Abstract 2343

Therapeutic Lymphoma Idiotype Vaccine Generated in Insect Cells Results in Mannose Receptor Targeting and Enhanced Immune Activation

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ABSTRACT

Treatment of lymphoma patients with tumor-specific immunoglobulin (idiotype, Id) coupled to the immunogenic carrier protein keyhole limpet hemocyanin (Id-KLH) has shown promising results in clinical trials. However, vaccines fail to elicit anti-Id immune responses in some patients, thus prompting the search for ways to improve the immunogenicity of Id-KLH vaccines. Current Id vaccine trials utilize tumor-specific Id proteins secreted by tumor-myeloma hybridomas, or recombinant Id proteins produced in mammalian lymphoid cells, bacteria, or insect cells. We now provide evidence that terminal mannose carbohydrate structures, characteristic of recombinant proteins produced in insect cells, lead to Id proteins with significantly enhanced immunostimulatory properties compared to Id proteins derived from mammalian sources. Monocyte-derived human dendritic cells (DCs) were incubated with fluorescently labeled Id proteins produced in insect or mammalian cell cultures. Insect cell-derived Id demonstrated substantially higher binding to DCs compared to the Id from a mammalian source by flow cytometry, and only the insect cell Id showed reduced binding when the DCs were preincubated with mannose receptor inhibitors. These results demonstrated that the insect cell-derived Id resulted in better targeting to DCs compared to the mammalian Id. When insect cell-derived Id proteins were coupled to KLH using glutaraldehyde and co-cultured with immature human DCs, increased expression of CD80 and CCR7 was observed by flow cytometry, indicating DC maturation. Upregulation of these markers was blocked by pre-treatment with anti-mannose receptor antibody. In tumor therapy studies, mice with 4-day established A20 murine B cell lymphoma were treated with 3 weekly injections of Id-KLH plus GM-CSF and followed for survival. A20 bearing mice treated with Id-KLH containing insect cell-derived A20 Id displayed improved survival compared with mice treated with hybridoma-derived A20 Id-KLH (61% vs. 46%, respectively). Anti-Id antibodies against A20 murine B cell lymphoma were assessed by ELISA following Id-KLH immunization. Both insect and mammalian sources of Id generated similar levels of anti-Id antibodies, showing no impairment in antibody responses due to the differences in glycosylation. Anti-A20 cytotoxic T lymphocyte (CTL) activity was measured in splenic T cells from Id-KLH-immunized mice. Here, induction of CD8$^+$ CTLs by insect cell-derived A20 Id-KLH was significantly greater than CTL induction following immunization with hybridoma-derived A20 Id-KLH (P=0.0061). To determine the importance of the T cell response in A20 tumor killing *in vivo*, mice were depleted of CD4$^+$ and CD8$^+$ T cell subsets, challenged with A20 tumor, and then vaccinated 4 days later as in the experiment above. Mice depleted of CD8$^+$ T cells all succumbed to tumor demonstrating a critical role for CD8$^+$ T cells in A20 tumor eradication. Our data comparing sources of recombinant Id protein tumor antigens used in therapeutic cancer vaccines suggest that post-translational modifications, namely terminal mannose residues, can significantly influence the immunological properties and eventual therapeutic efficacy of the product.

INTRODUCTION

- Tumor-specific immunoglobulin (idiotype, Id) can serve as a target for the active immunotherapy of B cell lymphomas.
- Vaccination with tumor-derived Id can elicit tumor-specific anti-Id antibodies, CD4$^+$ and CD8$^+$ T cells with anti-tumor effects.
- Id is conjugated to the immunogenic foreign carrier protein keyhole limpet hemocyanin (KLH) via glutaraldehyde to boost immunogenicity.
- Vaccination of follicular lymphoma patients with Id-KLH has shown promise in phase I/II clinical trial, and is now being studied in several phase III trials using Id proteins derived from tumor-myeloma hybrids or recombinant sources produced in either mammalian or insect cells.
- Mitumprotimut-T (FavId®, Specifid™, by Favrille, Inc.) is a novel therapeutic Id vaccine product produced via recombinant baculovirus (BV) infection of insect cells.
- Recombinant proteins produced in insect cells are reported to have altered glycosylation patterns (terminal mannose residues) that may contribute to immunogenicity, but direct comparisons of insect-derived and mammalian-derived tumor antigens are lacking.
- Therefore, we sought to systematically compare the immunologic properties of insect cell-derived vs. mammalian cell-derived Id proteins in a murine B cell lymphoma model and in cultures of human antigen-presenting dendritic cells.

Glycosylation Patterns of A20 Id Made in Insect Cells Vs. Mammalian Cells

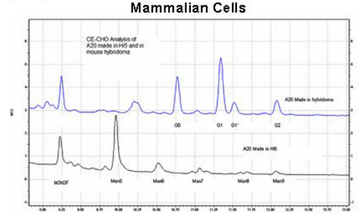

Figure 1: Insect and hybridoma derived A20 Id proteins were analyzed by the cIEF and CE carbohydrate methods. Only insect cell-derived Id has a high content of terminal mannose residues.

Insect Derived Idiotype Proteins Bind to Mannose Receptors on Human Dendritic Cells

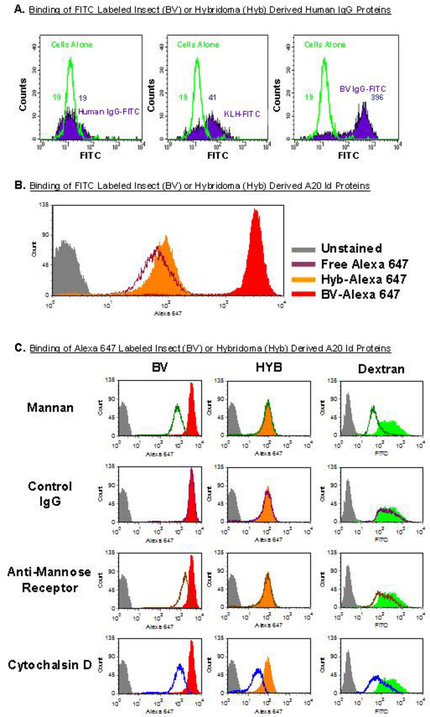

Figure 2: A. DC binding of FITC labeled KLH, hybridoma produced (Hyb) human IgG, and insect derived (BV) human IgG showed some binding of KLH, but no binding of human IgG (Hyb). Human IgG (BV) did show a strong signal for binding to human DCs. **B.** 50 µg of Alexa 647 labeled murine A20 Id produced in BV or Hyb were used to stain monocyte derived human DCs along with free Alexa. Insect derived Id shows substantially higher surface binding compared to hybridoma derived Id or unconjugated Alexa 647. **C.** 50 µg of BV derived A20 Id, hybridoma derived A20 Id, or FITC-Dextran were used to stain human DCs in the presence of various inhibitors. Cells were pretreated for 20 minutes with 3 mg/ml of mannan, 50 µg/ml of control IgG blocking antibody, 50 µg/ml of anti-mannose receptor blocking antibody, or 5 µg/ml of cytochalasin D. The blocking agents with the exception of cytochalasin D did not cause decreased binding of the hybridoma derived Id. In contrast, the BV derived A20 Id protein and the FITC-Dextran both showed decreases in binding in the presence of all the various inhibitors.

Insect Derived Idiotype Enhances Upregulation of Human Dendritic Cell Activation Markers

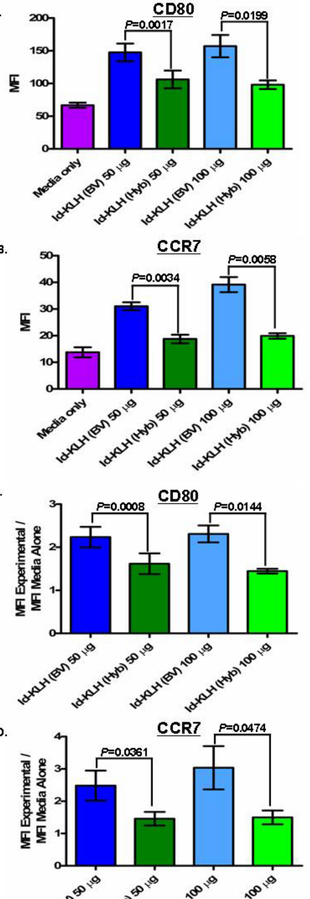

Figure 3: Insect derived Id proteins stimulate expression of DC activation markers over that seen with hybridoma-derived (Hyb) Id. The MFIs for surface markers (**A.**) CD80 and (**B.**) CCR7 were both significantly upregulated with the BV-derived Id conjugates compared to Hyb-derived conjugates. Unconjugated KLH alone showed surface marker levels similar to media alone controls (data not shown). (**C.**) CD80 and (**D.**) CCR7 surface expression levels are represented as MFI of experimental sample / MFI of media alone.

Blocking Mannose Receptor Inhibits the Upregulation of Human Dendritic Cell Activation Markers

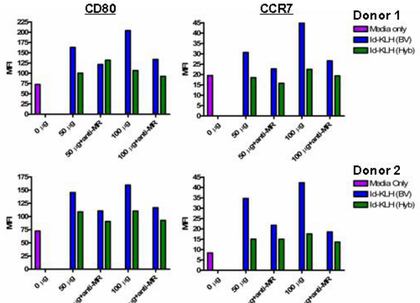

Figure 4: Surface expression of CD80 and CCR7 were evaluated after incubation with BV- or Hyb-derived conjugates with or without pretreatment using 50 µg/ml of anti-MR blocking antibody. The BV-conjugate treated samples showed a marked decrease in surface expression of both CD80 and CCR7 with anti-MR pretreatment.

Tumor Eradication is Superior with Use of Insect-Derived Idiotype and Maleimide Conjugation

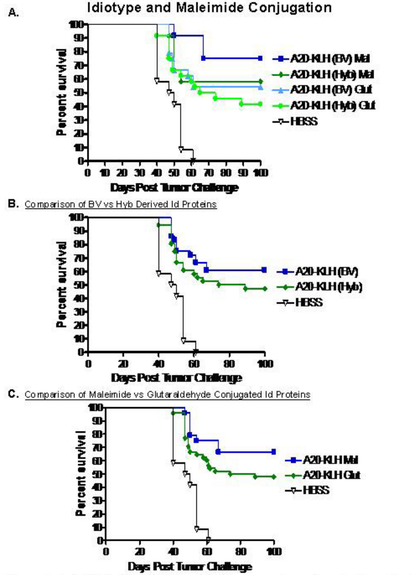

Figure 5: A. Individual A20-KLH (BV) and (Hyb) groups using each conjugation method demonstrating the (BV) maleimide conjugates resulted in the highest level of tumor eradication. **B.** A20-KLH (BV) and (Hyb) groups were pooled to show enhancement of survival with insect derived Id proteins. **C.** A20-KLH maleimide and glutaraldehyde treated mice were pooled to show superiority of maleimide conjugated Id proteins compared to glutaraldehyde conjugates.

CD8$^+$ T Cells are Required for Tumor Eradication

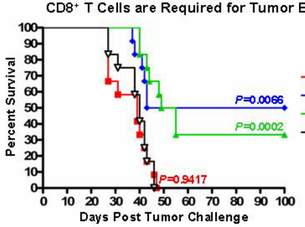

Figure 6: CD8$^+$ T cell are required for tumor eradication. *P* values represent comparisons between Id-KLH treated groups and HBSS unless otherwise indicated. The result shows a clear dependence on CD8$^+$ T cells.

Insect Derived Id Enhances Cytolytic Activity

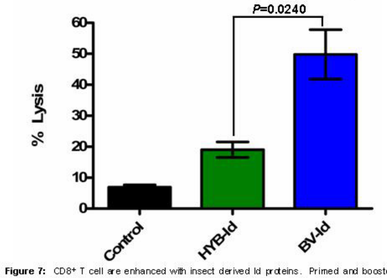

Figure 7: CD8$^+$ T cell are enhanced with insect derived Id proteins. Primed and boosted (2 immunizations) CTLs from spleen and lymph nodes were used at an E:T ratio of 1:1, both showed significant lysis compared to control. Additionally, the BV derived protein demonstrated statistically better lysis compared to Hyb produced Id.

Production of Anti-Idiotype Antibodies is not Hindered by the Presence of Mannose Residues, and is Enhanced with Maleimide Conjugation

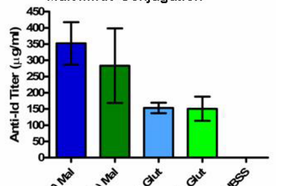

Figure 8: To evaluate the antibody responses generated by A20-KLH (BV) and (Hyb) vaccinated mice were bled and the immune sera anti-Id antibody titers were determined by ELISA. (target antigen = native, hybridoma-derived Id), using an A20 anti-Id monoclonal antibody as the standard. The BV derived Id proteins showed no reduction in anti-Id antibody titers, and the maleimide-conjugated proteins shown almost double the levels of anti-Id antibodies.

CONCLUSIONS

- The insect cell-derived idiotype (Id) proteins showed a mannose containing carbohydrate profile typical of antibodies derived from an insect cell source.
- Only insect cell-derived Id proteins demonstrated increased binding to human dendritic cells via the mannose receptor; the binding of mammalian-derived (hybridoma) idiotype proteins was low and did not change with the addition of mannose receptor inhibitors.
- Insect cell-derived Id proteins induced higher surface expression of dendritic cell activation markers CD80 and CCR7 than did mammalian-derived Id.
- Anti-mannose receptor antibodies could inhibit upregulation of DC activation markers.
- Vaccination of mice bearing A20 B cell lymphoma with insect cell-derived Id protein resulted in a higher proportion of survivors compared to hybridoma-derived Id (61% vs.49%), and conjugation to KLH using maleimide further enhanced the anti-tumor efficacy over the traditional glutaraldehyde method.
- CD8$^+$ T cells were critical for eradication of A20 lymphoma, and T cells primed with insect cell-derived Id showed enhanced tumor cytotoxicity.
- Insect cell-derived Id protein did not reduce the generation of anti-idiotype antibodies compared hybridoma-derived protein.
- Maleimide-conjugated Id-KLH, either insect or hybridoma-derived, generated approximately 2-fold higher anti-idiotype antibody titers compared to glutaraldehyde conjugates.
- Compared to mammalian cell-derived Id proteins, production of recombinant Id proteins via a baculovirus-insect cell system results in a structurally different antigen with improved immunogenicity, and this may lead to improved efficacy of the final Id-KLH vaccine product.

MATERIALS & METHODS

Production of A20 Id proteins: Mammalian A20 Id protein (IgG2a, κ) was affinity purified from culture media of a tumor-myeloma cell hybridoma. Recombinant A20 Id was produced by cloning the A20 Ig heavy and light variable regions into a baculovirus expression vector and infection of High5 insect cells. Nucleotide sequences of the tumor hybrid and recombinant Id genes were identical. Carbohydrate analysis demonstrated a typical N-linked glycosylation pattern for the mammalian-derived Id, but characteristic terminal mannose residues on the insect-derive protein (data not shown).

Activation of DCs with Id/KLH Preparations: Immature DC were incubated in growth media alone or with 50 µg/ml of A20/KLH (BV) or A20/KLH (Hyb) for 48 hours. Monoclonal anti-mannose receptor antibody at 50 µg/ml was added at the start of culture. Cells were harvested, and stained with FITC labeled anti-human CCR7, PE-labeled anti-human CD80, TC labeled anti-human CD45, and APC labeled anti-human CD14 monoclonal antibodies, and fixed in 4% PFA for flow cytometric analysis.

Animal Studies: Groups of 12 BALB/c mice were inoculated subcutaneously with 1x10^5 A20 cells on day 0. Mice were vaccinated on days 4, 11, and 18 with 50 µg A20-KLH (BV) or A20-KLH (Hyb), conjugated with either maleimide or glutaraldehyde. Each vaccination included 4 consecutive days of 55 ng GM-CSF given in the same location. Mice were sacrificed when tumors reached 1.4 cm in diameter, per institutional protocol. For T cell depletion studies, animals were injected i.p. with anti-CD8 or anti-CD4 antibodies, or control rat IgG on days –6, -5, -4, 0, and weekly thereafter, and depletions confirmed by flow cytometry. To evaluate the antibody responses generated by A20-KLH (BV) and (Hyb) vaccinated mice groups of 4-8 BALB/c mice were vaccinated on days 4, 11, and 18 with 50 µg A20-KLH (BV) or A20-KLH (Hyb), conjugated with either maleimide (Betting *et al*, Blood 105(11):146a,2006, full manuscript submitted) or glutaraldehyde. Each vaccination included 4 consecutive days of 55 ng GM-CSF given in the same site. Mice were bled 10 days after 3rd vaccination.

Anti-Id ELISA: F(Ab)$_2$ A20 hybridoma Id was coated onto Nunc Immunosorp plates at 5 µg/ml in carbonate buffer overnight at 4°C. Plates were washed and blocked with 5% non-fat dry milk, washed, and sera serially diluted along with an A20 anti-Id standard 1G6 (mouse IgG1) and incubated for 1 hour at room temperature, washed, and goat anti-mouse IgG (g specific)-HRP secondary detector antibody was added. Plates were washed, and developed with ABTS.

Generation of CTL: Post-vaccine spleen and lymph nodes were harvested and pooled from individual mice and made into single cell suspensions. Cells were cultured for 5 days with Mitomycin C treated A20 tumor cells. At the end of culture cells were purified via centrifugation in Histopaque. Live cells were counted and phenotyped to enumerate total number of surviving CD3$^+$CD8$^+$ T cells by Flow. 10^6 recovered cells were co-incubated with 10^4 D275 labeled A20 cells in 1 ml of 5% FCS/RPMI media for 3 hours at 37° C. 20 µl of PI was added to each sample and cells were assessed immediately by Flow. Percent lysis was calculated by the % of PI$^+$D275$^+$ A20 target cells/total D275$^+$ A20 targets.

Mannose Receptor Staining / Blocking: For human IgGs, immature monocyte-derived human DCs were incubated with 100 ng of FITC conjugated human IgG, KLH, or insect cell produced human monoclonal IgG1 antibody in the presence of 5% normal human serum for 20 minutes at 4°C. Cells were then washed twice in staining buffer and analyzed by flow cytometry. Cells cultured in absence of added FITC conjugated protein served as a negative control. Murine A20 Id (hybridoma or insect cell-derived) was labeled with Alexa 647 at equal molar ratios and 50 µg used to stain human DCs for 2 hours at 37°C in RPMI with 10% FCS and 1X PBS. The cells were washed 3x in ice cold FACS buffer and analyzed by flow cytometry. Some cells were also pretreated with various blocking agents for 20 minutes at 37°C to evaluate the affects on binding to mannose receptors on the cell surface.

Preparation of Immature Human DCs: Adherent PBMC were cultured in 2% human serum/RPMI media with 10 ng/ml recombinant human IL-4 and 50 ng/ml of GM-CSF. Cells were incubated for a further 6 days with a 50% cytokine media change every other day.