Primary T-Cell and Activated Macrophage Response Associated with Tumor Protection Using Peptide/Poly-N-Acetyl Glucosamine Vaccination

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ABSTRACT

The mode of peptide-based cancer vaccine administration critically affects the ability to achieve a clinically relevant tumor-specific response. We have previously shown (Cole et al., Clin. Cancer Res., 3: 867–873, 1997) that a specific formulation of the polysaccharide poly-N-acetyl glucosamine (p-GlcNAc, designated as F2 gel) is an effective vehicle for sustained cytokine and peptide delivery in vitro. The purpose of this study was to evaluate the efficacy of F2 gel/peptide vaccination in the murine EG.7-OVA tumor model and to elucidate potential mechanisms involved in the observed cell-mediated response. C57BL/6 mice were given injections of 200 μl in the base of tail/footpad using either F2 gel alone or 200 μg of: SIINFEKL minimal peptide (OVA) in PBS, OVA peptide/endoplasmic reticulum insertion signal sequence fusion (ESOVA) in PBS, OVA in F2 gel, or ESOVA in F2 gel. Splenocytes were tested 10 days later for a secondary response using a Cr51 assay as well as a primary CTL response using the lactate dehydrogenase cytotoxicity assay. Splenocytes from immunized mice were harvested at specific time points and assayed for cell surface and intra-cellular markers. On day 10 postvaccination, animals were challenged with EG.7-OVA murine thymoma cells. Tumor size and appearance were recorded. Vaccination with F2 gel/peptide (either OVA or ESOVA) resulted in a primary T-cell response (up to 25% tumor cell-specific lysis) and no tumor growth in 69% of the mice. By 48 h, the proportion of splenic T cells had increased 4-fold compared with B cells. Presence of an increased Th1 CD4 helper population was demonstrated by IFN-γ production. CD4 cells were activated at 24 and 48 h as shown by IL-2 receptor α chain expression (from 2% basal expression to 15.4% at 48 h). Activated splenic macrophages increased from 3 to 8% within 10 h, and their level of B7–2 expression doubled. Depletion of macrophages before vaccine injection abolished any tumor-specific primary CTL response. F2 gel/peptide tumor vaccine can prime the immune system in an antigen-specific manner by generating a measurable primary T-cell response with minimal peptide; this process involves macrophage presence and activation as well as induction of Th1 CD4 cells. This is the first demonstration of a primary CTL response generated with minimal peptide vaccination using a noninfectious delivery system. These results justify additional studies to better define the mechanisms involved in F2 gel/peptide vaccination in preparation for clinical trials.

INTRODUCTION

Progress in the application of IL-23 and autologous tumor reactive T-cell therapies for metastatic melanoma have suggested that enhancement of the cell-mediated response of the human immune system may lead to cancer regression (1–4). Melanoma-specific CTLs capable of mediating antitumor responses can be derived from tumor-infiltrating lymphocytes isolated from patient tumor samples (1–7). The ability of these T lymphocytes to lyse MHC-matched autologous and allogeneic tumor cells in vitro has made them useful as reagents for the identification of TAAAs and their MHC-restricted TAA epitopes (5, 6, 8–13). Additionally, these CTLs have made it possible to clone and characterize the T-cell receptor heterodimers responsible for tumor recognition (14, 15). Epitopes from antigenic proteins such as MART-1, MAGE-1, gp100, tyrosinase, and β-catenin have been identified by their ability to sensitize peptide-pulsed target cells to lysis by tumor infiltrating lymphocyte-derived CTL lines (6, 10, 12, 13, 16). The discovery of antigens recognized by CTLs and their potential capability to mediate in vivo tumor regression makes it possible, in principle, to carry out a systematic and rational design of antigen-specific cancer immunotherapies.

An increasing number of studies are reporting attempts to

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The abbreviations used are: IL, interleukin; LDH, lactate dehydrogenase; TAA, tumor-associated antigen; APC, antigen-presenting cell; p-GlcNAc, poly-N-acetyl glucosamine; F2 gel, formulation of p-GlcNAc (70% deacetylated and dissolved at a 2% polymer concentration) used in these studies; CM, complete medium [RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml)/streptomycin (50 μg/ml), 2 mM l-glutamine, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol]; FACS, fluorescence-activated cell sorting.
use the specificity of the T-cell receptor/TAA interaction to develop effective cancer vaccines (14, 15, 17–30). Direct immunization with TAA s and TAA epitopes to generate or augment in vivo antigen-specific T-cell recognition of tumor has been attempted by several groups (17, 18, 21–23, 25–29, 31). Antitumor responses have been observed in model systems by delivering peptides via viral vectors (25), with cholera toxin adjuvant (22), on exogenously pulsed APCs (28), with an endoplasmic reticulum insertion signal sequence fusion peptide (29), and by particle bombardment (30, 31). These studies have shown that peptide-based immunotherapy is capable of achieving protective as well as therapeutic antitumor responses in animal models (19, 22, 25, 27–29, 32, 33). Depending on the manner of delivery, peptides can actually induce T-cell tolerance (34, 35). Observed variabilities in the effectiveness of different methods of peptide delivery make it clear that the mode of delivery is a critical component of vaccine design.

Our objective has been to design a TAA peptide/cytokine-based cancer vaccine that can be administered at a location distant from the potentially immunosuppressive local tumor. To this end, we have focused our efforts on the well-characterized E.G7-OVA tumor model, which expresses a defined antigenic epitope, SIINFEKL (22, 25, 29, 36–39). This model allows us to observe the effects of sustained release of the SIINFEKL minimal peptide (OVA) on the immune system, evaluate any subsequent antitumor response, and elucidate some of the mechanisms involved in this process. There have been several successful attempts at generating an immune response in the EG.7-OVA model, which have necessitated the introduction of the whole chicken ovalbumin protein, a large and allogeneic immunogen (22, 29, 36–39). To date, however, there has been only a single instance of successful vaccination with a minimal (8-amino-acid) peptide using a recombinant vaccinia virus for delivery (25). Because a viral vector can have drawbacks in applications of vaccine technology to human clinical trials, we have investigated the potential of an alternative noninfectious delivery system, a specific gel formulation of p-GlcNAc. This biocompatible, highly purified polysaccharide has been previously shown (40) to be capable of concomitant local delivery as well as sustained release of TAA peptide and cytokine in vitro; it also elicits a local transitory inflammation in response to the release of cytokine in vivo. We refer to the particular formulation used in these studies, which is 70% deacetylated and dissolved at a 2% polymer concentration, as F2 gel. We have previously shown (40) that peptide was released at biologically relevant levels from the F2 gel in vitro; the next goal was to evaluate it as an in vivo peptide vaccine delivery system. We proceeded according to the following steps: (a) in vitro analysis of the ability of the F2 gel/peptide formulation to prime antigen-specific CTLs; (b) tumor challenge experiments to define the in vivo efficacy of F2 gel/peptide vaccination; and (c) evaluation of F2 gel/peptide effects on the cell-mediated immune response following vaccination and tumor challenge. The data demonstrate that the F2 gel/peptide vaccine formulation is able to elicit a primary in vitro cytotoxic response associated with macrophage activation. This response is shown to be effective at slowing the onset and progression of tumors.

**MATERIALS AND METHODS**

**p-GlcNAc F2 Gel Formulation.** p-GlcNAc is a polysaccharide polymer produced by a microalga and isolated from controlled aseptic cultures (40). The p-GlcNAc material has a high purity and consistency in composition and properties with an average $M_r$ of 2,000,000. The polymer has been tested following United States Food and Drug Administration biocompatibility guidance, including sensitization assay, skin irritation test, systemic cytotoxicity, mutagenicity, subchronic toxicity, and pyrogenicity. A specific p-GlcNAc gel formulation, referred to as F2 gel in the present study, was designed to optimize immune system activation. The F2 gel was prepared by chemical deacetylation of p-GlcNAc to 70%, the conversion of the polymer to a lactate salt, and dissolving it under sterile conditions to a final 2% polymer concentration.

**Cell Lines.** EL4, a murine thymoma, and EG.7-OVA, a transfectant of EL4 that expresses chicken ovalbumin (35), were obtained from the American Type Culture Collection as frozen stocks. Both cell lines were cultured in CM [RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (Summit Biotechnologies, Fort Collins, CO), penicillin (50 units/ml)/streptomycin (50 μg/ml), 2 mM l-glutamine, 0.1 mM MEM nonessential amino acids (Life Technologies, Inc., Gaithersburg, MD), 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO)]. They were grown at 37°C in a humidified 5% CO2 incubator and were adjusted to 1 × 105 cells/ml every 2nd day. Because MHC Class I expression decreases rapidly in both EL4 and EG.7-OVA cell lines in vitro (data not shown), cells were cultured for only 8 days before use in any assay.

**Mice.** Female C57BL/6 (H-2b) and BALB/c (H-2d) mice 6–8 weeks old were purchased from The Jackson Laboratory (Bar Harbor, ME). All of the studies were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” as proposed by the Committee on Care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. Mice were housed in the animal care facilities of the Medical University of South Carolina Department of Laboratory Animal Resources, which are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**Reagents.** SIINFEKL minimal peptide (OVA) was purchased from the peptide synthesis facility of the Medical University of South Carolina Biotechnology Resource Laboratory. An OVA peptide/endoplasmic reticulum insertion signal sequence fusion, RYMLGLLALAAVCSAMSIN-FEKL (ESOVA; Ref. 29), was purchased from Research Genetics, Inc. (Huntsville, AL). Ovalbumin protein (grade VII) for ELISA assays was obtained from Sigma. The following antibodies were purchased from PharMingen (San Diego, CA): anti-CD3 (T lymphocytes), anti-CD4 (T helper cells), anti-CD8 (T effector cells), anti-CD13 (macrophages), and the isotypic controls anti-IgG and anti-IgG2ακ were all FITC labeled. Anti-B220 (B lymphocytes), anti-CD25 (IL-2 R α chain), anti-CD86 (B7–2), anti-IFN-γ as well as their isotypic controls anti-IgG2ακ and anti-IgG2ακ—k.
IgG2βκ were all PE labeled. Anti-CD8 (T cytotoxic cells) and its isotopic control IgG2ακ were Cy-Chrome labeled.

**Immunizations and Splenocyte Harvest.** Peptides were mixed with either PBS or F2 gel. As described previously (29), a total of 200 μg of peptide was injected in each C57BL/6 mouse (50 μg in each hind footpad and 100 μg in the base of the tail). Spleens were harvested, placed in CM, and their cells dispersed with a 3-ml syringe plunger. The cell suspension was then filtered through a 70-μm cell strainer, and erythrocytes were lysed with ammonium chloride lysis solution [prepared by mixing 9 parts 8.3 g/liter ammonium chloride:1 part 20.59 g/liter Tris (pH 7.65) immediately before use]. Splenocytes were then washed and resuspended in medium or FACS buffer.

**Secondary in Vitro Cytotoxicity Measurements.** On day 0, mice were immunized as described above. Ten days later, spleens were harvested and, after lysis of erythrocytes, a single-cell suspension of splenocytes (6.5 × 10⁶ cells/ml in CM) was placed into 24-well plates (2 ml/well) with 2 μg OVA peptide. For allogeneic controls, 7 × 10⁶ naive BALB/c splenocytes were placed in culture with 6 × 10⁶ irradiated (1500 rad) naïve C57BL/6 splenocytes in a total volume of 2 ml/well of a 24-well plate. After 7 days in culture, splenocytes were tested in an in vitro Cr⁵¹-release cytotoxicity assay as described previously (21, 29). Briefly, 3 × 10⁶ target cells were labeled with Cr⁵¹ for 60 min at 37°C. For peptide-pulsed EL4 cells, 5 μg OVA peptide was added during radioactive labeling. After washing, 1 × 10⁶ target cells were seeded in triplicate in a 96-well plate with serial dilutions of in vitro stimulated effector splenocytes. Plates were incubated for 4 h at 37°C in a humidified 5% CO₂ incubator. Supernatants were harvested using the Skatron Supernatant Collection System (Skatron Instruments, Inc., Ster- ling, VA) and counted in a gamma counter. The percent maximum lysis of target cells was determined as follows:

\[
\text{Percent lysis} = \frac{\text{Experimental cpm release} - \text{spontaneous cpm release}}{\text{Maximum cpm release} - \text{spontaneous cpm release}}
\]

**Primary in Vitro Cytotoxicity Measurements.** Ten days after immunization, splenocytes were isolated from C57BL/6 mice to evaluate a primary response to EG.7-OVA cells. Negative controls for the specificity of this response were EL4 thymoma cells. EG.7-OVA and EL4 target cells were seeded in 96-well plates at 1 × 10⁶ cells per well in RPMI 1640 (Irvine Scientific) supplemented with 10% heat inactivated fetal bovine serum (Summit Biotechnologies). Splenocytes were added to effector:target ratios of 100:1, 50:1, 25:1, and 12.5:1. Plates were spun at 250 × g for 5 min and incubated at 37°C for 4 h. LDH activity was measured using the Cytotoxicity Detection Kit (LDH) from Boehringer Mannheim (Indianapolis, IN) according to the manufacturer’s recommended protocol. After centrifugation, supernatant was removed from all of the wells. LDH substrate was added to the supernatants, and plates were incubated for another 30 min before stop solution was added. Absorbance (A) was measured at 570 nm with a 630 nm reference filter on a Dynatech MRX Plate Reader (Dynatech Laboratories, Inc., Chantilly, VA). The average values for wells performed in triplicate were used for calculations after the medium controls were subtracted. The percent maximum lysis was calculated using the following formula:

\[
\text{Percent lysis} = \frac{\text{Experimental absorbance} - \text{spontaneous target lysis}}{\text{Maximum target lysis} - \text{spontaneous target lysis}}
\]

EL4 background lysis was always less than 3% for all of the conditions tested. The mean values at an E:T ratio of 50 were averaged for three independent experiments.

**Tumor Protection in Vivo.** C57BL/6 mice were immunized as described above. Ten days later, immunized mice were challenged by s.c. injection of 3 × 10⁵ E.G7-OVA cells in 200 μl HBSS. Tumor growth was followed by measuring the length and width of tumors on odd numbered days after challenge. Tumor volumes were calculated using the formula

\[
\text{Volume} = \text{Length} \times \text{width} \times \frac{\text{length} + \text{width}}{2}
\]

Each experiment was terminated when any mouse tumor reached 2800 mm³.

**Mannan Depletion of Macrophages.** C57BL/6 mice were given i.p. injections of 300 μl of a 10 mg/ml solution of mannan (Sigma, St. Louis, MO) in HBSS at days 3 and 1 before the start of vaccination experiments. Control mice were sacrificed at days 0, 3, 5, and 7 to confirm the absence of macrophages in the spleens and peritoneal cavity. This was accomplished by FACS analysis after staining with anti-CD13 antibodies for macrophages. Cytotoxic and helper T-cell populations were determined accomplished by FACS analysis after staining with anti-CD4 and anti-CD8 antibodies to be within normal ranges in these same animals. Mice were vaccinated according to the protocols described above.

**FACS Analysis.** Mice were immunized and challenged with tumor as described above. Animals were killed starting at day 1 after vaccination and ending on day 24 after tumor challenge (a total of 34 days). Spleens and draining inguinal lymph nodes were harvested in CM and washed with HBSS. Splenocytes were isolated according to the previously described protocol. Lymph node cells were separated using the same method without RBC lysis. All of the cells were washed and resuspended in FACS buffer (1% BSA, 0.02% Na Azide in 1 × PBS). Cells were incubated at 4°C in the dark for 45 min with FITC-, PE-, or Cy-chrome- labeled antibodies, then washed and resuspended in FACS buffer. Fluorescence was analyzed on a Coulter EPICS XL flow cytometer (Coulter, Miami, FL). For all of the calculations of percentage positive or mean fluorescence, gates were set on the corresponding isotypic controls, and backgrounds were subtracted from the experimental values. The ratio of FITC:PE fluorescence was calculated to obtain T-cell:B-cell ratio. The CD4:CD8 ratio was obtained by calculating the FITC: Cy-Chrome fluorescence; three-color analysis was used in this series to determine the percent of CD4-positive cells (FITC) that were also CD25-positive (PE). The CD8-positive cells were never CD25-positive above background levels.

**Statistics.** Statistical significance was determined with a paired two-tailed Student’s t test with a 95% confidence inter-

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val. All of the statistical calculations were performed using the Statview software package (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Secondary CTL Response to Immunization with Ovalbumin-derived Peptides in a F2 Gel Formulation. In vitro cytotoxicity assays were performed with secondarily stimulated murine splenocyte effector cells to explore the ability of peptides delivered in the F2 gel formulation to induce an antigen-specific CTL response. C57BL/6 mice were immunized with 200 μg of either OVA peptide or the ESOVA fusion peptide as described in “Materials and Methods.” Ten days following immunization, splenocytes were harvested and restimulated for 7 days in vitro with OVA peptide. These effector cells were then tested in a Cr51-release assay for their ability to lyse EL4 tumor target cells, either pulsed or not pulsed with OVA peptide.

In allogeneic controls, primed BALB/c (H-2d) effector splenocytes were able to lyse both peptide-pulsed and unpulsed EL4 (H-2b) target cells at equivalent levels (Fig. 1, A and B). Approximately equivalent levels of lysis were achieved with ESOVA delivered via either PBS or F2 gel, confirming the results of Minev et al. (up to 30% and 32% of maximum lysis respectively; data not shown; 29). In contrast to the findings of previous studies that used other delivery systems (19, 22, 25, 27–29), we were able to elicit significant lysis of target cells by immunization with the minimal 8-amino-acid SIINFEKL peptide (200 μg). BalbC splenocytes were used as positive controls for an allogeneic reaction (E). Specific lysis for each condition was performed three times, and representative experiments are shown.

Antigen-specific Primary CTL Response by F2 Gel/Peptide-Primed Splenocytes. Our results from the secondary CTL assays in Fig. 1 indicate that F2 gel/peptide vaccination may elicit an antigen-specific response. However, an assay of target lysis by fresh splenocytes would provide a more accurate assessment of the antitumor potential of the OVA-primed precursor population. To test for the generation of a primary CTL response, mice were immunized as described in the “Materials and Methods” section. At 10 days postvaccination, splenocytes were harvested and immediately tested in a LDH-release cytotoxicity assay. Vaccination with the F2 gel formulation mixed either with minimal OVA peptide or with ESOVA peptide resulted in a significant specific lysis of E.G7-OVA tumor cells (Fig. 2). This magnitude of effect was different for the two peptides tested (10.5% for OVA, 27% for ESOVA) and was significantly higher than the background lysis due to F2 gel alone or to PBS with peptides (P < 0.01). The antigen-specific effector cell frequency was significant enough postvaccination for the fresh splenocytes not to require further stimulation to detect in vitro reactivity against the relevant tumor. These results constitute the first reported example of a primary cytotoxic T-cell response generated against a minimal peptide in a non-viral delivery system.

Postvaccination Protection from Tumor Challenge. To test the effectiveness of F2 gel/peptide immune priming in generating an in vivo antitumor effect, we then used a s.c. E.G7-OVA tumor protection model. Mice were immunized with peptides delivered in either F2 gel or PBS; controls received F2 gel alone or were left untreated. Mice immunized with F2 gel formulation plus OVA or ESOVA were protected from subsequent tumor growth as compared with control groups, and tumor onset was delayed (Fig. 3A). Mice that received either PBS/OVA or PBS/ESOVA failed to show any evidence of tumor protection as compared with untreated controls. By day 13, more than 80% of untreated and F2-gel-alone-treated mice had developed tumors, whereas F2 gel/OVA and F2 gel/ESOVA groups had 0 and 29% tumor-bearing mice respectively. By day
whereas 60% of PBS OVA mice were tumor bearing (Fig. 3A), only 33% of F2 gel/OVA mice had developed tumors, Fig. 2 with a proportion of T lymphocytes to B lymphocytes in the spleen of 0.06 to 1.41 (P, 0.05; Fig. 3B). The increase in T-cell:B-cell ratio progressively disappeared over the course of 10 days. However, a significant population of activated T-helper cells was detected in response to F2 gel/OVA injection in both the spleen (13.3%) and lymph nodes (15.4%), which appeared as early as 24 h (Table 1) and persisted until day 10 post immunization. The F2 gel injection alone gave significantly higher activated T-helper cells in both the spleen (7.2%) and lymph nodes (11.8%) compared to nongel controls.

To further characterize the activated T-helper cells in F2 gel/peptide-vaccinated mice, we used FACS in conjunction with intracellular staining to analyze IFN-γ production (Fig. 4). The injection of F2 gel alone resulted in a slight increase of IFN-γ production in splenic cells (1.7%). This shift was substantially higher (14.9%) in T-helper cells of animals injected with the F2 gel/OVA peptide. The increase in IFN-γ was transient, inasmuch as it diminished by 24 h. F2 gel/ESOVA had a similar effect that appeared to persist until 24 h (15.4%), but not PBS/ESOVA, suggesting the importance of F2 gel in IFN-γ expression. Th1 cells are T-helper cells (i.e., CD4-positive) that expressed IL-2, IL-12, or IFN-γ. Various studies have measured a Th1 bias by cytokine production in culture supernatants, by intracellular production of cytokines, or by expression of the mRNAs for these cytokines.

Increase in Relative Macrophage Population and Level of Activation in the Spleens of Vaccinated Mice. Because macrophages can be excellent APCs for helper T cells, the in vivo effect of F2 gel/peptide treatment on splenic macrophages was investigated. Both F2 gel alone and gel with OVA peptide led to an increase in the relative number of macrophages in the spleen as early as 10 h after immunization, and the increase persisted at 24 h. The proportion of splenic macrophages nearly tripled in response to treatment (F2 gel alone increased from 3 to 7%, whereas F2 gel/OVA increased from 3 to 9%). This increase was paralleled by macrophage activation as seen by up-regulation of surface B7-2. The B7-2 costimulatory molecule was expressed on 68% (±0.06) of splenic macrophages in response to F2 gel alone and 79% (±0.30) in response to F2 gel/OVA (compared with basal levels of 32% (±4.6)). PBS did not induce any significant change in either the proportion or the activation level of macrophages.

![Graph showing the percentage maximum lysis](https://example.com/graph.png)

**Fig. 2** Antigen-specific primary CTL response by F2 gel/peptide primed splenocytes. Reactivity of C57BL/6 splenocytes from immunized animals against EG.7-OVA tumor cells. At 10 days postimmunization, splenocytes were harvested and assayed using the LDH method for specific cytotoxicity against EG.7-OVA cells. Nonspecific lysis against parent EL4 cells was determined to be below measurable levels. Mean lysis was calculated by combining the means of the average of triplicate wells for three independent experiments. SEs were calculated with n = 3.

To determine whether the increase in T cells in the spleen was due to a predominance of CD4 or CD8 cells, the CD4:CD8 ratio in the spleens were determined at 24 and 48 h (Table 1). Although the untreated, F2 gel alone, and PBS/OVA groups exhibited a nearly constant CD4:CD8 ratio of 1, animals injected with F2 gel/OVA had a progressively higher ratio of CD4:CD8 cells (1.38 at 24 h, 1.83 at 48 h). A similar effect was observed with F2 gel/ESOVA injection (1.49 at 24 h, 1.54 at 48 h), but not with PBS/ESOVA. In absolute percentages, helper and cytotoxic T cells represented 40 and 22.1%, respectively, of splenic lymphocytes compared with basal levels of 14.9 and 14.3%. This indicated that the CD8-positive cells also increased in absolute numbers, although not as substantially as CD4-positive cells. Therefore, the injection of F2 gel/peptide increased the presence of T cells in the spleen within 2 days, and this effect was due primarily to an increase in helper T lymphocytes.

Activation of T-cells in response to treatment was measured by using FACS analysis to compare the relative expression of the inducible IL-2 receptor α chain (CD25; Table 1). No activation of CD8+ cytotoxic T-cells was observed in either the spleen or the lymph nodes over the course of 10 days. However, a significant population of activated T-helper cells was detected in response to F2 gel/OVA injection in both the spleen (13.3%) and lymph nodes (15.4%), which appeared as early as 24 h (Table 1) and persisted until day 10 post immunization. The F2 gel injection alone gave significantly higher activated T-helper cells in both the spleen (7.2%) and lymph nodes (11.8%) compared to nongel controls.

T-Cell Response to Immunization. To study the basis of the observed CTL response, FACS analysis was used to observe cell surface markers distinguishing B and T splenic lymphocytes. As early as 24 h after the injection of the OVA minimal peptide in the F2 gel formulation, an increase in the proportion of T lymphocytes to B lymphocytes in the spleen [from 0.53 (±0.06) to 1.41 (±0.09)] was observed. The absolute numbers of B cells did not vary significantly. Thus, this observation did not represent a decrease in B cells but rather an increase in the number of T lymphocytes. By 48 h, there was a 3-fold increase of T cells over B cells [0.53 (±0.06) for untreated controls, with 2.25 (±0.35) for F2 gel/OVA]. The increase in T-cell:B-cell ratio progressively disappeared over the course of 10 days, and the levels of T cells in the spleens of animals injected with F2 gel/OVA peptide were not significantly greater than controls by day 12 (data not shown).

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Effect of Macrophage Depletion on Generation of a Primary Cytotoxic Response. To confirm the importance of macrophage presence in generating a tumor-specific CTL response after F2 gel/peptide injection, we depleted the peritoneal and splenic macrophage populations. This was accomplished by injection of mannan as described in the “Materials and Methods” section at day 3 and day 1 before vaccination. Macrophage absence (as well as normal levels of B and T cells) was confirmed by FACS analysis of i.p. and splenic lymphocyte populations. At day 10 postvaccination, animals were sacrificed for a primary LDH cytotoxicity assay. The results demonstrated that macrophage depletion almost completely abolished the ability of the F2 gel/OVA peptide vaccine to induce a primary CTL response (Fig. 5).

DISCUSSION

The cloning of the genes that encode TAAs and the description of their class I MH restricted epitopes provides a new opportunity for the design of cancer vaccines (2, 6, 9, 10, 12, 14–16, 41–43). Although numerous approaches using these peptides are currently being considered, direct peptide vaccination is gaining increasing attention. Attractive aspects of such an approach include (a) the specificity of the immune response to small tumor-specific epitopes; (b) the ability to present biologically relevant levels of TAA epitopes away from the immunosuppressive influences that exist at the tumor site; (c) the lack of need for ex vivo manipulation; (d) off-the-shelf accessibility; (e) control over amounts and types of reagents to be delivered; and (f) ease of administration. Recent preclinical and clinical studies using TAA epitopes now provide in vivo evidence that a cancer therapy approach based on TAA peptides can be effective (18, 19, 43). Additionally, several cytokines including GM-CSF, IL-12, and IFN-γ have been documented to elicit an antitumor effect by enhancing the functional activity of APCs, resulting in the stimulation of antigen-specific T lymphocytes when present at the local tumor site (44–49). In the context of a TAA peptide
vaccine, these cytokine(s) could be administered concomitantly with peptide to create a local microenvironment that would provide a potentially more effective milieu for antigen presentation. Moreover, very recent developments in peptide vaccines indicate that there exist potential applications for combinations of peptides and cytokines in the clinic (43, 44, 49). Using melanoma-derived antigens injected with IFA and simultaneous IL-2 treatment, Rosenberg et al. (43) have demonstrated a 42% response rate in patients with advanced disease, closely correlating with the cytotoxic response to the TAA. These studies have, therefore, provided a significant impetus for further development and design of TAA-derived peptides and cytokine-based cancer vaccines (19, 45, 50, 51).

The choice of vehicle used for peptide vaccine administration toward achieving an effective tumor-specific CTL response is as important as the components being delivered to the immune system. Optimal CTL activation may be dependent on the duration, amount, and availability of peptide for uptake by locally recruited APCs. Predictable and sustained levels of biologically available peptide in an enhanced antigen presentation environment are desirable to elicit an effective immune response (19, 24, 50, 51). Our approach has been to develop a vaccine with the F2 gel formulation described herein, which is able to provide sustained delivery of TAA-derived peptides and can be combined with cytokine(s) capable of enhancing in vivo APC uptake and presentation (40). This report represents the first demonstration of a primary in vitro response generated with minimal peptide vaccination using a noninfectious delivery system.

Although the F2 gel was initially conceptualized only as a
Peptide/p-GlCNAC Vaccination

...vehicle for peptide delivery, it is apparent from the enhanced postvaccination primary CTL response that the F2 gel vehicle may act in synergy with the TAA peptide to elicit some form of immune response (Fig. 2). The binding of polysaccharide chains to an evolutionarily highly conserved receptor on the macrophage surface (e.g., the mannose or scavenger receptors) can cause activation and B7-2 expression (52, 53). In the case of F2 gel/peptide vaccination, postinjection edema and potential capillary damage should lead to rapid recruitment of macrophages to vaccination site, providing an opportunity for contact with the F2 gel/peptide formulation, leading to contact with the mannose or scavenger receptors and subsequent macrophage activation. If this hypothesis is true, then these macrophages may represent a portion of the activated cells later found in large numbers in the spleens of the post-F2 gel/peptide vaccinated animals.

The presence of F2 gel/peptide-activated macrophages could contribute to T-helper cell activation and proliferation resulting in the increase of T cells in the spleens and the change in the B-cell:T-cell ratio. It is unlikely, however, that the initial T-helper response observed within 48 h was peptide-specific. In a naïve animal, there is only a very small population of T cells with receptors that could recognize the SIINFEKL epitope. In general, the activation of helper T cells could favor either the cell-mediated or the humoral branch of the immune system. The shift toward one or the other depends to a great extent on the cytokine microenvironment and the phenotype of the helper T lymphocytes (52, 53). Our results provide evidence that vaccination with the F2 gel formulation induces early IFN-γ production by T-helper cells and, therefore, Th1 cells (Fig. 4), which may be an important factor in the observed tumor challenge response (Fig. 3). Th1 effector cells could directly activate macrophages through cellular interaction or lead to an activation of tumor-specific CD8 cells by providing the appropriate cytokine environment. In this enhanced antigen presentation environment, the continued presence of OVA peptide being released from the F2 gel/peptide formulation could then account for the augmented expansion of a small, OVA peptide-specific, population of T cells leading to the observed primary CTL response by day 10. Both of these potential mechanisms could explain the delay or absence of solid tumor appearance and decreased tumor growth. Although the exact role that these activated macrophages play in generating an immune response with the F2 gel/peptide has yet to be defined, the fact that macrophage depletion before vaccination completely abrogates a primary tumor-specific CTL response confirms their importance.

Recent studies (54–57) on peptide-based cancer vaccines have focused on the role that APCs, such as dendritic cells, play in eliciting a successful antitumor response. Additionally, investigation of MUC-1 and breast cancer has shown the effectiveness of delivering TAAs directly to macrophages through the binding of a delivery matrix to mannose receptor. This was accomplished using a fusion protein with mannan, which resulted in an increased MUC-1-specific CTL population (57). One potential advantage of using the F2 gel in this setting is that it has the capacity to deliver biologically active peptides to APCs in a sustained manner, while eliminating the need for chemical fusion products. If our hypothesis concerning the priming of Th1 effector cells and increased macrophage activation is correct, this delivery system could further augment tumor rejection. To test some of these hypotheses, we plan to conduct additional studies on the depletion of specific lymphocyte populations such as macrophages and helper T cells at various time points.

In conclusion, F2 gel/TAA-based cancer vaccines offer promising ways of using the cell-mediated branch of the immune system to develop effective antitumor responses. A significant and tumor-specific CTL response is generated and decreases or completely prevents tumor growth in vivo. It is clear that F2 gel/peptide formulations can prime the immune system in an antigen-specific manner that involves macrophage presence and activation and possibly a T-cell shift toward the Th1 pathway. Because cancer may prevent an effective response against itself by suppressing the cell-mediated pathway, the mechanism of F2 gel/peptide action may entail a reversal or prevention of this process. Future studies will focus on clarifying mechanisms of vaccine action as well as optimizing the system with Th1 cytokines such as IL-2 or IL-12 and different polysaccharide formulations. These results justify additional studies defining vaccination mechanisms in preparation for pilot clinical trials.

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