A Novel Method to Display [gal α1, 3 gal] Antigens on Human Leukemic Cells for Preparation of Anti-leukemia Vaccines

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Abstract. *Background: Expression of the immunostimulatory xenoantigen αGal on malignant cells is being investigated as a means to formulate anticancer vaccines. Expression methods have been limited to gene transfer using viral vectors and enzymatic manipulation. We report here a novel method using polyethylene glycol (PEG) to induce plasma membrane fusion between malignant human hematological cells and αGal$^+$ porcine blood cells (PBC) in order to display αGal antigens on human cells. Materials and Methods: Freshly isolated white blood cells (WBC) were obtained from patients with malignant hematological disease and combined with diluted PBC. Cell mixtures were labeled with human CD mAbs, followed by IB4 lectin or M86 mAb to detect αGal antigens and then co-incubated with PEG. Back-gated, dual-color flow cytometry was used to detect αGal on human cells. Results: αGal antigens were detected on sizeable numbers of human WBC (~45%) after incubation with PEG. Antigen expression was profuse as assessed by the strong fluorescent intensity demonstrated by IB4-FITC and M86 labeling. Human cells combined with PBC without PEG were not reactive with IB4-FITC or M86. Conclusion: Our method provides an effective, highly reproducible means to efficiently express αGal antigens on cells obtained from patients with a spectrum of hematological malignancies. This method can provide a simple, safe alternative to viral-mediated gene transfer or enzymatic alteration to express αGal antigens on human tumor cells. By virtue of its simplicity, our technique presents a novel approach to the preparation of polyvalent autologous or syngeneic anticancer vaccines.*

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Despite improved treatments, hematological malignancies remain often incurable. Immune-based therapies such as antileukemia vaccines are in development. The xenoantigen (gal α1, 3 gal) (αGal) is being investigated as an immunostimulatory molecule in vaccine formulation (1-3). Humans lack the gene encoding αGal (*CGTA1*) and instead produce potent humoral and cellular immune responses against αGal$^+$ cells (4-6). Numerous studies have demonstrated the immunostimulatory properties of αGal antigens. Human tumor cell lines that express αGal *via CGTA1* gene transfer are lysed by human serum (7-9). Treatment with the recombinant enzyme EC 2.4.1.151 (α 1,3 galactosyltransferase) yields similar results (10). When αGal$^+$ tumor cells are explanted into an αGal immune host (*CGTA1* knock-out mouse) (*CGTA1* KO) their tumorigenicity is strikingly reduced in correlation with anti-αGal IgM antibody titer; also antibodies against other tumor neoantigens are induced. Both human and *CGTA1* KO anti-αGal antibodies cause robust cytolysis of αGal$^+$ tumor cells *in vitro* (1, 11-13).

Expression of *CGTA1* and EC 2.4.1.151 is phylogenetically limited. All mammals *except* catarrhine primates (*e.g.* humans) have a functional *CGTA1* and form αGal structures (4-6). IB4 lectin, which binds terminal α-D-galactosyl residues, has long been used to detect αGal (5, 14-15). Monoclonal antibodies (mAb) against αGal are now available that are more specific than IB4 lectin (15, 16).

Porcine cells express abundant αGal antigens (~25×10^6 residues per cell) and are rapidly lysed by human serum. Humans continually produce high levels of 'natural' anti-αGal antibodies; this is the basis for the iatrogenic transplantation phenomenon of hyperacute rejection (HAR) (6, 17-19). Characterizations of HAR and subsequent responses against αGal$^+$ cells provide a well-studied paradigm of human anti-αGal immunity.

The strategies used to formulate αGal-based antitumor vaccines (enzymatic modification with EC 2.4.1.151 or gene transfer with retroviral and adenoviral (Ad) vectors containing *CGTA1*) have shown success but have significant weaknesses (1, 2, 13, 20-22). Retroviral transduction requires proliferating

cells; also viral titer/gene expression can vary dramatically dependent upon the vectors employed and cells targeted. Retroviral gene therapy also poses the risk of proviral insertion, oncogene activation and development of fatal malignant disease (23, 24). Although Ad vectors efficiently infect non-proliferating cells, Ad toxicity is a common, complex and potentially fatal complication of Ad vector administration (25).

We report here a novel method to exhibit αGal antigens on human cells as a means to prepare polyvalent vaccines against hematological malignancies. Cells from patients with leukemia/lymphoma are co-incubated with polyethylene glycol (PEG) and porcine blood cells (PBC). PEG induces plasma membrane fusion interactions between human white blood cells (WBC) and PBC, with PBC membranes providing copious αGal antigens. Using multi-parameter flow cytometric analysis, the presence of abundant αGal antigens on human CD mAb$^+$ cells was demonstrated by labeling with IB4 lectin and the αGal-specific mAb M86.

Materials and Methods

Normal and malignant human hematological cells. Normal human peripheral blood mononuclear cells (NHPBMC) were obtained from healthy donors and isolated using LSM media (Mediatech, Manassas, VA, USA). Patient cells were obtained from blood, bone marrow, spleen, lymph node or tonsil tissue samples submitted for clinical diagnosis and isolated using LSM. Clinical diagnoses were rendered by the pathology laboratory according to College of American Pathologists and American Society of Clinical Pathology guidelines and included acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), B-cell chronic lymphocytic leukemia (B-CLL) and B-cell non-Hodgkin's lymphoma (B-cell NHL). Studies were performed with Institutional Review Board approval.

Porcine peripheral blood. Peripheral blood was obtained *pre-mortem* from healthy animals under an institutionally approved animal use and care protocol, anticoagulated with acid-citrate-dextrose and held at 4˚C. Complete blood counts and differentials were obtained on 5 representative animals.

Human WBC/PBC mAb and IB4-FITC labeling. Human cells were resuspended at a density of ~1.5×10^6 cells/ml in serum-free Dulbecco's Modified Eagle's Medium (SF-DMEM). PBC were diluted to yield a 2% v/v cell suspension (~1.2×10^6 WBC and 5.5×10^7 RBC/ml). Combined human cells (100 μl) and PBC (50 μl) were labeled as follows with human CD-PE mAbs. NHPBMC were labeled with CD45. Cells from patients with AML were labeled with CD13 or CD33, B-CLL or B-NHL with CD19 or CD20, pre-B ALL with CD19. To detect αGal antigens, samples were then labeled with 20 μg/ml IB4-FITC (Sigma-Aldrich, St. Louis, MO, USA) or with mAb M86 (Axxora, San Diego, CA, USA) followed by FITC secondary antibody. Isotype controls were used as appropriate.

Human cells were labeled with M86 or IB4-FITC *versus* PBC labeled with human CD mAbs to control for species cross reactivity/non-specific binding. Unlabeled cells were treated with PEG to check for nonspecific fluorescence. Negative controls included: combined human WBC/PRC labeled only with human anti-CD-PE mAbs and combined cells labeled with CD-PE mAbs and IB4-FITC or M86 but *not* treated with PEG. Human WBC labeled with human CD mAbs and PBC labeled with IB4-FITC or M86 were used as positive controls. Combined cells labeled as above but not treated with PEG were used to control for non-specific cell aggregation. Cell viabilities were obtained before and after PEG treatment using propidium iodide exclusion.

PEG fusion. The above samples were centrifuged and 1 ml of 37˚C 50% PEG (molecular weight 4,000; Sigma-Aldrich, St. Louis) in SF-DMEM was added to the cell pellet. Cells were incubated for 1 minute at 37˚C, washed with 10 ml of SF-DMEM, and resuspended gently in 1.5 ml SF-DMEM. Samples were immediately analyzed *via* flow cytometry.

Flow cytometric analysis. Samples were analyzed using a FACScan equipped with CellQuest software and calibrated with Calibrite Beads (BD Biosciences San Jose, CA, USA). Spectral overlap compensation was performed using human CD-PE mAb labeled cells and PBC labeled with IB4-FITC or M86. Percentages of dual-labeled cells were calculated using quadrant statistics and histogram statistics were used to determine geometric mean fluorescence intensity (GMFI).

Data/statistical analysis. Two methods of data analysis were used assess αGal expression. Dual-parameter histograms were used to calculate the % αGal$^+$ human cells in PEG-treated samples compared to PEG-untreated controls. Analysis was back-gated using human CD-PE mAbs. Alternatively, IB4-FITC and M86 mean GMFI were calculated from single-parameter FITC histograms gated on human CD mAb$^+$ cells and compared to controls. Excel (Microsoft Corporation, Redmond, WA, USA) was used to calculate mean and standard deviation for each data set. SPSS (SPSS Inc, Chicago, IL, USA) was used to determine *p*-values using Student's *t*-test and ANOVA.

Results

PBC and human mononuclear WBC populations were overlapping; analysis was gated on all WBC. PBC did not react with human CD mAbs nor were human WBC labeled by IB4-FITC or M86. PEG treatment did not increase autoflourescence and/or or non-specific mAb binding, nor did it significantly reduce cell viability (mean viability post PEG=85% , n=7) (data not shown).

NHPBMC from 4 healthy adult donors were assayed; one in replicate on 2 separate days. Samples obtained from thirteen patients with hematological malignancies were assayed. Of these, there were four AML's, three B-cell NHL's, three pre-B-cell ALL's (pediatric), two B-cell CLL's and one acute promyelocytic leukemia (PML). One NHL sample was assayed in replicate on 2 separate days. Three patient samples had poor viabilities and were excluded. The remainder of the normal and patient samples (≥90% viability) gave excellent results.

Table I. *A, Presence of αGal antigens on human WBC determined by IB4-FITC and M86 GMFI. Analysis was gated on human CD mAb+ cells. Control A: Mixed human WBC/PBC labeled only with human CD-PE mAbs. Control B: Duplicate sample labeled with human CD-PE mAb, IB4-FITC or M86 mAb without PEG. Test: Duplicate sample labeled and treated with PEG. IB4-FITC and M86 group mean GMFI are shown separately. PEG- treated samples gave highly increased GMFI compared to controls (IB4-FITC, p≤0.005; M86, p≤0.05). B, Detection of αGal on human WBC determined by co-expression of IB4-FITC or M86 and human CD mAbs. Mean basal dual+ cells is shown in the 'Labeled no PEG' column. Co-expression after PEG treatment is shown in the 'Labeled PEG-treated' column. Final dual+ values were calculated by subtracting % basal dual+ cells from total dual+ values. Large numbers of human WBC displayed αGal after co-incubation with PBC and PEG compared to samples without PEG (IB4-FITC, p≤0.005; M86, p≤0.005).*

A.	Control A No FITC label/no PEG	Control B FITC label /no PEG	Test labeled + PEG-treated
Mean M86 GMFI (n=16)	5	15	159
Mean IB4-FITC GMFI (n=9)	6	6	65
B.	**Labeled no PEG**	**Labeled PEG-treated**	**Final % dual+**
Mean IB4-FITC/human CD dual+ cells (n=16)	24%	72%	48%
Mean M86/human CD dual+ cells (n=9)	13%	60%	47%

After co-incubation with PBC and PEG, human cells displayed abundant αGal antigens as assessed by reactivity with IB4-FITC and M86. Test samples were strongly reactive with IB4; the average IB4 GMFI for test samples was 159 channels (range 35-394) and was significantly much higher than either negative control GMFI ($p \leq 0.005$ for both). Negative control A gave a mean GMFI of 5 channels (range 5-9) and control B gave a mean GMFI of 15 channels (range 3-29) (n=16 for all assays). No statistically significant difference was found between the IB4 GMFI for the two sets of negative controls ($p=0.075$) (Table I; Figure 1).

The mAb M86, which is specific for αGal antigens, was also used. M86 was included in 4 NHPBM and 5 patient cell assays. M86 and IB4-FITC gave almost identical results. Test samples were intensely reactive with M86; the average M86 GMFI for test samples was 65 channels (range 25-184) and much brighter than either negative control ($p \leq 0.05$). Both controls gave a mean GMFI of 6 channels (Table I; Figure 1).

The very low and consistent IB4 and M86 GMFI values determined for negative controls A and B are significant. As described, control A comprised human WBC+PBC labeled only with human CD mAb and B comprised a duplicate sample labeled with both human CD mAb and IB4-FITC or M86 *without PEG treatment*. These low GMFI values confirmed that human cells became reactive with IB4-FITC or M86 (thus demonstrating the presence of exogenous αGal antigens) only after co-incubation with PBC and PEG.

Two-color analysis was used to determine the percentages of human CD mAb+ cells that exhibited αGal after treatment. Large numbers of human CD mAb+ cells co-expressed IB4-FITC, ranging from 38% -94% (mean 72% , n=16). Control samples that were labeled and co-incubated but not treated with PEG yielded many fewer dual+ cells, ranging from 2% -39% (mean 24% , $p \leq 0.005$, n=16). After subtracting control (basal) dual+ cells, final percentages of IB4+ human WBC ranged from 15% -72% (mean 48% , n=16) (Table I; Figure 1).

M86 mAb labeling gave similar results; large percentages of human cells co-expressed M86, ranging from 17% -93% (mean 60% , n=9) after incubation with PEG and PBC. Controls showed far fewer dual+ cells (range 3% -31% , mean 13% , $p \leq 0.005$, n=9). Basal dual+ cells were subtracted from the total % M86+ human WBC to give final dual+ values (range 14% -77% , mean 47% , n=9) (Table I; Figure 1).

NHPBMC samples (n=5) analyzed separately gave almost identical results to patient samples (n=11). Final mean values of dual human CD+/IB4+ cells in the normal group were 34% , compared to 35% in the patient group ($p=0.973$). The proportion of M86+ human cells in the normal group (mean=33% , n=4) compared to the patient group (mean=56% , n=4) was also very similar ($p=0.292$) (data not shown).

Discussion

Our study shows that malignant human WBC can be easily and reproducibly modified to exhibit abundant αGal antigens using co-incubation with PEG and PBC. Our method was effective upon a wide variety of hematological cells (NHPBMC, myeloid and lymphoid blasts, and mature B-cell malignancies) obtained from various sources, including PB, BM, spleen and lymph node. Untreated human cells did not display αGal in contrast to ~46% of treated cells. IB4-FITC and the αGal-specific mAb M86 were used to detect αGal antigens and strongly labeled all porcine cells whereas neither labeled untreated human cells. None of the human CD mAbs used cross-reacted with PBC whereas they did

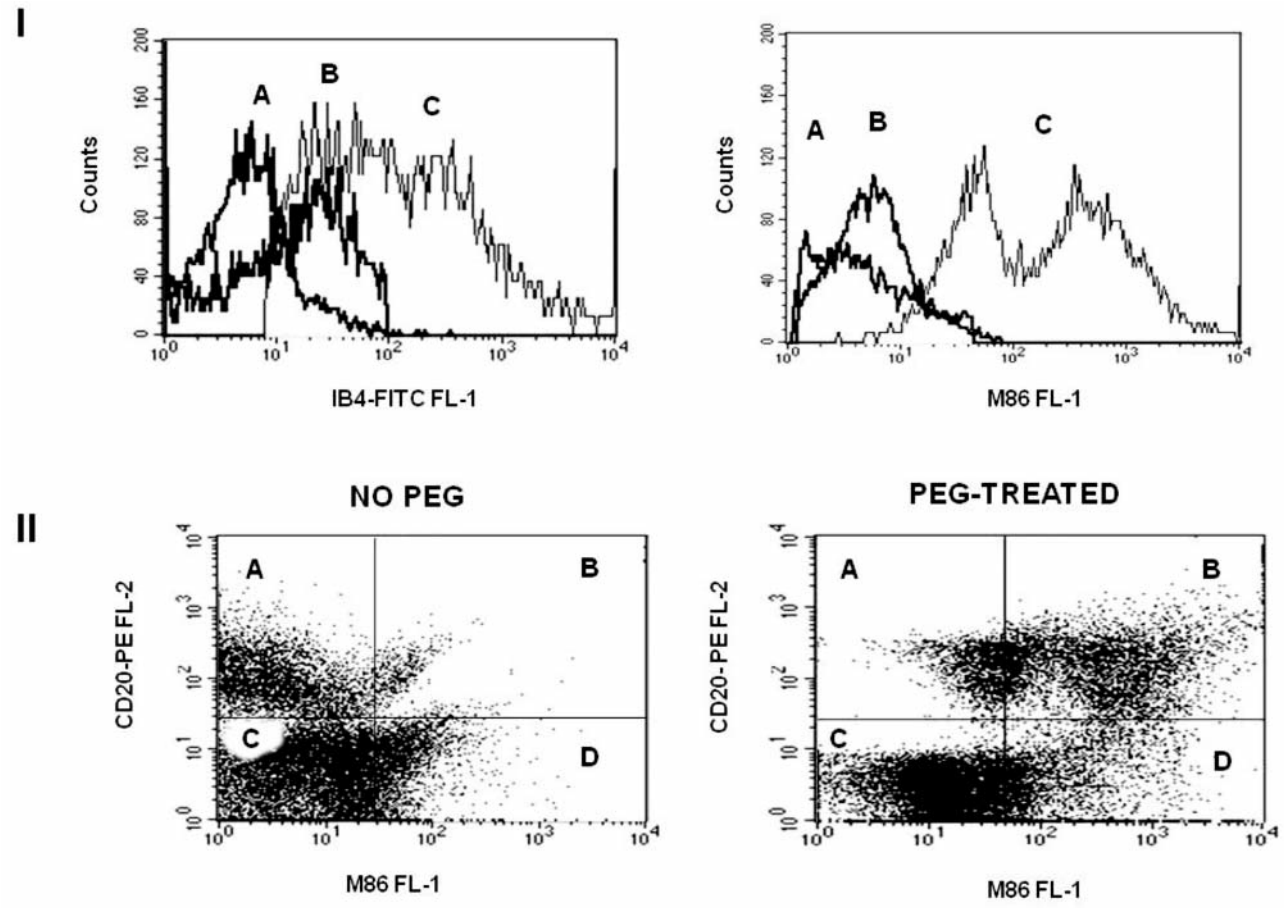

Figure 1. *Histograms from one representative patient sample (B-cell CLL) demonstrating the presence of αGal on CD20+ human cells after co-incubation with PBC and PEG. I, Overlays of single-parameter IB4 or M86 labeling of human cells: A, Mixed CLL/PBC cells labeled only with human anti-CD20-PE; B, duplicate sample labeled with CD20 and IB4-FITC or M86 but not treated with PEG; C, duplicate sample labeled and treated with PEG. II, Quadrant analysis of CD20-PE vs. M86-FITC labeling: A, CD20+ CLL cells only; B, CD20+ cells co-expressing M86; C, unlabeled cells and debris; D, M86+ PBC only.*

appropriately label human cells. This was expected, although some adhesion molecule mAbs (not used in this study) may cross react with porcine antigens, human CD mAbs as a rule do not label porcine cells (26, 27).

In preliminary studies, complete PBC cytolysis was caused by incubation with a 1:5 dilution of human serum for 30 min at 37˚C (data not shown). We have previously characterized similar lysis of GalT+ murine tumor cells by anti-αGal antibodies (11, 12). Our studies and others have shown that responses to αGal+ cells are dependent on abundant serum antibodies and complement rather than αGal antigen density. This premise is also supported by a plethora of xenotransplantation studies (2, 6). Thus, we anticipate that human tumor cells altered to display αGal antigens by our method will elicit potent, innate human anti-αGal responses and we are performing studies to examine these effects.

The IB4 lectin used to detect αGal structures binds other epitopes with terminal α-galactosyl residues, most significantly human blood group B and AB antigens (28). None of the samples in this study were from B or AB persons. We did not exclude these groups; rather we acquired samples as they became available and blood groups B and AB are infrequent in Western populations. Regardless, we used the αGal-specific mAb M86 to confirm the results from IB4-FITC labeling. M86 and IB4 gave very similar results in each sample, demonstrating the utility of αGal specific mAbs in future studies with patients of any blood group.

PEG likely induced redistribution of αGal glycoconjugates from PBC plasma membranes to human WBC plasma membranes. In addition to acting as a cell fusogen, various studies have demonstrated that PEG facilitates redistribution of plasma membrane-bound proteins between disparate cell types. Major histocompatability complex (MHC) proteins

are mobile in the plasma membrane of human/mouse heterokaryons. PEG induces exchange of MHC I and II, the IL2-α subunit, as well as CD48 and CD71 components between human T-cell lines. Using porcine cells and PEG, a recent study demonstrated that human mesenchymal stem cells could be fused with porcine adrenal chromaffin cells using PEG. The resulting hybridomas exhibited the porcine chromaffin-cell specific markers tyrosine hydroxylase and methionine enkephalin (29-31).

PEG treatment could have induced the formation of porcine/human WBC hybrids (32). Dual positive cells did not display significantly altered light scatter characteristics nor back gate to a distinct population. This suggests that human/porcine WBC hybrids were not formed. However, PEG may have induced fusion of human WBC with porcine erythrocytes. Porcine RBC were much more numerous than porcine WBC in the PBC suspensions, express copious αGal epitopes, are enucleate and several orders of magnitude smaller than human WBC. Thus, cell fusion could occur without significantly altering light scatter. We are performing further studies to determine whether cell hybrid formation contributed to our results.

Basal levels of double positive cells were present in control samples containing combined PBC/human WBC untreated with PEG, perhaps due to non-specific cell aggregation. However, human $CD8^+$ lymphocytes, monocytes and natural killer cells have all been reported to directly recognize porcine MHC and αGal antigens, respectively. These cellular responses are significant and in the xenotransplantation model cause delayed xenograft rejection (33-36). Given that significant antibody-independent cellular recognition of αGal occurs, it is very possible that co-incubation allowed some direct cell interactions between human WBC and PBC, despite relative short (one hour) incubations at room temperature.

Our study demonstrates that freshly isolated cells from patients with hematological malignancies can be easily altered to display αGal antigens by co-incubation with PBC and PEG. Our method was consistent, reproducible and successful using a broad variety of hematological cells, including NHPBMC, malignant lymphoblasts, myeloblasts and mature malignant B-cells obtained from various tissue sources. Our method to display αGal antigens on human tumor cells is simple and thus may offer technical advantages over the use of gene transfer or cell surface remodeling using recombinant EC 2.4.1.151, in addition to obviating the safety concerns that arise with viral gene therapy. We are performing further studies to the examine the degree to which our treatment renders cells from patients with hematological malignancies susceptible to the various immune responses evoked by αGal antigens, with the ultimate goal of using our method to prepare polyvalent tumor vaccines.

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