20 scFv and hFc proteins were detected by Western blotting of cell lysates using a 1/1000 dilution of rabbit anti-mouse (Sigma), as appropriate, before developing.

Production of A20 Ig and A20 scFv protein

Idiotype IgG2a, secreted from A20 cells, was concentrated from culture supernatant by precipitation with 50% saturated ammonium sulfate and purified on a HiTrap Protein G HP column (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. Purified A20 Ig was analyzed by Western blotting, as described.

For bacterial expression of A20 scFv, the pUC119.A20 plasmid was transformed into Escherichia coli strain BL21, grown from single colonies on an OD 600 of 0.9, and induced with 1 mmol isopropyl-D-thiogalactopyranoside (BDH Laboratory Supplies, Poole, Dorset, U.K.) for 18 h at 30°C. Bacterial pellets were sonicated for three cycles of 30 s in the presence of 8 ml urea (BDH Laboratory Supplies), followed by ultracentrifugation at 100,000 × g for 0.5 h. The protein was resolved by re-suspending in alkaline dialysis buffer (50 mM KH2PO4, 50 mM NaCl, 1 mM EDTA (pH 10.7) for 0.5 h, followed by titration to pH 8. The protein was dialyzed overnight in PBS, concentrated in a Vivapore 10/20 column (Sartorius, Epsom, U.K.), and purified using an ECL detection system.

Protein samples were fractionated by reducing SDS-PAGE (15%) and electrophoretically transferred onto nitrocellulose. The blot was blocked for at least 1 h at room temperature with blocking buffer (5% milk powder in PBS (PBS/0.1% Tween 20)), and probed for 1 h with the detecting Ab (HRP-conjugated anti-hFc (Sigma) or polyclonal antitoxetan serum from immune individual or 9E10 anti-myc monoclonal, diluted 1/20 in blocking buffer). The blot was washed three times in PBS and developed using an ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.) or incubated with an HRP-conjugated anti-hFc or HRP-conjugated goat anti-mouse (Sigma), as appropriate, before developing.

Retroviral transduction of XS52 cell line

Recombinant retroviruses were produced by transient CaPO4 transfection of 293T cells with rKat.A20.IRES.GFP or rKat.GFP as a negative control (Cell Genesys, Foster City, CA). The plasmid pKAt, which encodes the retroviral packaging genes. Retrovirus-containing supernatants were harvested after 48 and 72 h, filtered through 0.45-μm pores, and used immediately.

For retroviral transduction 1 × 105 XS52 cells/well were plated in a six-well plate and allowed to adhere. A total of 1 ml XS52 medium was
then replaced with 1 ml of viral supernatant containing 2 μg/ml polybrene (Sigma), and the cells were incubated overnight at 37°C/5%CO2. The retroviral supernatant was removed, the cells were incubated with fresh medium for 8 h, and the retroviral transduction process was then repeated.

Cloning of XS52 A20 scFv cell line

XS52 cells retrovirally transduced with rKat.A20.IRES.GFP were plated at decreasing concentrations from 5 cells/well, with a feeder layer of 105 irradiated NS47 cells/well (7000 rad), to a total volume of 200 μl XS52 medium in U-bottom 96-well tissue culture plates. The XS52 clones were initially screened for GFP expression using a fluorescent microscope and then analyzed by flow cytometry. One clone (XS52.A1.A20), which showed high expression of GFP as well as DC markers, was selected for use in T cell assays. Confirmation of A20 scFv transcription by XS52.A1.A20 was achieved by PCR amplification of total cellular cDNA using oligonucleotide primers specific for the A20/vig gene.

XS52 cells were also retrovirally transduced with rKat.B1.8.IRES.GFP (XS52.B1.8).

Flow cytometric analysis of cell surface markers

XS52.rKatA20RESGFP clones were stained with a panel of R-PE-conjugated mAbs against surface markers characteristic of DC including H-2Kd, H-2Dd, CD54 (BD PharMingen, Heidelberg, Germany), CD40, CD80, and CD86 (Serotec, Oxford, U.K.). Isotype-matched mAbs were used as controls. Immunostained cells were analyzed on a FACScan flow cytometer (BD Biosciences, Oxford, U.K.) using PCLysys software (BD Biosciences).

Adenoviral transduction of XS52 line

XS52 cells were plated at 2 × 106 cells in 2 ml XS52 medium. The cells were allowed to adhere and then infected with Ad.A20, Ad.A20hFc, or Ad.GFP at a multiplicity of infection of 250. A 18 h medium was replaced and the cells were used for T cell stimulation following a 72-h incubation. Successful adenoviral transduction was confirmed by the expression of the reporter gene GFP.

Infection of mice with adenoviral vectors encoding A20 scFv and generation of T cell lines against A20 scFv

Preliminary experiments using Ad.B1.8hFc established the optimal route and dose of adenoviral vaccine. To determine whether the administration of Ad.A20 constructs could induce Ag-specific immune responses, BALB/c mice were immunized with 107 PFU per mouse of Ad.A20 or Ad.A20hFc in 100 μl PBS/2% milk protein given s.c. and 50 μl injected i.m.

To evaluate specific immune responses to the A20 scFv, blood was collected at day 21 postinjection by cardiac puncture under terminal anesthesia and the spleens were obtained. Splenocytes were pooled according to the vaccine used and cocultured (2 × 106 cells/ml) with irradiated (3000 rad) adenovirus-infected XS52 cells (2 × 106 cells/ml) in a six-well tissue culture plate. T cells were restimulated weekly, initially with adenovirus-infected XS52 cells. When the T cell lines were found to specifically proliferate to XS52.A1.A20, an A20 scFv retrovirally transduced XS52 cell line, they were maintained by restimulation with these cells at weekly intervals, at an effector:stimulator ratio of 10:1, in the presence of recombinant human IL-2 (20 U/ml) (PeproTech, London, U.K.). XS52.A1.A20 (3000 rad) and 2 × 104 irradiated syngeneic splenocytes/well (7000 rad), to a total volume of 200 μl XS52 cells retrovirally transduced with rKat.B1.8.IRES.GFP were plated at 2 × 105 cells/well. In the case of XS52 peptide-pulsed cells, peptide was added to the cells with the 51Cr at a final concentration of 20 μg/ml. Purified anti-CD8 and anti-CD4 Abs (BD PharMingen) were added in some instances to the T cells 30 min before the addition of the target cells at a concentration of 20 μg/ml. Purified isotype controls were used at the same concentration. Effector cells were added at various E:T ratios to a final volume of 150 μl, with each ratio represented in triplicate. Maximum 51Cr release was determined from supernatants of cells that were lyzed with 100 μl PBS/2% Tween 20. Spontaneous release was obtained by incubating target cells in medium alone. The plates were incubated at 37°C/5% CO2 for 4 h. A total of 90 μl of supernatant was collected from each well and counted in a Topcount scintillation counter (Packard Instrument, Berkshire, U.K.). The percentage of lysis was calculated as follows: % lysis = ([specific release - spontaneous release]/maximum release – spontaneous release) × 100.

Proliferation assay

Stimulator cells consisting of irradiated XS52, XS52.A1.A20, XS52.B1.8 (3000 rad), and A20 cells (7000 rad) were plated out in triplicate in U-bottom 96-well plates at a concentration of 5 × 104 cells/well. T cells (1 × 105 cells/well) were added to each well and the cultures were incubated at 37°C/5% CO2 for 4 days. The percentage of proliferation was calculated as follows: % proliferation = (cpm test culture - cpm control cultures)/cpm control cultures × 100.

ELISPOT

T cells were also assessed in an ELISPOT assay using an IFN-γ ELISPOT kit (IDS, Tyne and Wear, U.K.). T cells were plated out in duplicate in the wells of a 96-well hydrophilic, high protein-binding Immobilon-P membrane plate (Millipore, Gloucester, U.K.) coated overnight with IFN-γ capture Ab, diluted in carbonate-bicarbonate buffer (pH 9.6). Irradiated XS52.A1.A20, XS52, and A20 cells were used as stimulators. After 48 h at 37°C/5% CO2, the plates were washed vigorously with PBS/0.05% Tween 20 to remove the cells. IFN-γ production by T cells was detected by a 3-h incubation with biotinylated detection Ab diluted in PBS/1% FCS, followed by a 1.5-h incubation with alkaline phosphatase-conjugated streptavidin. Spots were visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate buffer, and the number of spots was quantified using computer-assisted video image analysis (Image-Pro Plus; Media Cybernetics, Silver Spring, MD).

ELISA

Vaccine-induced anti-A20 scFv and anti-A20 Ig Ab responses were assessed by ELISA. The A20 proteins, diluted in borate buffer (100 mM boric acid, 150 mM NaCl, pH 8.5) were coated to the wells overnight at 4°C. Purified monoclonal mouse IgG2a (BD PharMingen) was used to check the specificity of the immune response. Dilutions of sera were made in PBS/2% milk protein with bound mouse Ig detected by HRP-conjugated goat anti-mouse Fc (Sigma). Isotype-specific detectors were used for the quantification of anti-idiotypic Abs of the IgG1, IgG2a, and IgG2b subclasses (AMS Biotechnology, Abingdon, U.K.). Plates were coated using BM Blue POD substrate (Roche, Mannheim, Germany).

Tumor challenge

Two groups of BALB/c mice were immunized once on day 0 with the recombinant adenoviruses Ad.A20 and Ad.A20hFc (107 PFU in 100 μl PBS split s.c. and i.m.). One group of mice was immunized with 50 μg PCACA2A0 i.m. on days 0, 21, and 42 (based on previous experiments with the plasmid p19). Two control groups were used, one vaccinated with Ad.B1.8hFc (107 PFU in PBS) and one with PBS alone. On day 42 1 × 105 A20 tumor cells were injected s.c. The mice were monitored for up to 130 days.
Statistical analysis
Quantitative results are expressed as mean ± SD. The log rank test was used to analyze the statistical differences between the vaccine groups.

Results
Generation of constructs expressing A20 scFv
Idiotypic sequences identified from the A20 lymphoma cell line were subcloned as a scFv into various expression cassettes (Fig. 1A). To generate recombinant idiotype-encoding adenoviral vectors for these studies the A20 scFv was subcloned into the adenoviral expression vector, pAdlOx. A second plasmid, pAdlOxA20hFc, was also constructed. This directs the expression of the A20 scFv as a fusion to the Fc fragment of IgG1. The xenogeneic Fc fragment was included as the result of studies elsewhere demonstrating the importance of foreign proteins in the induction of effective anti-idiotypic immunity (9, 13).

Generation and cloning of an XS52 cell line stably expressing the A20 scFv
To generate an A20 scFv-expressing target for use in T cell assays, XS52 cells were transduced with the retrovirus rKat.A20.IRES.GFP. The presence of the reporter gene GFP allowed confirmation by fluorescent microscopy of successful retroviral transduction and a simple means by which to clone idiotype-expressing XS52 cells. Several clones were established and further analyzed by flow cytometry for the expression of GFP (98%) and DC surface markers (class I (98%), class II (98%), ICAM-I (98%), B7.1 (98%), B7.2 (98%), CD40 (98%)). A representative clone, designated XS52.A1.A20, expressing high levels of DC markers was chosen for future use. Confirmation of the presence of A20 scFv DNA was achieved by PCR analysis using A20 scFv V\text{H} primers of total cellular cDNA isolated from XS52.A1.A20 cells.

Optimization of dosing schedule of scFv-expressing adenoviral vectors
To establish the optimal dosing schedule of scFv-expressing recombinant adenovirus, mice were vaccinated with Ad.B1.8hFc. This virus encodes a scFv specific for the hapten 4-hydroxy-3-nitrophenyl acetyl/4-hydrox-3-5-iodo-phenylacetyl, which is expressed as a fusion with hFc. Results of experiments with this vector demonstrated that the optimal dose for anti-B1.8 Ab response was 10⁸ PFU, split i.m./s.c., with a peak Ab response at day 49. Intravenous administration of the virus was less effective and repeat immunization over a short period of time was not beneficial. Nevertheless, after 23 wk of rest, boosting with the same adenoviral construct was effective (data not shown).

Vaccination with idiotype-encoding Ad vectors induces idioype-specific T cell immunity
Attempts to induce idiotype-specific T cell responses in vitro using splenocytes from naive mice were unsuccessful. To determine whether vaccination of mice with a single dose of an idiotype-encoding adenoviral vector is able to induce an idiotype-specific CTL response in vivo, BALB/c mice were immunized with 10⁹ PFU of Ad.A20 or Ad.A20hFc. Twenty-one days later pooled splenocytes from the immunized mice were cultured in vitro with XS52 cells infected with the recombinant adenoviral vector AdA20. In vitro T cell expansion using the A20 cell line was unsuccessful. Cultures were assayed for cytotoxic activity against a panel of target cells in a standard ⁵¹Cr release assay (Fig. 2). T cells from both Ad.A20- and Ad.A20hFc-vaccinated mice showed specific lytic activity against the idiotype-expressing cell line XS52.A1.A20, with negligible lysis of the untransduced XS52 line. The T cell lines were also able to significantly lyse the A20 lymphoma cell line used to derive the A20 scFv. Both adenoviral vectors induced similar levels of cytotoxic activity against XS52.A1.A20 and A20 cells. High, nonspecific cytotoxic activity was seen against adenoviral-transduced XS52 cells. There was

![Figure 1](http://www.jimmunol.org/)

![Figure 2](http://www.jimmunol.org/)
some cytotoxic activity of the T cells against the control scFv, B1.8.

The capacity of the adenoviral vectors to induce a proliferative response to the A20 scFv was also assessed. Splenocytes from immunized mice were restimulated in vitro as above and then tested for their ability to proliferate in response to irradiated stimulators. Strong specific proliferative activity was seen against the A20 scFv but not against XS52 cells nor XS52 cells expressing B1.8 (XS52.B1.8) or GFP (XS52.GFP) (Fig. 3). No proliferative response to the A20 tumor line, there was no detectable lysis of GFP-expressing cells (data not shown).

To determine the restriction element involved in the specific anti-idiotypic activity demonstrated by the T cell lines generated from the mice vaccinated with the A20 scFv adenoviral constructs, both cytotoxic and proliferative assays were conducted in the presence of monoclonal blocking Abs against CD4 and CD8 molecules. These Abs are known to block the interaction between CD8+ (class I MHC and CD4+ (class II MHC, leading to inhibition of proliferative and lytic activity of CD4/CD8-restricted T cells. Fig. 4A shows that the specific proliferative activity was completely inhibited by anti-CD8 Ab, with the proliferative activity unaffected by anti-CD4 Ab or isotype-matched control Abs. The specific lytic activity of these T cell lines was also inhibited by the addition of anti-CD8 Ab (Fig. 4B and C). Furthermore, the lytic activity of these T cell lines against the A20 tumor cell lines was also completely inhibited by the CD8 Ab, demonstrating the CD8+ involvement in this recognition (Fig. 4C). Phenotypic analysis revealed that CD8+ T cells accounted for 85% of both T cell populations, with the remainder of the cells being CD4+ (data not shown).

A20[106-114] DYWGQGTEL idiotype-derived peptide is the immunodominant H-2K<sup>d</sup>-restricted CD8<sup>+</sup> T cell epitope

Five potential H-2K<sup>d</sup>-binding peptides (Fig. 1B) were identified from the idiotypic sequence using the program developed by Parker et al. (28) and individually analyzed for their recognition by the T cell lines in a <sup>51</sup>Cr release assay. As shown in Fig. 5, only the A20[106-114] scFv-derived peptide, DYWGQGTEL, was recognized by both Ad.A20 (Fig. 5A) and Ad.A20hFc (Fig. 5B) T cell lines. To test whether T cell populations against the other four epitopes were present at very low frequencies in these T cell lines, we used an ELISPOT assay to evaluate each peptide for its ability to stimulate T cell lines. As depicted in Fig. 6, T cell lines derived from Ad.A20-vaccinated mice secreted IFN-γ in response to stimulation with XS52.A1.A20, XS52 cells pulsed with A20[106-114], and A20 cells. No IFN-γ production was recorded when the T cells were stimulated with XS52 cells pulsed with the
other four peptides. T cell lines derived from mice vaccinated with Ad.A20.hFc showed a similar pattern of IFN-γ/H9253 production against XS52.A1.A20, A20[106–114], and A20 cells (data not shown).

Generation of T cell clones

T cells lines from mice vaccinated with Ad.A20 and Ad.A20hFc were cloned using XS52.A1.A20 cells as stimulators and irradiated splenocytes as a feeder layer, in the presence of 100 U/ml recombinant human IL-2. The cells were restimulated on two further occasions and then screened in a 51 Cr release assay, with 14 of 96 clones tested able to lyse XS52.A1.A20 cells and not XS52 cells. These specific clones were expanded with irradiated XS52.A1.A20 cells and tested in a second 51 Cr release assay for their ability to specifically lyse XS52 cells loaded with the A20-derived peptides. Six successfully expanded T cell clones were able to lyse XS52 cells loaded with A20[106–114], XS52.A1.A20, and the A20 tumor cell line, but not XS52 cells loaded with the other four peptides. The specific lytic activity of one representative T cell clone, T.B1, derived from mice vaccinated with Ad.A20, is shown in Fig. 7A. This T cell clone was further analyzed in an ELISPOT assay. Again, specific IFN-γ release was demonstrated in response to stimulation by XS52.A1.A20 cells (Fig. 7B) and to stimulation by A20[106–114], but not the other peptides.

Vaccination with idiotype-encoding Ad vectors induces anti-idiotype Ab response

Serum was obtained from mice 21 days after vaccination with the adenoviral vectors and the idiotype-encoding plasmid, pVAC2.A20FrC. Anti-idiotype Ab responses were assessed by ELISA using both A20 Ig purified from tissue culture supernatant and A20 scFv purified from E. coli. Although the idiotype-encoding adenoviral vectors were equivalent at inducing T cell responses in the vaccinated mice, there was a clear difference in the ability of the vectors to induce an anti-idiotypic Ig Ab response, with mice vaccinated with Ad.A20hFc but not Ad.A20 or pVAC2.A20FrC developing Abs to the purified A20 Ig. Ad.A20 did induce an Ab response to the recombinant A20 scFv, but at a much lower titer than that induced by Ad.A20hFc. The isotype profile of this Ab response was also examined (Fig. 8). Vaccination of mice with Ad.A20hFc induced anti-A20 Ig IgG1, -2a, and -2b responses, which is consistent with

FIGURE 5. The A20[106–114] scFv-derived peptide (DYWGQGTEL) is recognized by cytotoxic T cell lines generated after immunization of mice with Ad.A20 (A) and Ad.A20hFc (B). The T cell lines were tested for their lytic activity against XS52 cells pulsed with 20 μg/ml of the five A20 scFv-derived peptides listed in Fig. 1, with XS52 and XS52.A1.A20 cells serving as negative and positive controls, respectively.

FIGURE 6. The A20[106–114] scFv-derived peptide (DYWGQGTEL) specifically induced IFN-γ production by the Ad.A20 T cell line, as detected by an ELISPOT assay. XS52 cells (10² cells/ml) were incubated the five A20 scFv-derived peptides at 20 µg/ml for 2 h before irradiation (3,000 rad). A total of 250 T cells were then cocultured for 48 h on an IFN-γ-coated plate, with 5,000 peptide-pulsed XS52 cells, XS52.A1.A20 cells, or XS52 cells, or 50,000 A20 cells (12,000 rad). Data represent the mean of duplicate samples, with error bars showing ± SD.

FIGURE 7. Specific activity against A20 scFv-derived peptides as measured by 51 Cr release assay at an E:T ratio of 25:1 (A), and IFN-γ secretion in an ELISPOT assay (B). This clone was generated from T cell lines isolated after vaccination of mice with Ad.A20, as described in Materials and Methods. XS52 and XS52.A1.A20 cells served as negative and positive controls in the assays. The specific lytic activity of the clone against the A20 lymphoma line is also shown.
a Th1- and Th2-type T cell response. In contrast, the anti-A20 scFv response was predominantly IgG2b (data not shown).

The specificity of the immune response was demonstrated by the lack of detectable Abs against an isotype-matched control murine Ig. We also examined the anti-A20 Ig response induced in mice vaccinated with Ad.B1.8hFc. While there was a 2-fold difference in the anti-IgG2b response (titer: 1/300 in mice vaccinated with Ad.B1.8hFc and 1/650 in mice vaccinated with Ad.A20hFc), similar levels of anti-A20 IgG1 and 2b Abs were induced.

**Adenovirus encoding A20hFc protects against challenge with a lethal dose of A20 cells**

To determine the capacity of adenoviruses expressing the A20 idiotype to induce protective antitumor immunity, mice were immunized with $10^7$ PFU of adenovirus and then challenged with a lethal dose of tumor cells. Another group of mice was vaccinated with the plasmid pVAC2.A20.FrC at a dose previously shown, in another lymphoma model (9), to protect against tumor challenge. Mice vaccinated with PBS or Ad.B1.8hFc, a recombinant adenovirus expressing a control scFv, were not protected from tumor challenge, nor were mice preimmunized with the plasmid pVAC2.A20.FrC (Fig. 9). Mice preimmunized with Ad.A20 had a significant delay in tumor development compared with the mice vaccinated with Ad.B1.8hFc ($p = 0.002$). However, $>40\%$ of the mice pretreated with Ad.A20hFc were effectively protected against A20 challenge ($p = 0.0003$), remaining tumor free for >250 days. Furthermore, mice pretreated with Ad.A20hFc had a significant survival benefit to those pretreated with Ad.A20 ($p = 0.022$).

**Discussion**

Previous studies have demonstrated that naked DNA vaccines encoding tumor idiotypes fused to xenogeneic proteins are capable of protecting mice against challenge with lethal doses of lymphoma cells (9, 13). Recombinant adenoviral vectors offer a potentially superior approach to plasmid vaccines. Such vectors are highly immunogenic and allow a greater efficiency of gene transfer than that of DNA vaccines. Adenoviral vectors encoding model tumor Ags are able to induce protective cellular and humoral immunity against several tumor Ags (19, 20). Therefore, we sought to evaluate the use of replication-defective, idiotype-encoding adenoviral vectors as a means to induce therapeutic anti-idiotypic humoral and cellular immunity.

In this study we have shown that vaccination of mice with a single dose of recombinant adenovirus encoding the A20-derived idiotype alone, or as a fusion to hFc, is capable of inducing potent idiotype-specific CD8$^+$ T cell responses. Only the adenovirus encoding the A20 fused to hFc is able to induce anti-idiotypic Abs able to recognize A20 Ig. Importantly, we were able to show strong idiotype-specific cytotoxic T cell activity; these T cells were also capable of lysing the A20 tumor cells themselves. The majority of murine models examining the use of idiotype vaccines have demonstrated the importance of induction of anti-idiotypic Abs in protecting mice against tumor challenge (13, 29). While T cells have been shown to play a role in protection in murine lymphoma models (14–18), no animal model to date has demonstrated the induction of idiotype-specific cytotoxic T cells following idiotype vaccination. One possibility is that some tumor idiotypes do not contain CTL-binding motifs. However, idiotype-specific CTLs have been clearly demonstrated against human idiotypes (10–12), and in some murine models the tumor protection cannot be fully attributed to an anti-idiotypic Ab response (15, 17, 18), suggesting that T cell-mediated immunity may play a role in tumor eradication. It is possible that the in vitro systems used to expand and identify idiotype-specific T cells in such studies may need to be optimized to demonstrate such an immune response. In our study, identification of idiotype-specific T cells relied on the use of a syngeneic DC line, XS52 retrovirally transduced to express the A20 idiotype. Interestingly, expansion of T cells from vaccinated mice in vitro using the A20 lymphoma cell line itself was not successful, possibly because lymphoma cells are poor APCs in comparison to DC.

T cells from mice vaccinated with adenoviral vectors were able to lyse, with equal efficiency, XS52 cells adenovirally transduced to express the idiotype, a control idiotype, or GFP, reflecting the induction of antiadenoviral-specific CTL response in vaccinated mice (data not shown). There was also some lysis of XS52 cells, retrovirally transduced to express a control scFv, B1.8, although the lytic activity of T cells against the A20 scFv was clearly stronger. This cross-reacting activity is not surprising, as the
B1.8 scFv and the A20 scFv have some sequence homology. Indeed, the B1.8 scFv peptide predicted by the Parker computer program to be the strongest H-2K\textsuperscript{d} binder is identical to A20[106–114] apart from the last two amino acids (DYNWGGQTTV). This cross-reactivity does not result in cross-protection; animals vaccinated with Ad.B1.8hFc were not protected from tumor challenge with A20 cells.

An important finding of our study was the identification of an immunodominant class I binding epitope in the idiotropic region of the A20 Ig. Five potential MHC class I (H-2K\textsuperscript{d})-binding peptides were identified using a peptide-binding prediction database. Subsequent analysis revealed that both T cell lines from the vaccinated mice were able to recognize XS52 cells pulsed with the A20[106–114] peptide, in both a cytotoxic and an ELISPOT assay. However, the T cell did not recognize XS52 cells pulsed with any of the other four peptides. Analysis of the peptide specificity of the T cell lines by the more sensitive ELISPOT assay took place after some weeks of in vitro culture and restimulation, which may have led to the expansion of the T cell population specific for the immunodominant peptide. Therefore, it remains to be demonstrated whether the A20[106–114] peptide is the only idiotypic peptide induced by vaccination, or if further peptide epitopes will be identified, by the use of assays such as ELISPOT, in T cell populations isolated from vaccinated mice before in vitro expansion.

Both Ad.A20 and Ad.A20hFc adenoviral vectors appear to be equally capable of inducing anti-idiotypic T cell responses. However, assessment of the T cell lines by ELISPOT after a long period of in vitro culture suggests that the frequency of idiotypic-specific T cells is greater in the T cell line generated from mice vaccinated with Ad.A20. Whether this reflects the in vivo frequency of anti-idiotypic T cells or is the result of long-term culture needs further investigation.

We have been able to generate T cell clones specific for the A20 idiotype which are also able to lyse the A20 tumor cells and a syngeneic DC line pulsed with the A20 immunodominant peptide, A20[106–114]. These T cell clones should enable further investigation of the use of idiotypic-specific T cells. It would be of interest to determine whether idiotypic-specific T cells, capable of lysing the parent lymphoma line, are also capable of treating tumor-bearing mice.

This study investigated the use of adenoviral vectors expressing the idiotype either alone or as a fusion to hFc. While both idiotypic-encoding adenoviral vectors induced similar idiotypic-specific T cell responses, the vector expressing the idiotype-hFc fusion is clearly superior at inducing an anti-idiotypic Ab response (Fig. 8). Before analysis the anti-idiotypic T cells were expanded in vitro with a DC line. The quantitative assessment of T cell responses before in vitro expansion, using sensitive assays such as ELISPOT, may elucidate any qualitative differences between the two vectors in their ability to induce anti-idiotypic T cell responses. Furthermore, although mice vaccinated with either Ad.A20 or Ad.A20hFc had a significant delay in tumor development compared with mice vaccinated with Ad.B1.8hFc (p = 0.002 and p = 0.0003, respectively), mice vaccinated with Ad.A20hFc had a clear survival advantage with >40% remaining tumor free for >120 days. It appears, then, that in accordance with other lymphoma models the induction of an anti-idiotypic Ab response is important for tumor protection in the A20 model. Interestingly, mice vaccinated with Ad.B1.8hFc developed cross-reacting Abs to the A20 Ig, with equivalent levels of anti-A20 Ig Abs of the IgG1 and -2a isotype, but half the titer of the IgG2b isotype, to that induced by Ad.A20hFc. This raises the possibility that it is Abs of the IgG2b isotype that are critical for tumor protection here. Future work will determine whether the anti-idiotypic humoral response is the only arm of the immune system contributing to tumor protection in mice vaccinated with Ad.A20hFc. Nevertheless, for some B cell tumors, such as myeloma, which do not express surface Ig, it seems likely that the induction of an idiotyp-specific cytotoxic immune response will be useful.

Interestingly, this study failed to demonstrate a protective benefit resulting from vaccination of mice with the plasmid pVAC2.A20.FrC, thus failing to confirm the results of King et al. (9), who demonstrated that mice vaccinated with an equivalent plasmid in an identical dosing schedule were protected from subsequent tumor challenge. Similar plasmids have been effective in other lymphoma models (8, 9, 13). The reason for this discrepancy is unclear. The plasmid used in this study was successfully expressed in eukaryotic cells, although there may be insufficient in vivo expression from the plasmid to induce effective immunity in this tumor model. However, the superiority of the adenoviral vector encoding the A20 scFv-hFc fusion over the plasmid vaccine in this model is clear.

Although it has been possible to identify class I epitopes against human B cell lymphomas, most T cell responses against murine lymphoma idiotypes have been limited to proliferative responses. The identification of lymphoma-derived T cell epitopes raises the possibility of using peptide vaccines, which have been relatively successful in certain tumors, such as melanoma (30, 31). A recent study of idiotypic sequences derived from human B cell lymphomas found >70% of predicted HLA-A2 binding peptides were derived from framework region motifs (32). Some of these peptides were able to induce tumor-specific CTL responses. If the majority of lymphoma-derived epitopes are from Ig framework regions this may obviate the need for patient-specific therapies. The A20[106–114] peptide identified in the present study overlaps the complementary-determining region-3 and the framework region, with the first H-2K\textsuperscript{d} anchor residue being derived from the complementary-determining region-3. Thus it appears, in this model at least, amino acid residues from the hypervariable region itself are important in T cell-mediated immunity to the idiotype. One potential concern with the use of adenoviral vectors in clinical trials is that preexisting immunity to the vector, as the result of natural exposure, will preclude successful adenoviral vaccination. Timmerman et al. (21) were able to demonstrate that prevaccination of mice with an irrelevant adenovirus vector did not impair tumor protection induced by subsequent vaccination with an idiotype-expressing adenovirus. Other groups working with model tumor Ags and adenoviral (19, 20) report similar findings.

In conclusion, our results demonstrate that recombinant idotype-encoding adenoviral vectors are more efficient at inducing anti-idiotypic immunity than DNA vaccines, and they also support the inclusion of genes encoding xenogeneic proteins in the development of such vaccines for clinical trials. Furthermore, we have demonstrated CTL induction and identified the peptide epitope recognized by these T cells. The role of CTL in tumor protection remains to be elucidated.

Acknowledgments
We thank A. Takashima, who kindly provided the XS52 cell line; Richard Jones (Covance, Harrogate, U.K.) for assistance in some animal experiments; Freda Stevenson (Southampton University Trust, Southampton, U.K.) for supplying the pVAC2 plasmid, and Steve Roberts (Paterson Institute for Cancer Research, Manchester, U.K.) for help with statistical analysis.

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