Immunization of Chimpanzees with Tumor Antigen MUC1 Mucin Tandem Repeat Peptide Elicits Both Helper and Cytotoxic T-Cell Responses[1]

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ABSTRACT

CTLs and antibody responses to the tumor-associated antigen MUC1 mucin can be detected in patients with adenocarcinomas of the breast, pancreas, colon, and ovary. However, neither response is generally effective at controlling disease. Methods to augment immunity to MUC1 are being designed, with the expectation that this will lead to an antitumor response. The key to eliciting potent immunity to tumor MUC1 may be in generating MUC1-specific T-helper cell responses, which, to date, have not been reported in cancer patients. We have recently demonstrated that a synthetic vaccine representing five copies of the MUC1 tandem repeat peptide can be used to prime MUC1-specific human CD4$^+$ T cells *in vitro*. Here, we extend these studies to test the immunogenicity and safety of the tandem repeat peptide in the chimpanzee, which has the identical MUC1 tandem repeat sequence to the human. To promote induction of Th1-type responses, we used the novel adjuvant LeIF, a *Leishmania*-derived protein that is known to stimulate human peripheral blood mononuclear cells (PBMCs) and antigen-presenting cells, to produce a Th1-type cytokine profile. We found that MUC1 tandem repeat peptide administered with LeIF elicited positive, albeit transient, proliferative T-cell responses to MUC1 in the PBMCs from four of four chimpanzees. Immunization induced MUC1-specific IFN-γ but not interleukin 4 expression in CD4$^+$ T cells from PBMCs and draining lymph nodes. MUC1-specific CTLs were also generated that did not induce detectable autoimmune dysfunction during the 1 year of observation. We conclude that the MUC1 tandem repeat peptide can be used to elicit both T-helper and cytotoxic cell responses to MUC1 in the primate and holds promise as a safe and effective cancer vaccine.

INTRODUCTION

MUC1 mucin is a membrane glycoprotein that is present on ductal epithelia of the pancreas, ovary, breast, lung, and prostate. In normal tissues, MUC1 mucin is heavily glycosylated with *O*-linked carbohydrates. In contrast, in adenocarcinomas, the degree of glycosylation is markedly reduced (1). The effect of underglycosylation is to expose the extracellular region of the protein, which consists largely of a tandemly repeating peptide sequence of 20 amino acids. Concurrent with this unmasking, CTLs and antibody responses that are specific for epitopes within the tandem repeat region of MUC1 are generated in cancer patients (2–7). However, neither immune response is effective at controlling disease. Establishing ways to augment immunity to tumor MUC1 could lead to an effective immunotherapy for a range of human adenocarcinomas (8, 9).

The key to eliciting potent immunity to tumor MUC1 may be in generating MUC1-specific T-helper cell responses, which, to date, have not been reported in cancer patients. T-cell help will likely augment CTL responses to MUC1 and promote antibody isotype switching, leading to more effective antitumor immunity. We speculate that T-helper cells that are specific for MUC1 may not be expanded in cancer because they do not encounter sufficient MUC1 antigen presented by professional APCs.[3] Circulating MUC1 protein is highly glycosylated, and the complex carbohydrate side chains may limit processing of the protein by APCs for presentation to T cells. In contrast, it is possible that unglycosylated MUC1 represents a more suitable form of the antigen for processing by APCs. The amount of free unglycosylated MUC1 in patient sera is negligible, however, because this form of the protein remains tumor cell associated. Providing MUC1 in a soluble unglycosylated form may circumvent this problem and lead to induction of MUC1-specific T-helper cell responses. In support of this notion, we recently have shown that naïve CD4$^+$ T cells from healthy donors can be primed *in vitro* to a synthetic MUC1 peptide of 100 amino acids, representing five unglycosylated tandem repeats (10). In contrast, the fully glycosylated MUC1 protein isolated from patient ascites did not stimulate specific T-helper cell responses (10).

Here, we extend our studies of MUC1 tandem repeat peptide as a potential cancer vaccine by testing its immunogenicity

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[3] The abbreviations used are: APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; IL, interleukin; LN, lymph node; KLH, keyhole limpet hemocyanin; SI, stimulation index.

Table 1 Immunization protocol

Series	Week	Procedure	Immunogen[a]	Route	Site
1	0	Immunize	MUC1 peptide and LeIF	s.c	Left leg
			KLH and LeIF	s.c.	Right leg
	2	Boost 1	MUC1 peptide and LeIF	s.c.	Left leg
			KLH and LeIF	s.c.	Right leg
	4	Harvest LNs[b]			
2	23	Boost 2	MUC1 peptide and LeIF	s.c.	Left leg
			KLH and LeIF	s.c.	Right leg
	25	Boost 3	MUC1 peptide and LeIF	s.c.	Left leg
			KLH and LeIF	s.c.	Right leg
	27	Harvest LNs			

[a] 500 μg of MUC-1 peptide or 500 μg of KLH were mixed with 100 μg of LeIF.
[b] Left and right inguinal LNs.

and safety in a relevant preclinical model, the chimpanzee. The 20-amino acid tandem repeat sequence of MUC1 is identical in chimpanzee and human, providing an ideal model for addressing questions of immunity and autoimmunity to a self-antigen that is nonmutated in cancer. We have previously demonstrated that immunization with cell-associated MUC1 mucin elicits CTLs in this model (11). We predict that induction of an MHC Class II-restricted MUC1-specific T-helper cell response following vaccination with peptide is unlikely to elicit autoimmune dysfunction *in vivo* because the normal MUC1-bearing epithelial cells do not express MHC Class II molecules. To promote induction of Th1-type responses, we used as an adjuvant a recombinant protein derived from *Leishmania braziliensis*, called LeIF (*L. braziliensis* homologue of the eukaryotic initiation factor 4A; Ref. 12). LeIF has been shown to stimulate human PBMCs and APCs to express a Th1-type cytokine profile and to produce IL-12 (12–14). We show that vaccination with MUC1 mucin peptide in combination with LeIF induced proliferative T-cell responses and expression of IFN-γ by $CD4^+$ peripheral blood and LN T cells in healthy chimpanzees. Immunization also induced CTL responses that were specific for MUC1, which were not associated with any detectable autoimmune disease in the 1-year period of observation. These studies suggest that a synthetic MUC1 tandem repeat peptide combined with an effective adjuvant has the potential to serve as a safe and efficacious cancer vaccine.

MATERIALS AND METHODS

Animals. Four adult chimpanzees were selected for use in this study, including three nulliparous females (identification nos. 638, 654, and 664) and one male (identification no. 575). Animals were housed and all *in vivo* experiments were performed at the Yerkes Regional Primate Research Center (Atlanta, Georgia), according to institutional standards and procedures for working with nonhuman primates.

Antigens. The MUC1 mucin peptide used for injection was 100 amino acids long and represented five repeats of a tandemly repeating 20-amino acid region of the MUC1 mucin extracellular domain $[(GVTSAPDTRPAPGSTAPPAH)_5]$. The peptide was synthesized on a Chemtech 200 machine using *N*-(9-fluorenyl)methoxycarbonyl chemistry and purified by high-performance liquid chromatography (10). In some *in vitro* assays, 80- or 140-amino acid peptides representing four and seven tandem repeats, respectively, were used. KLH (Sigma Chemical Co., St. Louis, MO) was used as a control immunogen. The full-length recombinant LeIF protein (M_r 45,000) used as adjuvant in immunization studies was supplied by Corixa Corp. Expression and purification of recombinant LeIF have been described elsewhere (14).

Antibodies. Monoclonal antibodies specific for human CD3 (conjugated to FITC and PerCP), CD4 (FITC), and CD8 (FITC) and isotype control antibodies were purchased from Becton Dickinson (San Jose, CA). Monoclonal antibodies specific for human cytokines IL-4 (phycoerythrin), IFN-γ (FITC and phycoerythrin), and appropriate isotype control antibodies were purchased from PharMingen (San Diego, CA). BC-2 monoclonal antibody specific for MUC1 mucin tandem repeat region was kindly supplied by Ian F. C. McKenzie (The Austin Research Institute, Victoria, Australia).

Immunization Protocol. All four animals received the same immunization and boost schedule, consisting of two series of two injections (Table 1). The first series included an immunization at week 0 and a boost at week 2; the second series included boosts at weeks 23 and 25. For each administration, 500 μg of MUC1 peptide were mixed with 100 μg of LeIF and injected s.c. in the left thigh in a region drained by the inguinal LN. At the same time, 500 μg of KLH mixed with 100 μg of LeIF were injected contralaterally s.c. into the right thigh as a positive control antigen (Table 1). Inguinal LNs were surgically collected at the end of each immunization series at weeks 4 and 27.

T-Cell Proliferation Assays. Proliferation assays were done essentially as described (15). Briefly, 1×10^5–2×10^5 PBMCs or LN cells were cultured in AIM V medium (Life Technologies, Inc., Grand Island, NY) with L-glutamine (Life Technologies, Inc.) in U-bottomed microtiter plates in triplicate in the presence or absence of 100 μg/ml KLH or MUC1 peptide or 5 μg/ml phytohemagglutinin for 3–7 days. [^{3}H]Thymidine (Amersham, Arlington Heights, IL) was added at a final concentration of 5 μCi/ml in the last 18 h, and proliferation was read on a β scintillation counter. Cell proliferation was recorded as cpm or as SIs (the cpm in response to antigen divided by the response to medium alone).

Detection of Intracellular Cytokines. Intracellular cytokine production was detected using a modification of a published protocol (16). Briefly, PBMCs or LN cells were cultured

Table 2 Proliferative responses of PBMCs to KLH and MUC1 in immunized chimpanzees[a]

Identification no.	Ag	Week of immunization																		
		0			2			4			8			25			27		
		No Ag	Ag	SI	No Ag	Ag	SI	No Ag	Ag	SI	No Ag	Ag	SI	No Ag	Ag	SI	No Ag	Ag	SI
575	KLH	1.2	1.4	1.2	1.7	2.5	1.5	0.3	2.5	8.0[b]	3.2	18.8	5.9[b]	4.4	11.7	2.6[b]	14.1	39.5	2.8[b]
	MUC1	1.2	1.6	1.4	1.7	1.1	0.7	0.3	2.2	7.1[b]	3.2	3.6	1.1	4.4	3.4	0.8	14.1	27.1	1.9
638	KLH	0.6	0.1	0.2	6.2	11.7	1.9	0.4	1.2	3.2[b]	1.1	7.1	6.3[b]	9.7	25.3	2.6[b]	12.5	97.4	7.8[b]
	MUC1	0.6	1.0	1.7	6.2	9.5	1.5	0.4	2.7	6.9[b]	1.1	9.7	8.7[b]	9.7	9.6	1.0	12.5	21.5	1.7
654	KLH	3.0	5.4	1.8	2.0	3.1	1.5	1.4	20.8	14.9[b]	1.7	11.6	6.8[b]	4.5	16.3	3.6[b]	0.5	16.0	32.8[b]
	MUC1	3.0	1.2	0.4	2.0	0.9	0.4	1.4	2.2	1.5	1.7	2.8	1.6	4.5	4.0	0.9	0.5	2.6	5.2[b]
664	KLH	2.0	7.5	3.8[b]	1.4	20.4	14.3[b]	4.3	16.9	4.0[b]	1.7	16.5	10.0[b]	13.2	21.9	1.7	1.1	4.5	4.1[b]
	MUC1	2.0	0.8	0.4	1.4	2.2	1.5	4.3	1.8	0.4	1.7	4.1	2.5[b]	13.2	32.7	2.5[b]	1.1	0.9	0.8

[a] PBMCs were cultured with 100 μg/ml KLH, MUC1 peptide, or no antigen for 3–7 days and proliferation measured by incorporation of [^{3}H]thymidine. Data are expressed as absolute cpm ($\times 10^{-3}$), and SIs are expressed as fold increase in proliferation in the presence of antigen. Ag, antigen.

[b] SI $\geq$ 2.5.

in 24-well plates at a density of 2×10^6 cells per well in AIM V medium and glutamine in the presence or absence of 100 μg/ml KLH or MUC1 peptide. After 7–10 days, cells were restimulated with 1×10^5 autologous EBV-transformed B cells that had been incubated overnight with or without antigen. Four h prior to labeling, cells were treated with 10 μg/ml brefeldin A (Sigma) to inhibit secretion of cytokines. Cells were resuspended in PBS containing 0.5% BSA and 0.1% sodium azide (cytometry buffer) and incubated with monoclonal antibody specific for cell surface antigen for 30 min. Cells were washed with cytometry buffer, fixed in 2% formaldehyde, and permeabilized in cytometry buffer containing 0.1% saponin (Sigma). Cells were then incubated with monoclonal antibody specific for cytokines for 45 min, washed, and fixed in 0.1% paraformaldehyde solution. Immunofluorescence was detected using a FACS Caliber flow cytometer (Becton Dickinson). Preliminary studies demonstrated that monoclonal antibodies specific for human IL-4 and IFN-γ cross-reacted with chimpanzee antigens, as determined by analysis of PBMCs stimulated with anti-CD3 and IL-2 followed by phorbol dibutyrate (Sigma) and ionomycin (Sigma), as described by the manufacturer (data not shown).

Cytotoxicity Assays. PBMCs were stimulated with 100 μg/ml MUC1 peptide in AIM V medium in the presence of 10 units/ml IL-2. After 5 days of stimulation, variable numbers of T cells were tested against 2000 target cells in a standard 4-h ^{51}Cr release assay (4). To generate target cells, we first subcloned MUC1 cDNA from the pDKOF.muc1 vector (17) into the pRc/CMV vector (Invitrogen, Carlsbad, CA). Autologous EBV-transformed B cells were transfected with pRc/CMV-MUC1 and stable transfectants selected using G418 (Life Technologies, Inc.). Stable B cell transfectants could only be established from animal 664, and hence, all cytotoxicity assays were done using cells from this animal. Additional target cells were the nontransfected parent cells incubated with or without 100 μg/ml MUC1 peptide overnight prior to the assay.

RESULTS

Immunization with MUC1 Peptide-induced T Cell-proliferative Responses. The primary focus of these experiments was to test the ability of MUC1 tandem repeat peptide to induce T-helper cell responses as a self-antigen in the chimpanzee. Our first evaluation of this response was T-cell proliferation. PBMCs were harvested before, during, and after vaccination and cultured with 100 μg/ml peptide antigen for 3–7 days, with proliferative responses measured by [^{3}H]thymidine incorporation. Immunization with a complex foreign protein KLH generated, as expected, strong T-cell proliferative responses in PBMC of all animals following the first immunization series, which was sustained to at least week 27 (Table 2). T-cell proliferative responses to MUC1 mucin 100-mer peptide were elicited in all animals to varying degrees. As expected, proliferative responses were much weaker than those elicited by KLH, reflecting the simple 20-amino acid repeating sequence of the peptide, which contains potentially a single immunogenic epitope. Proliferation of cells from animal 638 reached 9700 cpm at week 8, representing a SI of 8.7. Responses in animals 575 and 664 reached 2200 cpm and 4100 cpm, respectively, after the first boost (SI = 7.1 and 2.5, respectively). Responses in animal 664 increased to 32,700 cpm after the second boost (week 25, SI = 2.5) but were reduced following the final boost (week 27). Proliferative responses were detectable in animal 654 following the first boost (2800 cpm, SI = 1.6) and after the third and final boost (2600 cpm, SI = 5.2).

MUC1-specific IFN-γ Expression Was Induced in LN CD4 T Cells in Response to Vaccination. To more critically evaluate the MUC1-specific T-cell response elicited by immunization, we analyzed cytokine expression of cells isolated from LNs draining the site of vaccination. Three-color staining for CD3, CD4, and either IFN-γ or IL-4 was performed on LN cells following *in vitro* stimulation with antigen, as described in "Materials and Methods." Fig. 1 shows a representative analysis of cells from animal 654. T cells isolated from inguinal LNs after the first and second series of immunizations expressed IFN-γ in response to both KLH and the MUC1 peptide. The responses were mediated by CD4$^+$ T cells and were stronger in response to KLH, as compared to MUC1. After just one boost, 5.8 and 3.5% of CD4$^+$ T cells expressed IFN-γ in response to MUC1 peptide in the right and left inguinal LNs, respectively, compared to 16.3 and 5.6% for KLH in the same tissues (Fig. 1). The proportion of IFN-γ-expressing cells following the second

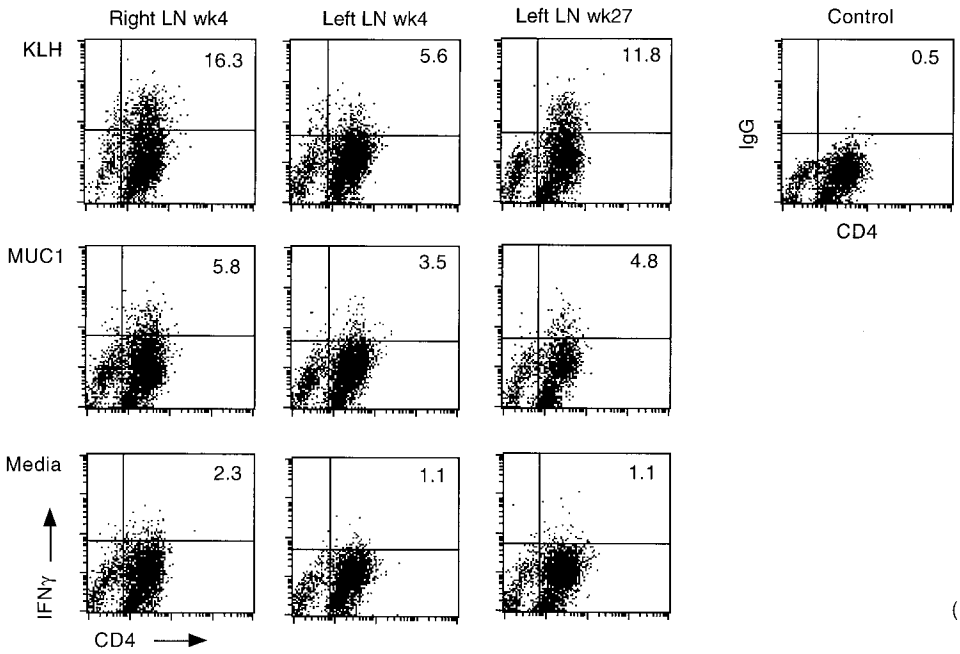

Fig. 1 Intracellular expression of IFN-γ in $CD3^+$ LN cells from animal 654 after the first (*wk4*) and third (*wk27*) boost with LeIF/MUC1 and LeIF/KLH. Cells were cultured with 100 μg/ml antigen or medium alone for 7–10 days and then restimulated with autologous EBV-transformed B cells incubated with antigen overnight prior to staining with monoclonal antibodies. Cells were gated on expression of CD3. Numbers represent percentage of $CD4^+$ T cells expressing IFN-γ. Also shown is a representative dot plot of LN cells stained with IgG isotype control antibody.

series of boosts was similar to the first (4.8% for MUC1, compared to 11.8% for KLH). Background IFN-γ expression in response to *in vitro* culture (medium) was minimal. Antibody labeling was specific because control IgG antibody did not generate a fluorescence signal (Fig. 1). In contrast to the expression of IFN-γ, intracellular expression of IL-4 was negligible in all samples (data not shown). These data indicate that immunization elicited Th1-type responses to antigen. Similar data were obtained from animals 664 and 638, whereas negligible cytokine expression was detected in response to MUC1 in animal 575 (data not shown).

MUC1 Immunization Induced IFN-γ Responses in Peripheral Blood T Cells. We next sought to determine whether effector cells induced by this vaccination protocol could be detected in the peripheral circulation. Peripheral blood lymphocytes were stimulated and stained for expression of surface CD3 and intracellular IL-4 or IFN-γ. Fig. 2*a* shows a representative profile from animal 664, which had a strong proliferative response to MUC1 in the PBMCs at weeks 8 and 25 (Table 2). As expected, the proportion of peripheral blood T cells expressing IFN-γ in response to KLH increased over the course of vaccination. Importantly, IFN-γ responses to MUC1 peptide also increased with time. The proportion of T cells in peripheral blood expressing IFN-γ in response to MUC1 peptide increased from 0.7% prior to vaccination to 1.5% after one boost and 3.0% after the third and final boost (Fig. 2*a*). Concurrently, IFN-γ was expressed by $CD3^-$ cells in the PBMCs (Fig. 2*a*). This population likely represents natural killer cells nonspecifically stimulated to release IFN-γ by the activated T-cell component. The response of $CD3^+$ T cells to medium alone also increased slightly from 0.8 to 1.4% over the course of vaccination. This is not surprising because we consistently find that PBMCs are more responsive to culture following immunization due to the activated state of T cells in the circulation. In contrast to the expression of IFN-γ, intracellular expression of IL-4 in the PBMCs in response to KLH and MUC1 was weak or not detectable (data not shown), consistent with a Th1-type response. The peripheral T-cell IFN-γ response corresponded to similar expression in cells isolated from the draining LN from the same animal. As shown in Fig. 2*b*, the proportion of T cells expressing IFN-γ in response to MUC1 peptide after the third boost was 3.4%, almost five times the background (medium) response. Interestingly, 8.0% of T cells in the left LN expressed IFN-γ in response to stimulation with KLH, despite immunization with KLH in the right thigh (Table 1). This response reflects the systemic response to KLH at week 27, as seen in the PBMCs (Table 2; Fig. 2*a*). Similar responses were detected following immunization of animals 654 and 638, with negligible cytokine response induced in animal 575 (data not shown). From these data, we conclude that immunization with MUC1 peptide elicited Th1-type responses in peripheral T cells, consistent with responses observed in the LN $CD4^+$ T cell fraction (Fig. 1).

Immunization with MUC1 Peptide and LeIF Elicited CTL Responses in PBMCs. We next wanted to determine whether immunization with MUC1 tandem repeat peptide could elicit CTL responses to MUC1, as had been seen in our previous studies using cell-associated MUC1 vaccines in chimpanzees (11). To test this, we attempted to transfect B-cell lines from all four animals with MUC1 cDNA, but we were only able to establish a stable MUC1-expressing cell line from animal 664. This animal had evidence of a Th1-type response to MUC1 in PBMCs and LN cells (Table 2, Fig. 2). PBMCs from animal 664 were stimulated once *in vitro* with soluble MUC1 peptide prior to testing against the autologous MUC1-transfected target. Results from two separate experiments are given in Fig. 3. Prior to vaccination, specific killing of the transfected target did not differ from the nontransfected parent cell line. The MUC1-specific CTL response increased over the course of vaccination, whereas killing of the parent cell line remained low (Fig. 3). At week 27, after the third boost, specific killings of the MUC1-

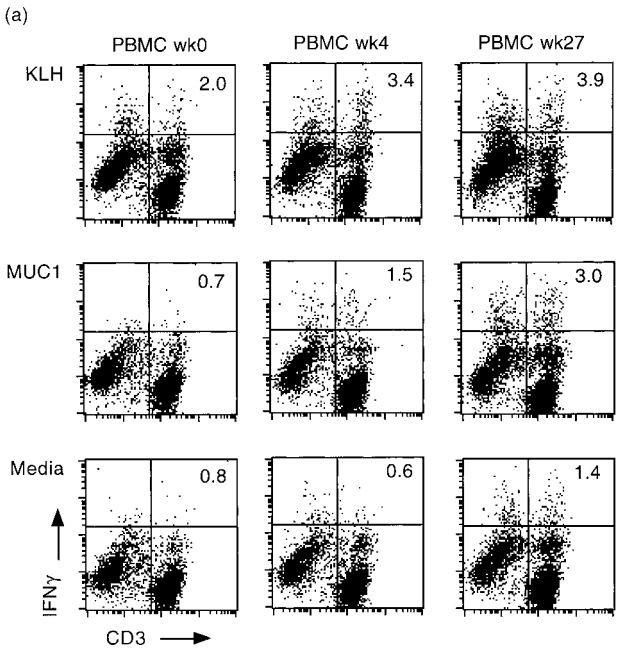

Fig. 2 Intracellular expression of IFN-γ in PBMCs (*a*) and LN cells (*b*) from animal 664 before immunization (*wk0*) and after the first (*wk4*) and third (*wk27*) boost with LeIF/MUC1 and LeIF/KLH. Cells were cultured with 100 μg/ml antigen or medium alone for 7–10 days and then restimulated with autologous EBV-transformed B cells incubated with antigen overnight prior to staining with monoclonal antibodies. *Numbers* represent percentage of $CD3^+$ T cells expressing IFN-γ.

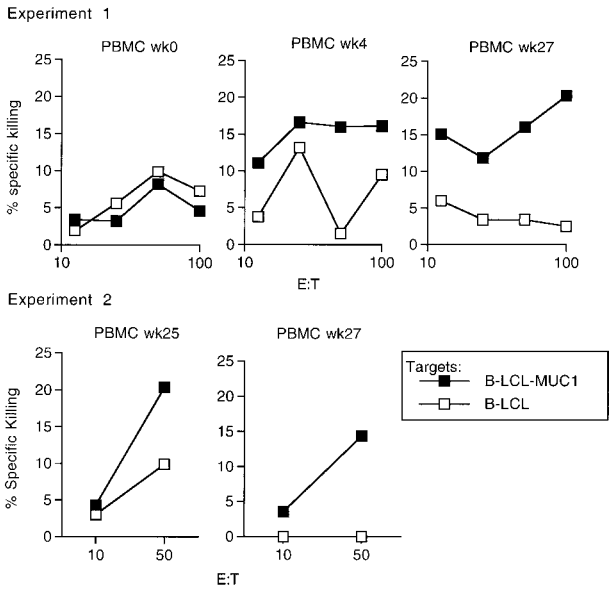

Fig. 3 Cytotoxic T-cell function of PBMCs from animal 664 before immunization (*wk0*) and after the first (*wk4*), second (*wk25*), and third (*wk27*) boost with LeIF/MUC1. Cells were stimulated for 5 days with 100 μg/ml MUC1 peptide in IL-2 and then tested against ^{51}Cr-labeled autologous EBV-transformed B cells transfected with MUC1 cDNA (*B-LCL-MUC1*) or untransfected EBV-transformed B cells (*B-LCL*).

transfected cell line at an E:T ratio of 50:1 were 16 and 14% for each experiment, as compared to 3.4 and 0% for the parent cell line (Fig. 3, *Experiment 1* and *Experiment 2*, respectively). This level of cytotoxicity probably closely reflects the *in vivo* CTL activity because the PBMCs were assayed only 5 days after a single *in vitro* restimulation. Moreover, cytotoxic activity was directed against naturally processed MUC1, suggesting that

Table 3 Summary of immune responses to MUC1 in immunized chimpanzees

Identification no.	T-cell responses			Ab responses[d]
	Proliferation[a]	IFN-γ expression[b]	CTL function[c]	
575	+	−	NT	−
638	+++	+	NT	−
654	+	++	NT	−
664	++	++	+	−

[a] SI: + ≥2.5 at one time point; ++, ≥2.5 at two time points; +++, ≥5.0 at two time points.

[b] −, not detected; +, 1–2% IFN-γ^+ cells; ++, 3–6% IFN-γ^+ cells.

[c] NT, not tested; +, CTL function detected following immunization.

[d] Sera tested by ELISA (6). −, not detected. Ab, antibody.

CTL responses induced by peptide immunization may be biologically active against MUC1-expressing tumor cells. Interestingly, incubation with MUC1 peptide did not alter the degree of killing of the parent cell line (data not shown). These data indicate that, in addition to T-helper responses, immunization with the MUC1 peptide-induced CTL responses in this animal. A summary of the immune responses to MUC1 elicited by administration of MUC1 peptide with LeIF adjuvant for all four animals is provided in Table 3.

Chimpanzees Remained Clinically Normal Despite Immune Responses to MUC1. To test for autoimmune dysfunction, we measured serum amylase levels from all animals at regular intervals during the protocol. Serum amylase is a measure of pancreatic function and serves as an indicator for destruction of MUC1-expressing tissues. Levels of serum amylase were within normal limits for all animals at all times. No abnormalities were detected in serum chemistry panels taken at regular

intervals during the study, and all animals remained clinically normal. A transient leukocytosis was detected in three animals (identification nos. 575, 638, and 654) at different intervals during the study, which did not appear to be associated with any experimental procedure. This leukocytosis was not detected in animal 664, which had a CTL response to MUC1 following vaccination (Fig. 3). Twelve months after the completion of the study, all animals remained clinically normal.

DISCUSSION

The aims of this study were to evaluate safety and efficacy of the MUC1 mucin tandem repeat peptide and the ability to direct the immune response toward a Th1 type by using a newly described adjuvant. These *in vivo* experiments in the nonhuman primate extended our previous findings demonstrating that MUC1 tandem repeat peptide is effective at priming $CD4^+$ T cell responses *in vitro* in humans (10). Moreover, others have reported MUC1-specific T-helper responses in PBMCs of healthy multiparous women seen upon repeated stimulation with the tandem repeat peptide *in vitro* (18). The *in vitro* data suggested that unglycosylated tandem repeat MUC1 peptide may be useful as an immunogen *in vivo*, both to induce T-helper cell responses and to augment existing CTL immunity to tumor MUC1.

The nature of the T-cell response we elicited to MUC1 and KLH in immunized chimpanzees was a Th1-type response. IFN-γ but not IL-4 was produced by $CD4^+$ T cells from immunized chimpanzee PBMCs and LNs upon restimulation with antigen. CTL responses to MUC1 appear also to be inducible with this vaccine protocol, although for technical reasons, we could study it only in one animal. These findings are consistent with the adjuvant LeIF having been reported to skew the immune response to antigen toward a Th1-type profile (12–14). The T-cell responses elicited to MUC1 were relatively weak, but this may be due more to our methods of monitoring rather than a true reflection of the *in vivo* immunity. For example, studies using the same intracellular cytokine analysis of T-cell responses to human cytomegalovirus, a virus that elicits a sustained immune response in persistently infected humans, indicated that, frequently, $<0.5\%$ of peripheral blood $CD8^+$ T cells express IFN-γ in response to stimulation with the immunodominant viral peptide epitope (19). Hence, our data, showing responses in the order of 3–5% $CD4^+$ LN T cells expressing IFN-γ in response to MUC1 peptide, are consistent with induction of MUC1-specific T-cell responses.

Ideally, a cancer vaccine should induce both T- and B-cell immune responses. Surprisingly, vaccination failed to induce antibody responses to MUC1 in any animal, despite the presence of helper T-cell responses (Table 3). In contrast, antibody titers to KLH reached 1:1280 to 1:2560 in sera of all animals by the end of the second immunization series (data not shown). In a previous study, the same tandem repeat peptide administered with oil-in-water adjuvant elicited MUC1-specific antibody responses detected by ELISA in one of four chimpanzees (15). It is conceivable that, in this study, the skewed Th1-type response prevented antibody production to MUC1 in immunized chimpanzees or, alternatively, that the amount of peptide used was not sufficient to activate antigen-specific B cells.

Previous studies using the same MUC1 tandem-repeat peptide as an immunogen in chimpanzees failed to elicit T-cell proliferative responses, although only a limited vaccination course was tested (15). Animals were immunized with a single injection of antigen-pulsed dendritic cells followed by one boost of antigen in oil-in-water adjuvant. It is possible that immunity to MUC1 would have been elicited had additional boosts been given (15). However, in this study, a single immunization and boost were sufficient to induce detectable T-cell responses to MUC1 in all animals (Table 2). A similar pentameric MUC1 tandem repeat peptide, administered with *Bacillus* Calmette-Guérin adjuvant, has been tested in a Phase I trial in cancer patients (20). Vaccination induced detectable T-cell responses in some patients, as measured by an increase in CTL precursor frequency. It is likely that combining the tandem repeat peptide with improved adjuvants such as LeIF will enhance the immunity to MUC1 in patients.

We have recently shown that MHC Class II-restricted $CD4^+$ T-cell responses that are specific for MUC1 can be generated *in vitro* from naïve human PBMC (10). As in this study, primed T cells produced IFN-γ and had moderate cytolytic activity. The response was directed toward a sequence within the tandem repeat region presented by HLA-DR3 (10). We also have preliminary data to suggest that HLA-DR4 is a restricting element for peptides in the MUC1 tandem repeat region. We were not able to unequivocally determine MHC allele usage in the chimpanzees in this study. However, PCR patterns showed evidence of either HLA DR3 or HLA DR4 in each of the animals studied (data not shown). It is possible, therefore, that the MUC1-specific CTL and T-helper responses detected in the immunized chimpanzees were restricted by HLA DR3 or HLA DR4, as observed in the human.

Several investigators have demonstrated that MUC1-based immunogens can induce tumor rejection in murine models. Synthetic MUC1 peptides conjugated to KLH induced significant protection from MUC1-expressing tumor cell challenge in immunized mice (21). Interestingly, protection was mediated by antibody responses, as opposed to T-cell responses to the immunizing peptide (21). In contrast, mice immunized with dendritic cells transduced with a recombinant adenovirus vector to express MUC1 developed specific CTLs that protected against MUC1-positive tumor challenge (22). Dendritic cell-based fusion vaccines also induced rejection of established MUC1-expressing tumors in MUC1-transgenic mice, with no apparent autoimmunity (23). Other investigators have used MUC1 tandem repeat peptide conjugated to the carbohydrate polymer mannan to skew responses to the Th1 type. Mannan-MUC1 fusion antigen induced protective CTL responses and high levels of IFN-γ secretion in a murine tumor model (24). Unfortunately, the strong T-cell immune response to MUC1 induced with this vaccine in mice was not detected in humans. In a clinical trial using the same immunogen, the predominant response was humoral, with high titer anti-MUC1 antibody produced in 13 of 25 patients but only limited T-cell responses (25). This emphasizes the need to test vaccine approaches in a relevant preclinical model such as the chimpanzee or the MUC1 transgenic mouse (23, 26).

Because of a carefully selected immunogen, we did not expect to see evidence of autoimmunity, and our data show that there was none. The main reason is that, although there is no mutation in the MUC1 gene, the protein expressed on tumor cells differs both structurally and immunologically from MUC1 expressed on normal tissue (1). Profound underglycosylation of

tumor-associated MUC1 promotes generation of tumor-specific epitopes within the tandem repeat region, which are recognized by both antibody and CTL. These epitopes are not found on normal cells, and even if they could be generated under certain conditions, the whole MUC1 protein would not be accessible to the immune system due to its strictly polarized expression on the lumenal side of ductal epithelial cells. Furthermore, processed peptides from the tandem repeat region that could be presented in MHC Class I molecules on the basolateral surface of normal ductal cells do not bind Class I molecules very efficiently and are, thus, not likely targets (5). MHC Class II-restricted T cells also should not target normal ductal epithelium because these cells do not express MHC Class II molecules. These studies, therefore, support the use of MUC1 tandem repeat peptide immunogen as a vaccine to induce antitumor responses in cancer patients and provide the basis for further development and testing of vaccine formulations that will stimulate the highest level of immunity and broadest participation of immune effector mechanisms.

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