Characterization of a Sustained-Release Delivery System for Combined Cytokine/Peptide Vaccination Using a Poly-N-Acetyl Glucosamine-based Polymer Matrix


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ABSTRACT

Identification of tumor-associated antigens (TAAs) and their class I MHC-restricted epitopes now allows for the rational design of peptide-based cancer vaccines. A biocompatible system capable of sustained release of biologically relevant levels of cytokine and TAA peptide could provide a more effective microenvironment for antigen presentation. Our goal was to test a sustained-release cytokine/TAA peptide-based formulation using a highly purified polysaccharide [poly-N-acetyl glucosamine (p-GlcNAc)] polymer. Granulocyte-macrophage colony-stimulating factor (GM-CSF; 100 μg) and MART-1_27-35 peptide (128 μg in DMSO) were formulated into p-GlcNAc. Peptide release was assayed in vitro using interleukin 2 production from previously characterized MART-1_27-35-specific Jurkat T cells (JRT22). GM-CSF release was assayed via ELISA and proliferation of M-07e (GM-CSF-dependent) cells. Local bioavailability of MART-1_27-35 peptide for uptake and presentation by antigen-presenting cells was demonstrated for up to 6 days (>0.5 μg/ml). More than 1.0 μg/ml GM-CSF was concomitantly released over the same period. Biocompatibility and local tissue response to p-GlcNAc releasing murine GM-CSF was determined in C57BL/6 mice via s.c. injection using murine GM-CSF (0.2 μg/ml) in 200 μl of a 2.5% polymer gel. Significant lymphocytic and eosinophilic infiltration was observed 2–7 days after injection with polymer containing murine GM-CSF. The results of our studies show that this biocompatible system is capable of a sustained concomitant release of biologically active peptide and cytokine into the local microenvironment. These findings support further studies to validate a p-GlcNAc delivery system vehicle for a cytokine/TAA peptide-based cancer vaccine.

INTRODUCTION

CTLs that specifically recognize and respond to both autologous and allogenic tumor cells in a MHC-restricted manner can be isolated in vitro (1–3). These T cells are capable of mediating antitumor responses in select patients (4–6) and provide evidence that manipulation of the human immune system can result in the regression of cancer. Recent progress in our knowledge of antigen processing/presentation and techniques for the isolation of peptides presented in a MHC-restricted fashion has led to the identification of TAAs recognized by T lymphocytes (7–13). Several of the genes encoding for TAAs have been cloned, their class I MHC-restricted epitopes described, and in some cases the functional specificity of T-cell receptor heterodimer recognition characterized (14–17). These findings support the concept that CTL-mediated tumor regression in vivo can be explained by T-cell recognition of specific 9- or 10-amino acid peptides bound to MHC class I molecules presented on the surface of cancer cells and have been a major step forward in cancer vaccine development (18).

Recent work, performed in murine models using TAA epitopes, provides in vivo evidence that a cancer therapy approach based on relevant TAA peptides may be effective and justify development and design of peptide-based cancer vaccines (19–21). An attractive aspect of this approach is the ability to present biologically relevant levels of TAA epitopes away from the potentially immunosuppressive influences that may exist at the tumor site. Additionally, several cytokines including GM-CSF, IL-12, and IFN-γ have been documented to elicit an antitumor effect when present at the local tumor site (22–26). In the context of TAA peptide vaccine, these cytokines could be administered concomitantly with peptide to create a local microenvironment which would provide a potentially more effective milieu for antigen presentation.

Our objective, therefore, has been to develop a vaccine which uses TAA-derived peptides in combination with cyto-
kines which are potentially capable of enhancing in vivo APC uptake and presentation so as to achieve a more effective tumor-specific CTL response. Optimal CTL activation may be dependent on mode of presentation, antigen localization and concentration, and overcoming anergizing mechanisms by the tumor (19, 21, 27). As such, both the duration and availability of peptide for uptake by locally recruited APCs are significant variables in this approach. Sustained levels of biologically available peptide in an enhanced antigen presentation environment are potentially desirable for an augmentation in immune response (19, 28, 29). The vehicle, therefore, used for peptide vaccine administration may be of critical importance.

Several possible modalities for peptide vaccination have been described, including Freund's adjuvant (19), encapsulation of peptide in liposomes (30), or modification as lipopeptides (31). A newer approach, however, is now possible with the recent availability of a highly purified, biocompatible polysaccharide matrix: p-GlcNAc. The goal of this study was to characterize the p-GlcNAc-based matrix as a sustained-release vehicle for a cytokine/TAA peptide-based vaccine.

**MATERIALS AND METHODS**

**p-GlcNAc Polymer.** p-GlcNAc was provided as either a sterile fully deacetylated lyophilized disc able to fit in the bottom of a standard 24-well plate or as a hydrated 2.5% sterile gel for injection. p-GlcNAc is a highly purified polysaccharide polymer produced by a fermentation process and isolated from controlled aseptic cultures grown on a defined culture medium under conditions that are consistent with Good Laboratory Practice (GLP) guidelines (Marine Polymer Technologies, Danvers, MA). It has passed the Food and Drug Administration biocompatibility testing including USP class VI sensitization assay, irritation test, systemic toxicity, cytotoxicity, mutagenicity, subchronic toxicity, and pyrogenicity. For in vitro testing, rhGM-CSF was reconstituted in HBSS at a concentration of 200 ng/ml. Sterile p-GlcNAc polymer was provided in a fully deacetylated form (to minimize hydrophobicity) with 0.5 ml of reconstituted GM-CSF dissolved (for a total of 100 μg of GM-CSF) into 1.5 ml of the p-GlcNAc polymer prior to lyophilization. MART-1,27-35 peptide (0.1 ml of peptide/DMSO solution at 1280 μg/ml for a total of 128 μg of MART-1 peptide) was solubilized into postlyophilization porous matrix. In vivo studies used mGM-CSF (Genzyme Diagnostics, Cambridge, MA; 200 μl delivered per animal) dissolved into 0.5 ml of a 2.5% hydrated p-GlcNAc gel for injection (with a final concentration of 0.2 μg/ml GM-CSF).

**Cell Lines.** Jurkat 22 (JRT22) cells were maintained in culture with RPMI 1640 and 10% FBS at 37°C. These cells have been previously characterized for class I MHC-restricted recognition of MART-1,27-35 epitope (17). T2 cells (17) were maintained in RPMI 1640 with 10% FCS. M-07e GM-CSF-dependent cells (32) were maintained in RPMI 1640 and 10% FCS with 100 units/ml GM-CSF.

**Peptide Synthesis.** Peptides (kindly provided by Dr. Y. Kawakami, NIH, Bethesda, MD) were synthesized with a solid-phase method using a multiple peptide synthesizer (Gilion Co., Inc., Worthington, OH) and purified by HPLC on a C8 column (VYDAC, Hesperia, CA) with 0.05% TFA/water-acetonitrile.

The MART-1,27-35, peptide (AAGIGILTV) is located in a hydrophobic putative transmembrane domain in MART-1 (14).

**Detection of MART-1 Peptide by HPLC.** HPLC-mass spectrometry data were obtained using the Medical University of South Carolina Mass Spectrometry Research Resource Facility (courtesy of Drs. Kuruppu Dharmasiir and Daniel R. Knapp). Briefly, supernatants from one set of 24-h peptide release assay samples stored at −4°C were thawed at room temperature and analyzed by HPLC-electrospray mass spectrometry using a Finnigan LCQ instrument. RPMI 1640 with 1.0 μg/ml MART-1 peptide and RPMI 1640 alone were used as positive and negative controls, respectively. HPLC was performed using a 2 mm × 10-cm C18 column with a gradient of acetonitrile/0.05% TFA in 0.05% aqueous TFA at a flow rate of 200 μl/min.

**Peptide Release Assay.** Peptide or peptide/mGM-CSF containing lyophilized p-GlcNAc matrices were incubated in 1.0 ml of RPMI 1640 in 24-well plates at 37°C, with the p-GlcNAc supernatant changed to fresh media every 24 h. Twenty-four-hour samples were stored at −4°C until further testing. T2 cells were then preincubated for 2 h with either peptide (1 μg/ml in RPMI 1640) or p-GlcNAc supernatant. After incubation, the cells were washed twice with PBS and then added to effector JRT22 cells at a 1:1 ratio for a total of 1 × 10⁶ cells/ml in a 24-well plate. The ability of the peptide-pulsed T2 cells to stimulate IL-2 release from JRT22 cells was then assessed using the ELISA (Endogen, Inc., Cambridge, MA). Internal controls were performed for each assay using ELISA standards for IL-2 detection, and positive controls containing RPMI 1640 with 10% FCS and either 1 μg, 10 ng, 100 pg, or 1 pg of MART-1 peptide/ml. A standard curve was then generated correlating units of IL-2 released by the JRT22 cells to the amount of MART-1 peptide present. The correlation of IL-2 produced in response to MART-1 peptide was approximately 1000–1200 pg/ml of IL-2 per 1 μg/ml MART-1 peptide present in the supernatant. This assay was modified to assess local bioavailability of peptide release from the matrix by either placing T2 cells directly onto the matrix in 1 ml of RPMI 1640 with 10% FCS in a 24-well plate for 12 h or coincubated T2 cells with the supernatant-containing peptide released from the matrix into culture supernatant at 37°C. The cells were then harvested, washed twice, and then added to the effector cells as described previously.

**GM-CSF Release Assays.** GM-CSF or peptide/GM-CSF containing lyophilized p-GlcNAc matrices were incubated in 1.0 ml of RPMI 1640 in 24-well plates at 37°C, with the p-GlcNAc supernatant changed to fresh media every 24 h. Twenty-four-hour samples were stored at −30°C until further testing. The presence of rhGM-CSF was then assessed by proliferation of M-07e cells in response to GM-CSF and was verified using the ELISA (Endogen, Inc.). Briefly, for the proliferation assay, 7.5 × 10⁴ M-07e cells [which are derived from a human megakaryoblastic leukemia cell line and are dependent on either human GM-CSF or human IL-3 for growth and survival (32)] were seeded per well in a 24-well plate and grown for 3 days in 1.5 ml of common medium containing either 0, 1, 10, or 50 units/ml of human recombinant GM-CSF (Genetics Institute, Cambridge, MA) or the pGlcNAc test supernatants. At day 3, M-07e cells were counted and, by comparing these cell numbers with those of the standard curve, equivalent units of GM-CSF produced in 24 h were derived. Comparable data were obtained.
**Fig. 1** One-week time-course release tested in 24-h aliquots of rhGM-CSF (A) and MART-1_{27-35} peptide (B) from separate lyophilized p-GlcNAc polymer matrices into 1 ml of culture supernatant at 37°C. rhGM-CSF with 0.5 ml of reconstituted GM-CSF dissolved (0.5 ml of 200 μg/ml GM-CSF in PBS for a total of 100 μg of GM-CSF) in 1.5 ml of p-GlcNAc polymer was evaluated for in vitro release. Both a cellular proliferation assay with M-07e GM-CSF-dependent cells and the ELISA were used for quantitation. GM-CSF data presented are from a representative ELISA. MART-1_{27-35}, (0.1 ml of peptide/DMSO solution at 1280 μg/ml for a total of 128 μg of MART-1 peptide) p-GlcNAc polymer was evaluated for IL-2 production by JRT22 cells in response to class I MHC-restricted peptide presentation by T2 cells.

Using both methodologies, with values for GM-CSF release assayed with the ELISA with common standard curves used in our results and analysis.

**Biocompatibility and Local Tissue Response.** C57BL/6 (The Jackson Laboratory, Bar Harbor, Maine) mice were given s.c. injections of 0.2 ml of either saline, mGM-CSF (0.2 μg/ml) in HBSS, 2.5% p-GlcNAc polymer gel, or 2.5% p-GlcNAc polymer gel containing mGM-CSF (0.2 μg/ml; Genzyme Diagnostics). The local injection site and gel were harvested at days 2 and 7 after injection and processed with standard H&E paraffin blocks and stains. The histopathological sections were evaluated in a blinded fashion by one pathologist and graded for evidence of the physical presence of the 9-mer MART-I peptide and cellular infiltrates into the s.c. tissues, matrix interface and matrix per se, and vascularity. Two mice per condition were used, with the experiment repeated once.

**RESULTS**

**Detection of MART-1 Peptide by HPLC.** To provide evidence of the physical presence of the 9-mer MART-1 peptide prior to biological functional assays, we evaluated one set of 24-h supernatants used in the subsequent assays for the presence of peptide by HPLC-electrospray mass spectrometry. Control MART peptide gave a HPLC peak with the correct molecular mass (MH+ = m/e 814.5). A peak at the same retention time with the same molecular mass was observed in the p-GlcNAc (with MART-1 peptide) supernatant samples. No such peak was present in a control sample of RPMI 1640 alone (data not shown).

**Release of 9-mer Peptide and Cytokine from p-GlcNAc Matrices.** Although the pGlcNAc polymer had undergone extensive Food and Drug Administration biocompatibility testing, for it to be used as a vaccine delivery vehicle we needed (a) to define the methods required to allow incorporation and release of peptide and cytokine from the polymer matrix and (b) to determine the time course of release of these biological agents into the local environment. To this end, several formulations of polymer were initially tested with either peptide or cytokine alone. rhGM-CSF was reconstituted in HBSS at a concentration of 200 μg/ml. Sterile p-GlcNAc polymer was provided in a fully deacetylated form (to minimize hydrophobicity) with 0.5 ml of reconstituted GM-CSF dissolved (for a total of 100 μg of GM-CSF) into 1.5 ml of the p-GlcNAc polymer prior to lyophilization. The final lyophilized cytokine containing polymer was in the form of a disc which would fit into the bottom of a 24-well plate. MART-1_{27-35} peptide has significant hydrophobic amino acid residues and therefore required DMSO (Sigma Chemical, St. Louis, MO) for solubilization (0.1 ml of peptide/DMSO solution at 1280 μg/ml for a total of 128 μg of MART-1 peptide) into the p-GlcNAc polymer which had already undergone lyophilization. Release of rhGM-CSF and MART-1_{27-35} peptide from the lyophilized p-GlcNAc polymer matrix into 1.0 ml of culture supernatant at 37°C was then evaluated in vitro. rhGM-CSF release was assayed as described using a cellular proliferation assay with M-07e GM-CSF-dependent cells and ELISA. MART-1_{27-35} release was evaluated using IL-2 production in response to class I MHC-restricted peptide presentation by T2 cells as described previously. Biologically active MART-1_{27-35} peptide recognized by JRT22 was released from the lyophilized polymer matrix for up to 5 days (>0.5 μg/ml) into the 1.0-ml supernatant when tested at 24-h intervals with a total peptide recovery of approximately 26.8 μg (Fig. 1). Similarly, more than 1.0 μg/ml GM-CSF recognized by both bioassay and ELISA testing was released for up to 7 days, with an approximate total recovery of 54.4 μg.

**Release of 9-mer MART-1 Peptide and rhGM-CSF Cytokine from a Combined Formulation p-GlcNAc Matrix.** Concomitant formulation and release of rhGM-CSF and MART-1_{27-35} peptide from a single polymer matrix was then tested. Using the same formulation methodology, 0.5 ml of rhGM-CSF (reconstituted in HBSS at a concentration of 200 μg/ml) was dissolved into the p-GlcNAc polymer prior to lyophilization, with 0.1 ml of peptide/DMSO solution (1280 μg/ml in DMSO) dissolved into the lyophilized disc containing...
rhGM-CSF. Using the same assay methodology (Fig. 2), biologically active MART-1\textsubscript{127-35}, peptide recognized by JRT22 was released for up to 3 days (>10 ng/ml), whereas more than 0.1 µg/ml GM-CSF was released for over 6 days from the same matrix. It is notable that although the same amount of MART-1\textsubscript{127-35} peptide was formulated into the combined matrix as was used with the peptide-alone matrix, a level of magnitude less peptide release was detectable by bioassay from the combined matrix. In addition, the peptide was detectable for 3 rather than 5 days. Release of rhGM-CSF appeared to be of the same relative magnitude for both solitary and combined matrices (with a more rapid drop off after day 4 in the combined matrix).

Local Bioavailability of Peptide at the p-GlcNAc Matrix Interface for Uptake and Presentation by APCs. The availability in the local microenvironment of peptide at the p-GlcNAc matrix/tissue interface for uptake and presentation by APCs was considered to be potentially a more biologically relevant parameter to consider than merely the presence of peptide in an *in vitro* supernatant. Given this and the reduced release rate of peptide from the matrix in the initial cytokine/peptide matrix supernatant assay, we assessed the local bioavailability of peptide for APC uptake from the combined rhGM-CSF/MART-1\textsubscript{127-35} matrix using APCs (T2 cells) pulsed directly onto the matrix. T2 cells were either pulsed directly onto the matrix for 12 h at 37°C or coincubated with the supernatant containing peptide released from the matrix into culture supernatant at 37°C prior to separate coincubation with JRT22 cells. Up to a 5-fold augmentation (Fig. 3) in peptide available for presentation by T2 cells at the local matrix interface was observed, with a prolongation in duration from 2 to >6 days.

Histopathological Evaluation of Local Tissue Response to p-GlcNAc Releasing mGM-CSF. Biocompatibility and local tissue response to p-GlcNAc releasing mGM-CSF was next determined using mGM-CSF (0.2 µg/ml final concentration) dissolved into a 2.5% hydrated p-GlcNAc polymer gel. C57BL/6 mice were given s.c. injections of 0.2 ml of either normal saline, mGM-CSF in normal saline, polymer gel containing saline alone, or polymer gel containing GM-CSF. The local injection site was harvested and evaluated in a blinded fashion using standard H&E stain histopathologically at days 2 and 7 after injection. The tissue sections were evaluated for lymphocytic infiltrate, eosinophilic infiltrate, and vascularity.

Fig. 2 One-week time-course concomitant release tested in 24-h aliquots of rhGM-CSF (A) and MART-1\textsubscript{127-35} peptide (B) from a combined formulation p-GlcNAc matrix into 1 ml of culture supernatant at 37°C. Both a cellular proliferation assay with M-07e GM-CSF-dependent cells and the ELISA were used for GM-CSF quantitation. GM-CSF data presented are from a representative ELISA. MART-1\textsubscript{127-35} (0.1 ml of peptide/DMSO solution at 1280 µg/ml for a total of 128 µg of MART-1 peptide) p-GlcNAc polymer was evaluated for IL-2 production by JRT22 cells in response to class I MHC-restricted peptide presentation by T2 cells.

Fig. 3 Local bioavailability of peptide for APC uptake from the combined rhGM-CSF/MART-1\textsubscript{127-35} matrix using APCs (T2 cells) pulsed directly onto the matrix. T2 cells were either pulsed directly onto the matrix for 12 h at 37°C (T2 positive; ■) or coincubated with the supernatant containing peptide released from the matrix into culture supernatant at 37°C (T2 negative; □) prior to assaying for IL-2 production via separate coincubation with JRT22 cells.

No response was observed for the normal saline injection or normal saline containing mGM-CSF. A minimal inflammatory response to the matrix was noted with saline alone by day 7, with the most marked parameter increase being eosinophilic infiltrate (>10/hpf). In contrast, marked inflammatory changes were noted by day 2 in response to the matrix with GM-CSF, with significant lymphocytic and eosinophilic infiltration observed within the s.c. tissues and matrix interface and significant edema observed in the s.c. tissues at the time of harvest. This inflammatory response was persistent but diminished by day 7 (Fig. 4).

DISCUSSION

The present study characterizes a recently available highly purified polysaccharide polymer, p-GlcNAc (33), as a vehicle for the delivery of cytokine and/or peptide to a local tissue site. This polymer is biocompatible, relatively simple to formulate into hydrated gel or lyophilized disc, and capable of delivering sustained levels of biologically active peptide and/or cytokine into the local microenvironment. We are interested in using TAA-derived peptides in combination...
with cytokines potentially capable of enhancing APC uptake and presentation as a mode of antitumor therapy. These findings support further studies to validate a p-GlcNAc delivery system vehicle for a cytokine/TAA peptide-based cancer vaccine.

The cloning of the genes that encode TAAs and the description of their class I MHC-restricted epitopes now provides new opportunities for the design of cancer vaccines (7–18). Numerous approaches of interest using these peptides are currently being considered, including the use of recombinant viruses to infect normal cells and express recombinant TAA, direct immunization into muscle of DNA encoding for antigens (34, 35), or pulsing TAA peptides onto APCs directly (36). An additional approach also now possible is a direct peptide vaccination. Potential advantages of a peptide-based vaccine include the potential lack of need for ex vivo manipulation, off the shelf accessibility, control over amounts and types of reagents delivered, and ease of administration. Recent evidence in vivo that cancer therapy using putative TAA peptides per se may be an effective approach has provided further impetus for this concept (19–21). Several cytokines, including GM-CSF, IL-12, and IFN-γ, have been documented to elicit an antitumor effect and possibly enhance the functional activity of APCs, resulting in the stimulation of antigen-specific T lymphocytes when present at the local tumor site (22–26). In the context of a TAA peptide vaccine, these cytokines could be administered concomitantly with peptide to create a local cytokine environment that would provide a potentially more effective milieu for antigen presentation. Our approach, therefore, has been to develop a vaccine which uses TAA-derived peptides in combination with cytokines which are potentially capable of enhancing in vivo APC uptake and presentation so as to achieve a more effective tumor-specific CTL response. Optimal CTL activation may be dependent on the duration, amount, and availability of peptide for uptake by locally recruited APCs with predictable and sustained levels of biologically available peptide in an enhanced antigen presentation environment potentially desirable for an augmentation in immune response (19, 28, 29). In this setting,
the reliable delivery of peptide for uptake by locally recruited APCs are significant variables requiring a dependable vehicle for delivery.

We have successfully demonstrated the ability to formulate a 9-mer TAA peptide and GM-CSF cytokine into the p-GlcNAc polymer in a manner which allows the subsequent release of both biologically active peptide and cytokine into the local environment for 3 to 7 days (Fig. 2). Consideration of this system would suggest three possible modalities in vivo by which TAA peptide could be made available to locally recruited APCs: (a) release of the peptide from the polymer into the local milieu; (b) peptide bound at the polymer surface but available for uptake by APCs present at the polymer interface; and (c) degradation/phagocytosis of the polymer containing peptide with possible macrophage presentation of the TAA (37). The latter is made even more attractive by the fact that in vivo degradation of p-GlcNAc-containing materials is via the combined effects of lysozyme and N-acetylglucosaminidase. Both of these enzymes are present in human tissue macrophages during phagocytosis, with their action leading to the generation of sugar monomers (38-40). The initial in vitro assay looking at the release of peptide/cytokine into supernatant at best can only address the first mechanism, and even then is clearly artificial. The second mechanism was initially addressed with the further evaluation of the local bioavailability of peptide for uptake and presentation by APCs in direct contact with a combined rhGM-CSF/MART-1,27,35, p-GlcNAc matrix. Using this assay modification demonstrated (Fig. 3) a notable augmentation in the amount and duration of peptide present at the matrix interface available for presentation by APCs. The final mechanism remains to be determined in an in vitro model.

Other properties of interest with respect to the polymer were the biocompatibility and ease of formulation/administration as a putative vaccine. To this end, biocompatibility and local tissue response to p-GlcNAc releasing mGM-CSF was determined in C57BL/6 mice via a simple s.c. injection of 2.5% gel polymer containing mGM-CSF (0.2 μg/ml). Significant lymphocytic and eosinophilic infiltration was observed on days 2-7 with polymer containing mGM-CSF as compared to polymer alone (Fig. 4), of a duration consistent with the in vitro release profile of the lyophilized p-GlcNAc. In comparison, s.c. injection of mGM-CSF in HBSS (0.2 μg/ml) elicited no significant response (data not shown). These findings would suggest a localized inflammatory response secondary to the persistent presence of mGM-CSF. Also worthy of note is the ease of administration via hypodermic needle as a 2.5% hydrated gel and the relatively minimal inflammatory response in vivo to the polymer.

This work supports the consideration of additional studies to validate a p-GlcNAc delivery system vehicle for a cytokine/ TAA peptide-based cancer vaccine. Additional preclinical studies will be required to determine whether this conceptual approach is capable of achieving a more effective tumor-specific CTL response as the result of immunization with relevant TAA/ cytokine. Numerous variables specific to the vaccine, including optimal peptide dose level, cytokine used, and site of administration, have yet to be addressed. Furthermore, a direct comparison between this novel polysaccharide and other potential vehicles, including liposomes (30) and/or lipopeptides (31), would be useful to determine which one provides a distinct advantage in terms of formulation, administration, or response.

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