Novel Approach to the Formulation of an Epstein-Barr Virus Antigen-Based Nasopharyngeal Carcinoma Vaccine[▽]

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Epstein-Barr virus (EBV) is associated with several malignant diseases including nasopharyngeal carcinoma (NPC), a common neoplasm throughout southeast Asia. Radiotherapy and chemotherapy can achieve remission, but a reemergence of disease is not uncommon. Therefore, there is a need for specific therapies that target the tumor through the recognition of EBV antigens. In NPC, latent membrane protein 1 (LMP1) and LMP2 offer the best opportunity for specific targeting since they are typically expressed and T-cell determinants in each of these proteins have been defined. We have attempted to maximize the opportunity of incorporating every possible CD4 and CD8 determinant in a single formulation. We have achieved this by generating a scrambled protein incorporating random overlapping peptide sets from EBNA1, LMP1, and LMP2, which was then inserted into a replication-deficient strain of adenovirus (adenovirus scrambled antigen vaccine [Ad-SAVINE]). This report describes the construction of this Ad-SAVINE construct, its utility in generating LMP1 and LMP2 responses in healthy individuals as well as NPC patients, and its capacity to define new epitopes. This formulation could have a role in NPC immunotherapy for all ethnic groups since it has the potential to activate all possible CD4 and CD8 responses within EBNA1 and LMPs.

Epstein-Barr virus (EBV) is a member of the herpesvirus family and is one of the most common human viruses. It occurs worldwide, and most people become infected with the virus sometime during their lives. EBV is associated with a range of neoplasms. These include various B- and T-cell non-Hodgkin's lymphomas such as posttransplant lymphoproliferative disease (PTLD), Hodgkin's lymphoma (HL), and several lymphoepithelioma-like carcinomas, of which nasopharyngeal carcinoma (NPC) is the archetype (1). The association of the virus with these malignancies and its oncogenic potential have been well established (19).

Worldwide, NPC is characterized epidemiologically by foci of relatively high endemicity in certain geographic regions including southern China, Hong Kong, Taiwan, the Philippines, Singapore, Vietnam, Kenya, Tunisia, Sudan, and Uganda. The reason for the focal distribution of NPC is uncertain, although genetics and environmental factors have been suggested to be causes (14, 49).

Currently, the mainstay for the treatment of NPC is radiation and chemotherapy. Indeed, this treatment is frequently successful when the extent of the tumor is small and confined. However, when disease is advanced at diagnosis and where

metastatic spread has become apparent, more radical treatments may need to be adopted, including surgery. In either case, all these treatments are associated with severe short- and long-term side effects including secondary malignancies (16). Hence, there is a need for specific therapies that target the tumor itself rather than therapies that are associated with the destruction of normal tissue.

Virus-associated malignancies offer a distinct advantage in this regard since therapy can be directed specifically toward viral proteins expressed in the tumor, thus avoiding collateral damage to normal tissue. This has been dramatically demonstrated in the case of PTLD, where the adoptive transfer of EBV-specific cytotoxic T lymphocytes (CTLs) activated in vitro by using autologous lymphoblastoid cell lines (LCLs) has resulted in a resolution of disease with a very low frequency of side effects (9, 18, 40). In this case, it is likely that the effector cells infused into these patients are directed mainly toward the dominant EBV nuclear antigen 3A (EBNA3A), EBNA3B, and EBNA3C. The concept of immunological intervention as a treatment option for NPC is greatly enhanced by a range of previously reported studies that indicated the presence of transport-associated proteins (TAP1 and TAP2) and major histocompatibility complex class I and class II in NPC (23, 37, 42, 48), all of which are required for efficient CTL recognition. In NPC, EBNA1, latent membrane protein 1 (LMP1), and LMP2 offer the best opportunity for specific targeting since these are the only EBV proteins expressed in this malignancy. This is particularly so in the case of immunotherapy since defined CD4⁺ and CD8⁺ T-cell determinants in each of these proteins have been defined (12, 15, 20, 31). However, the CTL response in the case of the LMPs is relatively weak (particularly LMP1), and the glycine-alanine repeat sequence within

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EBNA1 may affect immunological processing (29), although this may not be the absolute barrier that was first hypothesized (46). Recent studies have provided some encouragement that immunotherapeutic intervention may be a realistic treatment option for NPC (4, 5, 7, 8, 10, 30, 43, 45). For example, Straathof et al. (43) and Comoli et al. (10) adoptively transferred effector cells expanded *in vitro* by using LCLs to activate CTLs in patients with advanced NPC, resulting in some cases in the resolution of disease, although in other cases, efficacy was limited and transient (3). Those studies, however, have provided a promising hint that the immunotherapeutic control of NPC might be feasible.

Indeed, recent studies have shown that multiple human leukocyte antigen (HLA) A2-restricted LMP1 CTL epitopes, when used as a polyepitope vaccine in a poxvirus vector, efficiently induced a strong CTL response, and this response could reverse the outgrowth of LMP1-expressing tumors in HLA-A2/Kb mice (13). The poxvirus-based LMP1-polyepitope vaccine tested in these studies contained only HLA A2-restricted epitopes, and targeting just one HLA allele will not be suitable for all ethnic groups. If a CTL-based therapy for NPC is to be universally applicable, the target epitopes must bind to a range of HLA alleles preferably present at a high frequency in patient populations and include determinants irrespective of whether they have previously been defined.

It is likely that the essential difference between the very successful treatment of patients with PTLN and the partial success in the case of NPC is that in the former case, immunodominant targets are available, while in the latter case, only relatively weak responses are seen even for healthy individuals. The present communication has arisen in an attempt to maximize the possibility of activating a response toward the three proteins present in NPC rather than skewing the effector population toward the immunodominant EBNA3A, -B, and -C proteins. We have achieved this by generating a "scrambled-antigen vaccine" (referred to as SAVINE) incorporating random overlapping peptide sets from EBNA1, LMP1, and LMP2. This SAVINE has been incorporated into a replication-deficient adenovirus (Ad5/F35) as a 6.9-kb insert (Ad-SAVINE). An important feature of the Ad-SAVINE strategy is that it provides a platform for the activation of all possible immunological determinants (including helper cells and CTLs) within EBNA1, LMP1, and LMP2 and should be applicable to all populations for which NPC is endemic. This report describes the construction of this Ad-SAVINE construct and its utility in generating LMP1 and LMP2 responses from peripheral blood mononuclear cells (PBMCs) from healthy individuals and NPC patients.

MATERIALS AND METHODS

All of the work presented in this publication was approved by the institutional Human Research Ethics Committee, which conforms to the Declaration of Helsinki. Human blood from healthy individuals and NPC patients was collected with the appropriate approved consent form.

Construction of the synthetic NPC Ad-SAVINE construct. The SAVINE vaccine was designed and constructed by using methods previously used to construct a human immunodeficiency virus SAVINE (47). The SAVINE incorporates the LMP1 sequence from the Cao isolate of EBV identified from a Chinese NPC (GenBank accession number AF304432), while the LMP2a and EBNA1 protein sequences were obtained from a common EBV A strain sequence assembled from EBV B95-8 (GenBank accession number AJ507799). Deletions were made

to the EBNA1 and LMP1 sequences corresponding to the Gly-Ala repeat and Arg-Gly amino acids 95 to 377 in EBNA1 and five copies of the 11-amino-acid tandem repeat at amino acids 260 to 315 in LMP1. The separated sequences of EBNA1 and LMP1 were then regarded as separate proteins during the rest of the design process.

To design synthetic SAVINE cDNA, the protein sequences were fragmented into overlapping peptides (30 amino acids overlapping by 15 amino acids), which were reverse translated into codon-optimized DNA by using the first and second most common mammalian codons alternating differently in peptide overlaps to reduce cDNA sequence duplication. The DNA sequences were then randomly "scrambled" on the computer, and a SAVINE sequence was assembled. The only restriction to this random reassembly of a 30-amino-acid sequence was that no two DNA sequences were joined so that they would encode a larger fragment (<30 amino acids) corresponding to the original parent protein sequence. Additional restriction enzyme site start and stop codons as well as a Kozac sequence were added to aid translation and subsequent cloning.

The SAVINE cDNA was synthesized by using long oligonucleotides from Invitrogen, asymmetric PCR, stepwise overlap extension, and restriction site-directed ligation as described previously (47) to make a 6,863-bp cDNA fragment (Fig. 1). The cDNA was then cloned into pShuttle (Clontech) and used to generate a recombinant replication-deficient adenovirus vaccine (Ad-SAVINE) based on the altered fiber adenovirus Ad5/F35 by using previously reported techniques (11, 34).

Establishment and maintenance of cell lines. LCLs were established from EBV-seropositive donors by exogenous virus transformation of peripheral B cells by using the B95-8 virus isolate in the presence of cyclosporine (Novartis). These cells were routinely maintained in RPMI 1640 medium (Gibco Invitrogen Corp., Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin plus 10% fetal calf serum (FCS).

To generate phytohemagglutinin (PHA) blasts, PBMCs were stimulated with PHA (CSL Ltd., Melbourne, Australia), and after 3 days of culture, growth medium containing MLA 144 supernatant and highly purified recombinant human interleukin-2 (rIL-2) was added. PHA blasts were propagated by a twice-weekly replacement of rIL-2 and MLA supernatant for up to 6 weeks.

Generation of EBV-specific CTL cultures. (i) **Autologous LCL activation.** LMP-specific CTLs were generated from a panel of healthy virus carriers and NPC patients by stimulating PBMCs with autologous EBV-transformed LCLs. Briefly, 2×10^6 PBMCs were cocultured with gamma-irradiated LCLs in a 24-well plate at a responder-to-stimulator ratio of 2:1 for 7 days. These cultures were restimulated weekly with gamma-irradiated autologous LCLs (ratio, 30:1), and the growth medium was supplemented with rIL-2 (20 IU/ml).

(ii) **Ad-SAVINE activation.** CTLs were established by using PBMCs infected for 1 h at 37°C in 5% CO₂ with the Ad-SAVINE construct at a multiplicity of infection (MOI) of 50:1 and added to uninfected PBMCs at a 1:2 ratio in RPMI 1640 medium with 10% FCS and 20 IU/ml rIL-2. Cultures were restimulated weekly (for 3 weeks) with gamma-irradiated autologous LCLs that had been infected with the Ad-SAVINE construct at an MOI of 50:1. These infected LCLs were cocultivated with the cultured cells at a ratio of 1:30, and the growth medium was supplemented with rIL-2. In some cases, a cytomegalovirus (CMV) construct was used as a control of specificity (Ad-CMVpoly). Briefly, CTLs were established by using PBMCs infected for 1 h at 37°C with the Ad-CMVpoly construct (50) at an MOI of 50:1 and added to uninfected PBMCs at a 1:2 ratio in RPMI 1640 medium with 10% FCS and 20 IU/ml rIL-2. Cultures were restimulated weekly with gamma-irradiated autologous LCLs that had been infected with the Ad-CMVpoly construct at an MOI of 50:1. These infected LCLs were cocultivated with the cultured cells at a ratio of 1:30, and the growth medium was supplemented with rIL-2.

(iii) **Peptide activation.** CTLs were established from PBMCs that were stimulated with peptide-coated gamma-irradiated autologous LCLs (peptide concentrations of 10^{-5} M, 10^{-6} M, and 10^{-7} M). Cultures were restimulated weekly (for 3 weeks) with gamma-irradiated autologous LCLs that had been coated with the peptide at a ratio of 1:30.

⁵¹Cr release assays. The ability of LMP-specific CTLs to induce lysis was analyzed in a standard 5-h chromium 51 release assay by using an effector/target ratio of 20:1. Autologous PHA target cells were incubated with LMP peptides for 1 h at 37°C in 5% CO₂, washed once, and then labeled with ⁵¹Cr for 1 h at 37°C in 5% CO₂, washed twice, and resuspended at 10^5 cells/ml in RPMI medium plus 10% FCS. A total of 10^4 cells were added to a 96-well plate, and effector CTLs were added at 20:1 effector/target ratios. The cells were incubated at 37°C in 5% CO₂ for 4 h before pelleting the cells, and 25 µl of supernatant was collected into Luma-Plate 96-well plates (Packard Instrument Co.). The plates were dried and read by use of a TopCount microplate scintillation counter (Packard Instrument

FIG. 1. Schematic description of the construction of the Ad-SAVINE vaccine. Shown is the construction of a recombinant adenovirus (Ad5/F35) that expresses a synthetic DNA encoding a scrambled protein that contains LMP1, LMP2, and EBNA epitopes. To design the synthetic SAVINE cDNA, the protein sequences were fragmented into overlapping peptides (30 amino acids [AA] overlapping by 15 amino acids), which were reverse translated into codon-optimized DNA. The DNA sequences were then randomly “scrambled” and joined together such that no two DNA sequences were joined so that they would encode a larger fragment (<30 amino acids) corresponding to the original parent protein sequence. The cDNA was then cloned into pShuttle and used to generate a recombinant replication-deficient adenovirus vaccine based on the altered-fiber adenovirus Ad5/F35. SOEing, stepwise overlap extension; E. coli, *Escherichia coli*.

Co.), and the percent specific lysis was calculated by the following formula: [(mean specific release $\pm$ mean spontaneous release)/mean total release] $\times$ 100.

ELISPOT assays. The precursor CTLs were determined by using enzyme-linked immunospot (ELISPOT) technology to detect gamma interferon (IFN- γ) secretion as described previously (2). Briefly, 96-well mixed cellulose ester membrane plates (Millipore, Bedford, MA) were coated overnight at 4°C with 100 μ l/well of 10 μ g/ml anti-IFN- γ capture monoclonal antibodies (clone 1-D1K; Mabtech, Stockholm, Sweden) in phosphate-buffered saline (PBS). Cells (2×10^5 PBMCs or 5×10^4 cultured CTLs) were added per well in triplicate in the presence of synthetic peptides (10^{-5} M to 10^{-12} M). For negative controls, cells were incubated without peptide. The plates were incubated at 37°C overnight and then washed prior to the addition of 100 μ l/well of 1 μ g/ml anti-IFN- γ biotin (clone 7-B6-1; Mabtech, Stockholm, Sweden) in PBS and incubated at room temperature for 2 h. After incubation, plates were washed again, and 100 μ l/well of 1 μ g/ml streptavidin-alkaline phosphatase conjugate was added and incubated at room temperature for 1 h. After a final wash, BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium developing substrate solution (Sigma, St. Louis, MO) was added at 100 μ l/well and kept at room temperature until individual IFN- γ -producing cells were detected as dark spots. Spots were

counted automatically by using an AIDELISPOT reader (Autoimmun Diagnostika GmbH, Strassberg, Germany) and were expressed as spot-forming units.

Phenotypic analysis and intracellular cytokine staining (ICS). The phenotype of the activated cultures was determined by labeling cells with fluorochrome-labeled mouse anti-human antibodies specific for T-cell markers. Cells (2×10^5) were washed once in fluorescence-activated cell sorter (FACS) buffer (1 $\times$ PBS, 1% FCS) and resuspended in 50 μ l FACS buffer. CD4-phycoerythrin (Beckman Coulter), CD8-allophycocyanin (APC) (Pharmingen, Becton Dickinson), CD3-APC-Cy7 (Beckman Coulter), and CD56-PC5 (Beckman Coulter) were added to the cells at a 1/20 dilution, incubated on ice for 30 min, washed once, resuspended in FACS buffer, and analyzed with a FACSCanto apparatus (Becton Dickinson).

CTLs were assessed for their ability to secrete IFN- γ in response to incubation with LMP peptides by ICS. A total of 5×10^5 CTL were resuspended in 100 μ l of FACS buffer with LMP peptide (10^{-5} M to 10^{-9} M, final concentration) and incubated for an hour. GolgiPlug (BD Biosciences, San Diego, CA) was then added, and cells were incubated at 37°C in 5% CO $_2$ for 5 h, pelleted, and washed twice in 200 μ l FACS buffer. Cells were stained for surface antigens (CD4-fluorescein isothiocyanate and CD8-APC) for 30 min at 4°C and resuspended in

fixation/permeabilization solution according to the manufacturer's protocol. Finally, cells were stained with anti-human IFN- γ -phycoerythrin (Pharmingen, Becton Dickinson).

Synthesis of peptides. Peptides synthesized by the Merrifield solid-phase method were purchased from Mimotopes (Melbourne, Australia), dissolved in dimethyl sulfoxide, and diluted in serum-free RPMI 1640 medium for use in standard assays. The purities of these peptides were tested by mass spectrometry and showed >70% purity.

Panels of 15-mer peptides (overlapping by 10 amino acids) were synthesized, covering the entire amino acid sequence of LMP2 from the prototype EBV strain B95-8. Twenty peptide pools comprising 8, 9, or 10 15-mer peptides were prepared so that each 15-mer peptide was represented in two pools, as previously described (17). These peptide pools were used to identify a new CTL epitope within LMP2.

To identify the minimally recognized LMP2 epitope sequence, additional peptides varying in length from 8-mer, 9-mer, 10-mer, and 11-mer peptides were created.

Growth inhibition assay. A modification of a previously described LCL growth inhibition assay (35, 38), in which the ability of Ad-SAVINE-activated T cells to inhibit the growth of autologous LCLs was tested, was used as an additional functional assay. Autologous LCLs were seeded (in triplicate) in round-bottom wells at serial dilutions running from 10,000 cells/well with or without the addition of 10,000 Ad-SAVINE-activated cells/well. In some cases, Ad-SAVINE-activated cultures were depleted of CD4⁺ or CD8⁺ T cells (by Dynabeads), and 10,000 cells/well were plated with LCLs as mentioned above. Culture medium was changed weekly, and the growth, or otherwise, was scored visually at 3 weeks, with large pellets of actively growing cells indicating the successful growth of LCL and the presence of debris in wells suggesting an inhibition of growth by T cells.

Depletion with Dynabeads. PBMCs from donors were depleted of CD4⁺ or CD8⁺ cells by using Dynabeads (Dyna, Biotech, Australia). Briefly, 10⁷ cells were resuspended in 1 ml of PBS-2% FCS in the presence of CD4 or CD8 Dynabeads and incubated for 30 min at 4°C. The cells were then placed into a magnet, and the CD4⁻ or CD8⁻ depleted fraction was used for the growth inhibition assays.

RESULTS

Generation of an Ad-SAVINE construct. Although it is well recognized that the CTL response to LMPs is relatively weak, previous reports from our own laboratory and other laboratories revealed that a memory response to these proteins can be activated with either defined LMP epitopes, LCLs, or an LMP polypeptide (13, 39, 41). However, these methodologies are limited because they either are restricted to specific HLA alleles (peptide epitopes and LMP polypeptide) or include dominant responses (LCLs) to non-LMPs, which are likely to mask the NPC-relevant responses (20, 36). The Ad-SAVINE vaccine has been developed in an attempt to incorporate all possible immunological determinants (CD4 and CD8) with specificity directed toward NPC and potentially applicable to all HLA types. Briefly, the Ad-SAVINE incorporates scrambled 30-mer overlapping sequences of EBNA1, LMP1, and LMP2, which were subsequently reverse translated into DNA and used to create a recombinant replication-deficient adenovirus, as outlined in Fig. 1.

Activation of an LMP-specific CTL response in PBMC from immune healthy donors by using the Ad-SAVINE construct. This study has focused on a comparison between autologous LCLs and the Ad-SAVINE construct since both are potentially applicable to all HLA types and the former has been used in a number of clinical trials. In the present report we have compared the ability of the Ad-SAVINE construct to activate LMP1 and LMP2 responses compared to the response using autologous LCLs of six healthy EBV-seropositive individuals. These cultures were phenotyped on day 20, and their activity

FIG. 2. Activation and expansion of LMP-specific CTL responses after stimulation with Ad-SAVINE. PBMCs from a healthy donor (donor 1 [HLA A2, A2, B44, and B60]) were cocultured with autologous PBMCs infected with Ad-SAVINE or autologous EBV-transformed LCLs at a responder-to-stimulator ratio of 2:1 for 7 days and stimulated weekly with LCLs infected or not with Ad-SAVINE. LMP-specific T-cell reactivity was subsequently assessed on day 20. (A) Cells were phenotyped by staining with antibodies for CD3, CD4, CD8, and CD56 and analyzed by flow cytometry. (B) ELISPOT assay for ex vivo detection of IFN- γ secretion by a CD8⁺ T-cell population following stimulation with LMP peptides. (C) Using in vitro cytotoxicity assays, autologous PHA blasts coated with individual peptides or uncoated were used as targets for these assays, with an E-to-T ratio of 20:1. See Table 1 for definitions of peptide abbreviations.

was assessed in terms of IFN- γ secretion by CD8⁺ T cells and lysis of peptide-coated autologous PHA blasts in ⁵¹Cr release assays. In the example shown, most of the cells were CD8⁺, while the levels of the CD4⁺ component were lower (Fig. 2A). However, for the majority of donors, the dominant phenotype was CD4⁺ (see Table 2). The functional assays using ⁵¹Cr release and IFN- γ revealed that stimulation with the Ad-SAVINE construct consistently resulted in higher levels of activation than autologous LCLs. Figure 2 summarizes the results of a single representative comparison between the abilities of autologous LCLs and the Ad-SAVINE construct to activate an HLA A2-restricted response in PBMCs from a

TABLE 1. LMP1 and LMP2 epitopes and their HLA restrictions^a

Peptide sequence	Abbreviated sequence	Specificity	HLA restriction	Reference
YLLEMLWRL	YLL	LMP1	A2	22
YLQQNWWTL	YLQ	LMP1	A2	22
ALLVLYSFA	ALL	LMP1	A2	12
IEDPPFNSL	IED	LMP2	B60 or B40011	27
CLGGLTMV	CLG	LMP2	A2	26
LLSAWILTA	LLS	LMP2	A2	25
LTAGFLIFL	LTA	LMP2	A2	27
LLWTLVV	LLW	LMP2	A2	27
FLYALALL	FLY	LMP2	A2	24
PYLFWLAAI	PYLF	LMP2	A23/A24	21
APYLFWLAA	APYL	LMP2	B55	This paper
MGSLEMVPM	MGS	LMP2	B35	44
SSCSCPLSK	SSC	LMP2	A11	27
TYGPFVMCL	TYG	LMP2	A24	27

^a Shown are a list of LMP1 and LMP2 epitopes that were used in functional studies, abbreviations used in the tables and figures, and their HLA restrictions.

healthy immune donor (donor 1 [HLA A2, A2, B44, and B60]). As can be seen in Fig. 2A, cultures are dominated by CD3⁺ cells, and for this individual, the level of the CD8⁺ component was higher in Ad-SAVINE cultures than in corresponding cultures activated with the autologous LCLs. Although for this particular individual, many of the CD3⁺ cells activated with the autologous LCLs were CD56⁺, considerable variation was found in other cultures set up from this individual and from other individuals. Functional studies of these cultures were assessed by ELISPOT assays and ⁵¹Cr release assays by using peptides selected on the basis of the HLA of donor blood and the

reported LMP1 and LMP2 responses associated with each of these HLA types (Table 1). In the example shown in Fig. 2B (ELISPOT) and Fig. 2C (⁵¹Cr release), in which the HLA A2 responses were evaluated, multiple LMP1 and LMP2 specificities were activated with the Ad-SAVINE construct, while the LCLs tended to activate a more focused response. A higher-level response was also seen with epitopes restricted through other HLA types when activated with Ad-SAVINE than with autologous LCLs. In some instances, the response was tightly focused through a restricted number of epitopes, and this is likely to be related to the previously defined HLA-restricted epitopes, which, in the case of some alleles, is limited. A summary of all the data (phenotype and response by ⁵¹Cr release) from all healthy donors is included in Table 2.

Activation of LMP-specific CTLs using PBMCs from NPC donors by using the Ad-SAVINE construct. Having demonstrated the utility of the Ad-SAVINE construct in activating LMP responses for all of the healthy seropositive individuals tested, we sought to determine whether this formulation could also be used to activate LMP responses from NPC patients. The activities of Ad-SAVINE- and LCL-stimulated cultures from 10 different NPC patients were assessed in terms of the lysis of peptide-coated autologous PHA blasts in ⁵¹Cr release assays. Of these, LMP responses were detected for six NPC patients. As with healthy individuals, the level of activation of defined epitopes was consistently higher with the Ad-SAVINE than with autologous LCLs. Figure 3A shows one representative experiment for an NPC patient who responded to only one peptide (patient 1 [HLA A11, A24, B62, and B75]), whereas Fig. 3B shows a representative experiment for an NPC patient

TABLE 2. Activation of PBMCs from healthy donors and NPC patients with Ad-SAVINE^a

Donor or patient	HLA	Ad-SAVINE-stimulated CTLs		% of cultures with indicated phenotype	
		Peptide(s) tested	Peptide(s) with a positive response	CD3 ⁺ CD4 ⁺	CD3 ⁺ CD8 ⁺
Donors					
1	A2, A2, B44, B60	YLL, YLQ, ALL, IED, CLG, LLS, LTA, LLW, FLY	YLQ, IED, CLG, LLS, FLY	25	65
2	A2, A2, B35, B57	YLL, YLQ, IAL, CLG, FLY, HPV	YLL, IAL, CLG, FLY, HPV	33	56
3	A24, A29, B44, B44	TYG, ILL	TYG	72	18
4	A2, A29, B44, B60	YLL, YLQ, ALL, CLG, LLS, LTA, LLW, FLY, IED	CLG, LLS, FLY, IED	65	24
5	A2, A2, B14, B44	YLL, YLQ, ALL, CLG, LLS, LTA, LLW, FLY	CLG, LLS, FLY, LLW	58	32
6	A2, A24, B57, B62	YLL, YLQ, ALL, CLG, LLS, LLW, LTA, FLY, IAL, TYG	YLL, LLS	40	29
Patients					
1	A11, A24, B62, B75	SSC, TYG, PYLF	SSC	32	53
2	A11, A24, B18, B62	SSC, TYG, PYLF	SSC, TYG, PYLF	74	24
3	A3, A68, B7, B55	APYL	APYL	66	23
4	A2, A26, B27, B55	YLL, YLQ, ALL, LLL, TLL, LLV, CLG, LTA, LLS, FLY	No response	66	28
5	A11, A24, B51, B51	SSC, TYG, PYLF	SSC	80	15
6	A1, A26, B55, B62	APYL	APYL	58	33
7	A2, A11, B5, B51	SSC, FLY, TVC, CLG	SSC	NA	NA
8	A2, A11, B48, B75	YLL, YLQ, ALL, LLL, TLL, LLV, CLG, LTA, LLS, FLY, SSC	No response	37	34
9	A24, A30, B18, B51	TYG, PYLF	No response	NA	NA
10	A24, A34, B56, B56	TYG, PYLF	No response	NA	NA

^a PBMCs from healthy donors (donors 1 to 6) and NPC patients (patients 1 to 10) were activated with Ad-SAVINE, and stimulated cultures were phenotyped and tested for reactivity against EBV peptides on day 20. A positive response indicates more than 10% specific lysis in ⁵¹Cr release assays. See Table 1 for peptide abbreviations. NA, not applicable.

FIG. 3. Activation and expansion of LMP-specific CTLs from NPC patients after stimulation with Ad-SAVINE. PBMCs were cocultured with autologous PBMCs infected with Ad-SAVINE or autologous EBV-transformed LCLs at a responder-to-stimulator ratio of 2:1 for 7 days and stimulated weekly with LCLs infected or not with Ad-SAVINE. LMP-specific T-cell reactivity was subsequently assessed on day 20 by using in vitro cytotoxicity assays; autologous PHA blasts coated with individual peptides or uncoated were used as targets for these assays, with an E-to-T ratio of 20:1. (A) NPC patient 1 (HLA A11, A24, B62, and B75). (B) NPC patient 2 (HLA A11, A24, B18, and B62). See Table 1 for definitions of peptide abbreviations.

that had multiple responses (patient 2 [HLA A11, A24, B18, and B62]). However, in contrast to healthy individuals, no response could be detected for four of the NPC patients tested. This is likely to be due, at least in part, to the fact that in most instances, the patients were non-Caucasian with HLA types for which few epitopes had been defined. It could also be possible that apparent responses were damped by the effect of the drugs used for the treatment of these patients. A summary of all the data (phenotype and response by ^{51}Cr release) from all NPC patients is included in Table 2.

Utility of Ad-SAVINE in activating responses in healthy individuals and NPC patients with an HLA type incompatible with previously defined HLA types. To date, most of the epitopes have been defined with Caucasian donors. However, problems can arise when extrapolating epitope-based therapies to non-Caucasian patients, which may involve HLA types outside the spectrum of epitopes normally associated with Caucasian patients, e.g., NPC patients from Asia. To assess the use of the Ad-SAVINE formulation, which includes the complete sequence of the three relevant EBV proteins, PBMCs from an

FIG. 4. Representative data from the ELISPOT assays using overlapping peptides. Ad-SAVINE-activated cultures from virus-seropositive healthy donor 2 (A) or NPC patient 3 (B) were screened with the complete set of LMP2 overlapping peptides, and the cells that produced IFN- γ were detected by an ELISPOT assay. The number of spot-forming cells (SFC) from test wells were counted by using an AIDELISPOT reader.

Asian NPC patient (patient 3 [HLA A3, A68, B7, and B55]) and a healthy individual (donor 2 [HLA A2, B35, and B57]) were activated with the Ad-SAVINE formulation and screened in an ELISPOT assay by using a complete set of overlapping peptides from LMP2. Representative data from ELISPOT assays using overlapping peptides for LMP2 are presented in Fig. 4. In the case of the healthy individual (donor 2) (Fig. 4A), reactivity was associated with two peptide pools (pool 1 and pool 11). The single peptide present in both pools was MGSL EMVPM (HLA B35 restricted), which corresponded to a reactivity defined previously (44). In the case of NPC patient 3, reactivity was associated with pools 6 and 13 (Fig. 4B). The common peptide present in both of these pools is PVIVAPY LFWLAAIA, which includes the previously described epitope PYLFWLAAI (21), restricted by an HLA type (HLA A23/24) not present in this patient. This suggests that PVIVAPY LFWLAAIA includes a CTL response restricted through a non-HLA A23/24 allele. To confirm that there is indeed an undefined epitope within this sequence and to define its HLA restriction, we screened activated cultures from NPC patient 3 in a ^{51}Cr release assay against a series of allogeneic PHA blasts sharing a single allele with this NPC patient in the presence or absence of the peptide (Fig. 5A). Representative results show the killing of two allogeneic peptide-coated PHA blasts (patients 4 and 6), each of which shares HLA B55. In order to confirm this HLA restriction, PBMCs from this patient (patient 3) and another HLA B55 NPC patient (patient 4) were stimulated with PVIVAPY LFWLAAIA and tested in a ^{51}Cr release assay against PHA blasts sensitized with PVIVAPY LFWLAAIA and PYLFWLAAI. PHA blasts from both NPC cases showed that the active epitope was clearly included

FIG. 5. HLA B55-restricted CTL recognition of an LMP2 epitope. (A) The CTL effectors from NPC patient 3 were tested against autologous PHA blasts and allogeneic PHA blasts presensitized with synthetic peptide or left uncoated. Allogeneic donors (donor 10 [HLA A3, A31, B35, and B37], donor 11 [HLA A1, A3, B8, and B35], patient 11 [HLA A32, A68, B35, and B44], donor 12 [HLA A1, A2, B7, and B57], donor 13 [HLA A1, A2, B7, and B37], patient 4 [HLA A2, A26, B27, and B55], and patient 6 [HLA A1, A26, B55, and B62]) shared only one allele with this NPC patient. A high level of killing was detected in donors that share the B55 allele. (B) This was confirmed in a ^{51}Cr release assay with another B55 donor (patient 4 [HLA A2, A26, B27, and B55]), who showed reactivity against the peptides PYLFWLAAI (PYLF) and PVIVAPYLFWLAAIA.

within PVIVAPYLFWLAAIA and that only part of the reactivity was seen in PYLFWLAAI (Fig. 5B).

Fine-mapping of LMP-2-specific T-cell response within PVIVAPYLFWLAAIA. As indicated above, there is an HLA-B55 epitope within the PVIVAPYLFWLAAIA sequence. In order to define the minimal epitope contained in this 15-mer sequence, a series of 11-mer, 10-mer, 9-mer, and 8-mer overlapping truncations was synthesized and screened in a ^{51}Cr release assay (Fig. 6A). The results indicate that activity resides in four 11-mer, three 10-mer, two 9-mer, and two 8-mer sequences. To further define the minimal epitope, the two active 9-mer sequences (VAPYLFWLA and APYLFWLAA) and 8-mer sequences (APYLFWLA and PYLFWLAA) were titrated by using 10-fold dilutions in a ^{51}Cr release assay (Fig. 6B). The titration results indicate that maximal activity resides with the 9-mer peptide APYLFWLAA. It is interesting that maximal activity is associated with a 9-mer sequence that includes alanine at both the amino- and carboxy-terminal positions, as was previously shown for this HLA allele (6).

To confirm the activity of this novel epitope, PBMCs from

NPC patient 3 were cultivated in the presence of APYLFWLAA, and the functional activity was assessed by ELISPOT assay, ^{51}Cr release assay, and intracellular staining for IFN- γ -producing CD8 $^{+}$ T cells. The results confirm the ability of this peptide to activate a peptide-specific response (Fig. 7). Indeed, ICS staining shows that this response is dominant, and about 37% of CD8 $^{+}$ T cells showed reactivity to this peptide (Fig. 7C). Furthermore, the APYLFWLAA-specific T cells killed autologous LCLs and recognized a B55-matched allogeneic LCL in a ^{51}Cr release assay (Fig. 7B). In addition, these effectors recognized autologous LCLs in an ELISPOT assay (Fig. 7A).

Effect of CD4 $^{+}$ and CD8 $^{+}$ T-cell depletion of Ad-SAVINE-activated PBMCs on the inhibition of growth of autologous LCLs. As indicated above, in some instances, PBMCs activated with Ad-SAVINE result in cultures in which CD4 $^{+}$ cells predominate. To determine the potential effector role of CD4 $^{+}$ and CD8 $^{+}$ cells in such cultures, unfractionated, CD8 $^{+}$, or CD4 $^{+}$ T-cell-depleted activated PBMCs were tested for their abilities to limit the growth of various numbers of autologous LCLs. PBMCs were activated with the Ad-SAVINE construct, and after 2 weeks, either CD4 or CD8 depletions were performed, and these cells were used in the limiting growth assay. These experiments involved two donors whose activated cultures regularly resulted in a predominance of CD4 $^{+}$ cells (donor 3 [HLA A24, A29, B44, and B44] and donor 7 [HLA A28, A32, B12, and B12]). The experiment presented in Table 3 was repeated twice, and on each occasion, similar results were obtained with both donors. The results indicated that LCL cultures grew at all cell numbers in the absence of added activated cells. Unfractionated Ad-SAVINE-activated cultures restricted the growth of autologous LCLs (Table 3) but not allogeneic LCLs (data not shown). An investigation of the phenotype responsible for this inhibition revealed that most of the inhibitory activity was included in CD8-depleted cultures. Indeed, when this assay was performed by using allogeneic LCLs sharing a single class II allele, comparable results were obtained, while on the other hand, little inhibition was seen when using allogeneic LCLs without any class II sharing (data not shown). Overall, it appears that in instances where there is a predominance of CD4 $^{+}$ cells following Ad-SAVINE activation, the CD4 component is capable of limiting the expansion of autologous LCLs.

In order to make sure that the effect seen was not related to an adenovirus response, cultures were activated with the CMV-adenovirus construct (Ad-CMVpoly) and used in the outgrowth assay. Table 4 shows a minimal inhibition of outgrowth of autologous LCLs when activating responses with this control adenovirus. Furthermore, no inhibition of outgrowth of allogeneic LCLs was seen (data not shown). Moreover, when these cultures were used in a ^{51}Cr release assay, Ad-SAVINE-activated cultures killed autologous LCLs, while Ad-CMVpoly-activated cultures showed a much reduced killing of autologous LCLs. In addition, only the Ad-SAVINE-activated cultures could recognize and kill LMP- and EBNA1-primed autologous PHA blasts (Fig. 8). Thus, the effect seen with Ad-SAVINE is specific for the sequences included in the SAVINE construct and is not associated with an adenovirus response or other nonspecific mechanisms.

FIG. 6. Mapping of a minimal novel epitope sequence using in vitro assays. Ad-SAVINE-activated cultures from NPC patient 3 were stimulated weekly, and reactivity was tested on day 20. (A) Fine-mapping of the novel epitope was achieved by using a series of truncations (11-mer, 10-mer, 9-mer, and 8-mer sequences) spanning the 15-mer peptide in a ⁵¹Cr release assay. (B) The two 9-mer and two 8-mer sequences that gave the highest responses were then tested in a ⁵¹Cr release assay using serial dilutions of the potential epitopes.

DISCUSSION

EBV gene expression in NPC and HL is limited to EBNA1, LMP1, and LMP2. Potentially, each of these proteins is available for CTL targeting, although the response to LMP1 is generally weak (32), and the functional response to EBNA1 is controversial. In spite of the relative precursor frequency of the CTL response to each of these proteins, the notion that successful therapy might be based on boosting each of these (or selected) responses to levels not seen in either healthy individuals or NPC patients should be considered. The boosting of these responses has been achieved by an LMP-specific peptide (30), by autologous LCLs (8, 43), and by the stimulation of PBMCs from NPC and HL patients by use of an LMP2 Ad5/F35 construct (4). Furthermore, the Ad5/F35 polyepitope was previously shown to boost LMP responses in vitro (11). The Ad-SAVINE formulation described here has the advantage over each of these methods in that it can potentially activate all CD4⁺ and CD8⁺ responses to EBNA1, LMP1, and LMP2 in the absence of other competing EBV immunodominant responses. It has the further advantage that it should be applicable across all populations independent of HLA type and avoids any potential oncogenic sequences within LMPs.

The first set of experiments was designed to show that the Ad-SAVINE formulation was capable of activating LMP responses in healthy individuals as well as NPC patients. The phenotypes of the activated cultures were variable between

different healthy individuals and patients, ranging from those in which the CD4 response was dominant (4/6 healthy donors and 6/7 NPC patients for which the phenotype was available) to others in which CD8⁺ cells were dominant (2/6 healthy donors and 1/7 NPC patients for which the phenotype was available). Since few CD4 EBV-specific epitopes have been defined, it is not possible to speculate on the EBV-specific component included in these CD4-dominant cultures. However, since SAVINE has the potential to activate all CD4 responses, presumably some of these CD4⁺ cells are indeed EBV specific. It should be pointed out as well that in the one case investigated, an EBNA1 response (HPV in donor 2) was detected in Ad-SAVINE-activated cultures (Table 2). However, since there are relatively few defined CD8 epitopes within this protein, it might be premature to speculate on its significance.

In terms of the EBV specificity of activated cultures, the results of a representative experiment indicate that the degree of activation (ELISPOT and ⁵¹Cr release assays) was consistently higher in the case of the Ad-SAVINE construct than in the case of autologous LCLs. In the case of healthy HLA A2 individuals, multiple LMP specificities were activated. This activation of a broad response is likely to be important for immunotherapy for NPC since the degree of LMP1 and LMP2 expression tends to be variable between patients, and a system that focuses on a broad response is

FIG. 7. Activation of the CTL lines specific for the novel epitope. Cultures from NPC patient 3 specific for this novel peptide were generated and tested in ELISPOT (A), ⁵¹Cr release (B), and ICS (C) assays, confirming that APYLFWLAA is the minimal epitope within PVIVAPYLFWLAAIA. Also, the APYLFWLAA-specific T cells were able to recognize autologous LCLs in an ELISPOT assay (A) and to kill autologous LCLs and a B55-matched allogeneic LCL from another patient (patient 4) in a ⁵¹Cr release assay (B).

more likely to be effective than one focusing on a single response.

One of the advantages of the Ad-SAVINE construct is that it can potentially be used to treat patients from diverse ethnic

TABLE 3. Incidence of LCL growth with and without added Ad-SAVINE-activated autologous PBMCs^a

No. of LCLs (10 ³)	No. of cultures with LCLs/total no. of cultures			
	Without activated T cells	10 ⁴ Ad-SAVINE-activated PBMCs		
		Unfractionated	CD4 depleted	CD8 depleted
10	3/3	3/3	3/3	3/3
5	3/3	3/3	3/3	0/3
2.5	3/3	1/3	3/3	0/3
1.25	3/3	0/3	3/3	0/3
0.6	3/3	0/3	0/3	0/3
0.3	3/3	0/3	0/3	0/3
0.15	3/3	0/3	0/3	0/3
0.075	3/3	0/3	0/3	0/3

^a Autologous LCLs were seeded (in triplicate) at serial dilutions running from 10,000 cells/well with or without the addition of 10,000 Ad-SAVINE-activated cells/well that were depleted or not depleted of CD4⁺ or CD8⁺ T cells. Culture medium was changed weekly, and growth was scored visually at 3 weeks, with large pellets of actively growing cells indicating the successful growth of LCLs and the presence of debris in wells suggesting an inhibition of growth by T cells.

backgrounds without any knowledge of HLA type since all of the immune determinants from the three EBV proteins are included. The next set of experiments was designed to screen Ad-SAVINE-activated cultures with an overlapping LMP2 peptide set (15-mer peptides overlapping by 10 amino acids) to determine whether new responses could be activated. In the single NPC patient investigated, maximal activity was detected in two pools (Fig. 4B), and the activity was subsequently defined as a B55-restricted LMP2 epitope, APYLFWLAA. This sequence was confirmed by ⁵¹Cr release assay, ELISPOT assay, and ICS staining and conforms to the previously reported HLA B55 binding motif (<http://bio.dfci.harvard.edu/Tools/rankpep.html>). Although other minor reactivities were seen, their significance was not investigated. In either case, this experiment is important in that it demonstrates that it might be possible to activate responses independent of a knowledge of the HLA type and in the absence of previously defined LMP epitopes and can be used as a method of identifying new

TABLE 4. Incidence of LCL growth with and without added Ad-SAVINE- or Ad-CMVpoly-activated autologous PBMCs^a

No. of LCLs (10 ³)	No. of cultures with LCLs/total no. of cultures		
	Without activated T cells	With 10 ⁴ Ad-SAVINE-activated PBMCs	With 10 ⁴ Ad-CMVpoly-activated PBMCs
10	3/3	3/3	3/3
5	3/3	3/3	3/3
2.5	3/3	1/3	3/3
1.25	3/3	0/3	3/3
0.6	3/3	0/3	3/3
0.3	3/3	0/3	2/3
0.15	3/3	0/3	2/3
0.075	3/3	0/3	1/3

^a Autologous LCLs were seeded (in triplicate) at serial dilutions running from 10,000 cells/well with or without the addition of 10,000 Ad-SAVINE-activated cells/well or 10,000 Ad-CMVpoly-activated cells/well. Culture medium was changed weekly, and the growth was scored visually at 3 weeks, with large pellets of actively growing cells indicating a successful growth of LCLs and the presence of debris in wells suggesting an inhibition of the growth by T cells.

FIG. 8. Specificity of the response of Ad-SAVINE- and Ad-CMVpoly-activated cultures. Ad-SAVINE- and Ad-CMVpoly-activated cultures from a healthy donor were tested for specificity in a ^{51}Cr release assay against autologous PHA blasts coated with LMP and EBNA1 epitopes. See Table 1 for definitions of peptide abbreviations.

epitope sequences (CD4^+ or CD8^+). It is interesting that this HLA allele is present at a high frequency in Asian populations, particularly in Taiwan and China (33). Specific CTLs activated with this novel peptide were functionally active in $\text{IFN-}\gamma$ production and cell target lysis assays, as shown in Fig. 7.

To determine the significance of the CD4^+ T-cell expansions following Ad-SAVINE stimulation, an autologous outgrowth assay was performed by using unfractionated or CD4^+ - or CD8^+ -depleted cultures. The results displayed in Table 3 indicate that the unfractionated PBMCs activated with the Ad-SAVINE construct were capable of limiting the growth of autologous LCLs but not HLA-unrelated allogeneic LCLs (data not shown) and that most of this activity could be attributed to the CD4^+ component of this population. This result highlights the advantage of the Ad-SAVINE approach over the polyepitope approach, since to date, relatively few CD4^+ CTL epitopes for possible inclusion in a polyepitope have been defined. Although the CD4^+ response is dominant in activated cultures from both most healthy individuals and NPC patients, we have not investigated the relative importance of the CD4^+ cells in cultures where CD8^+ is the dominant phenotype using this inhibition-of-growth assay. Ongoing experiments will help clarify the role of CD4^+ cells in cultures stimulated with Ad-SAVINE.

The results displayed in Table 4 indicate that when cultures were expanded and stimulated with another recombinant adenovirus (Ad-CMVpoly), a minimal inhibition of outgrowth was seen on autologous LCLs, while a strong inhibition was seen with Ad-SAVINE-activated cultures. It should be pointed out that when the activities of these cultures were tested in a ^{51}Cr release assay, only the Ad-SAVINE-activated cultures could recognize and kill EBV peptide-coated PHA blasts (Fig. 8). All of these experiments indicate that the effect seen is specific for EBV and it is not associated with an adenovirus-directed response. Furthermore, a previous report using the same replication-deficient adenovirus in which CMV had been encoded demonstrated that adenovirus did not inhibit the ability of this construct to activate a CMV-specific response (28).

A vaccine with the Ad-SAVINE formulation would appear

to have distinct advantages over other current modalities under development. Thus, for instance, the adoptive transfer of LMP-specific T cells is limited by the requirement for specialized facilities and the time required for activating autologous CTLs. Direct vaccination with the adenovirus polyepitope overcomes some of these problems but is limited to those individuals for whom epitopes have been defined and incorporated into this construct. On the other hand, the Ad-SAVINE formulation would appear to have the capacity of universal utility. In the current study, SAVINE was incorporated into a replication-deficient adenovirus. In the future, it will be necessary to compare this vector with others including poxviruses.

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