Cytotoxic Activity of CD48 Monoclonal Antibodies Against Human Lymphoma Cells

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Abstract: CD48 is a cell surface, glycosylphosphatidylinositol-linked glycoprotein, and a potential target for treatment of leukemia and lymphoma. Two anti-CD48 mAbs, murine HuLy-m3 and human IgG1-N2A, were compared in cellular assays using a human lymphoma cell line (Raji) for their ability to inhibit cell growth and induce apoptosis. *In vitro* studies revealed both HuLy-m3 and IgG1-N2A mAbs were able to induce potent growth inhibition, reflected by a reduction in viable cells of approximately 70% compared to controls after 90 h. Furthermore, Raji cells treated with IgG1-N2A showed evidence of apoptosis, including increased ethidium bromide uptake, cell shrinkage and chromosomal DNA degradation.

INTRODUCTION

A number of targets for antibody immunotherapy have been identified, including CD20 [1,2], CD21[3], CD19 [4] and CD52 [5]. CD48 is another potential target, a glycosylphosphatidylinositol (GPI)-anchored adhesion molecule expressed on all peripheral blood lymphocytes [6-8]. CD48 is expressed at higher levels (~2×10^5 CD48 binding sites/cell) on a human Burkitt's lymphoma cell line, Raji [9] and on most acute myeloid leukemia cells with phenotype $CD34^-/CD13^+/CD33^+$ [10]. Both normal and malignant white blood cells express CD48 on their membrane surface, but greater than 95% of $CD34^+$ haematopoietic stem cells do not express CD48.

Many of the original monoclonal antibodies (mAbs) against CD48 were of murine origin. The anti-CD48 IgM murine mAb WM63 was demonstrated in a clinical trial to elicit a significant but temporary reduction in circulating leukocytes in chronic lymphocytic leukemia (CLL) patients [11]. Another murine anti-CD48 mAb (HuLy-m3) was evaluated in a preclinical trial for anti-tumor activity, and was shown to lead to long-term survival of *scid* mice injected with Raji cells, however was not able to mediate significant antibody dependent cellular cytotoxicity (ADCC) [9]. A chimeric anti-CD48 mAb, with the variable region of HuLy-m3 fused with a human IgG1 constant region showed improved ADCC effector function in a further preclinical study [12].

Recently, a fully human anti-CD48 mAb (IgG1-N2A) was isolated from a human immunoglobulin gene library using phage display technology. IgG1-N2A mAb binds to an epitope distinct from the epitope of HuLym3 mAb, and may have advantages in anti-CD48 related immunotherapy [13].

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MATERIALS AND METHODS

Antibodies

The hybridoma cell line producing the murine anti-CD48 HuLy-m3 mAb (IgG2a) [14] was cultured in hybridoma serum-free medium (Invitrogen) and the mAb was purified and conjugated to biotin [13]. A CHO cell line (CHO-N2A) was used for human anti-CD48 mAb (IgG1-N2A) production [13]. A fluorescein-isothiocyanate (FITC) labeled goat anti-human IgG (Fc specific) and FITC-conjugated streptavidin were purchased from Sigma for immunofluorescence staining.

Cell Culture

The Burkitt's lymphoma cell line, Raji, was obtained from the American Type Culture Collection (CCL-86). Cells were maintained in growth medium (modified DMEM & Coon's F12 medium supplemented with 10% (v/v) fetal bovine serum).

Flow Cytometry Analysis of Antibody Binding Raji Cells

Raji cells were cultured in growth medium for 2 days to a cell density of 1×10^6 cells/ml. Approximately 5×10^6 Raji cells in growth medium were added to each well of a 6 well plate with human anti-CD48 mAb IgG1-N2A and incubated for 30 min, 1 h, and overnight or biotinylated HuLy-m3 mAb and incubated for 30 min at 37 °C. The cells were centrifuged at 300 × g for 5 min and the cell pellet was resuspended in 150 µl of phosphate buffered saline (PBS) with 3% (w/v) bovine serum albumin (BSA). Cells were then incubated for 30 min on ice with goat anti-human IgG (Fc specific) FITC conjugate or strepdavidin FITC conjugate. Following an extensive wash with PBS containing 1% (w/v) BSA, cells were resuspended in PBS with 3% (w/v) BSA. The stained cells were analyzed using MoFlo MLS flow cytometer (Cytomation) with Coherent Innova 90-5 argon laser

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(488 nm) and Green D530/40 filter (510-550nm). The viable cells were gated and analysed by the Summit software (Cytomation). A total of approximately 10^4 cells were analyzed for each sample.

Confocal Imaging

Raji cells were prepared as for flow cytometry analysis, and 5×10^6 cells in 0.5 ml growth medium were mixed with IgG1-N2A (10 μg/ml) and HuLy-m3 (25 μg/ml) and incubated for 3 h. Cells were washed 3 times with PBS with 1% (w/v) BSA and then incubated with secondary antibodies, goat anti-human Fc-FITC conjugate or Streptavidin-FITC conjugate respectively. The cells were incubated on ice for 1 h, washed 3 times with 1% (w/v) BSA in PBS before observation using a BioRad MRC1024 confocal laser scanning microscope (CLSM) with Argon laser (488 nm) and a Leica DMRB microscope (×40/1.00-0.50 Oil lens). FITC labeled Raji cells were captured with 488 nm excitation and 522 DF35 emission filter and with 512 × 512 pixel image format.

Determination of Cell Viability

Raji cells in growth medium seeded at a density of 1×10^5 cells/ml and containing 5 μg/ml IgG1-N2A mAb, 20 μg/ml HuLy-m3 mAb and human IgG-1 isotype control mAb respectively were cultured for various time at 37 °C in a humidified incubator in a 5 % (v/v) CO_2 environment. Raji cells cultured in media with no antibody addition were used as negative control. Cells were dispensed into a hemocytometer and cell viability was assessed by trypan blue exclusion. Viable and dead cells were visualized and quantified using bright-field microscopy. The numbers of viable and nonviable cells in each sample were summed to quantify the total cell population. Cells were counted every day for 6 days after exposure to the mAbs.

Detection of the Induction of Apoptosis

Cell Membrane Permeability

Flow cytometry was performed for cell membrane permeability studies. Raji cells were incubated with IgG1-N2A (5 μg/ml) or HuLy-m3 (25 μg/ml) mAbs for 72 h at 37 °C. Cells (5×10^6) were washed with PBS containing 1% (w/v) BSA and re-suspended in PBS with 3% (w/v) BSA before staining with 5 μg/ml ethidium bromide (EB) for 5 min at room temperature. The cells were sorted using the MoFlo MLS flow cytometer (Cytomation) with Coherent Innova 90-5 argon laser (488 nm) and D670/40 filter (650-690 nm). A total of approximately $1\text{-}2 \times 10^4$ cells from each sample were analyzed.

DNA Fragmentation Analysis

Raji cells were cultured with IgG1-N2A or HuLy-m3 mAbs for 72 h at 37 °C before whole cell DNA extractions. The untreated cells and cells treated with isotype antibody were used as negative controls. The cells (10^6) were harvested by centrifugation and resuspended in 50 μl PBS. The cells were lysed by addition of 50 μl phenol/chloroform/isoamylalcohol (25:24:1, v/v/v), 10 μl bromophenol blue dye and centrifuged for 5 min at 12000 × g. The whole cell DNA in aqueous phase were loaded and electrophoresed on a 0.75% (w/v) agarose gel. The DNA fragments were stained with ethidium bromide and photographed by exposure to UV light.

RESULTS AND DISCUSSION

Anti-CD48 mAb Interaction with Raji Cells

Raji cells, a malignant lymphoma cell line, express a number of membrane surface proteins, which have been identified as targets for immunotherapy including CD20, CD21, CD22, CD40, CD45, CD54, CD55, CD59, CD80, CD81, CD84 and CD86 [7,15,16]. CD48 is expressed on the surface of Raji cells at a higher level (approximately 2×10^5 binding sites/cell) compared to that on normal human B cells (4×10^4 binding sites/cell) [9]. Flow cytometric analysis of Raji cells using HuLy-m3 mAb as the primary antibody showed 98% of the cells to be CD48 positive (Fig. **1**). In a time course study of IgG1-N2A mAb binding to Raji cells by flow cytometric analysis, incubation for up to 24 h showed the mAbs remained attached to Raji cells (Fig. **2**).

Further examination by CLSM confirmed that the anti-CD48 antibodies were bound to the cell surface and not internalized (Fig. **3**). The prolonged maintenance of mAbs on the target cell surface facilitates ADCC, CDC and other effector functions. For example, the anti-CD48 murine/human chimeric mAb provided a positive result in the ADCC assay, showing approximately 64% specific lysis of Raji cells [12].

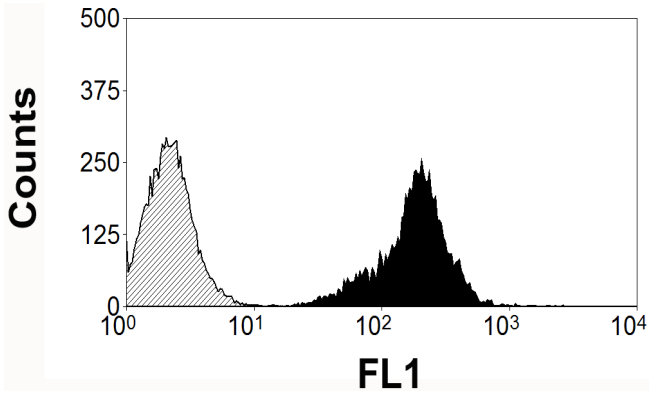

Fig. (1). Analysis of CD48 expression on Raji cell by FACS. Approximately 5×10^6 Raji cells were incubated with biotinylated anti-CD48 mAb HuLy-m3 followed by incubation with streptavidin FITC conjugated. The results show an overlay histogram, with more than 98% Raji cells to be CD48 positive (shaded) compared to the control (hatched, incubation with streptavidin-FITC conjugate only). The X-axis, labeled as FL1 (fluorescence log 1) is the level of fluorescent intensity and the Y-axis is a measure of cell number.

Viability and Growth Inhibition

To determine the effects of incubation of anti-CD48 mAbs with Raji cells over time, HuLy-m3 and IgG1-N2A mAbs were incubated with Raji cells for up to 144 h in cell culture. Significant reductions in viable cell populations were observed for murine HuLy-m3 and human IgG1-N2A mAbs. Inhibition of cell growth was evident at 48 h with IgG1-N2A mAb, whereas inhibition by HuLy-m3 mAb occurred after 48 h (Fig. **4**). Inhibition of cell growth by both IgG1-N2A and HuLym3 mAbs was evident after 60 h compared to controls. After 96 h, cell viability of HuLy-m3 and

IgG1-N2A mAb-treated Raji cells was reduced to approximately 30% of that of the controls.

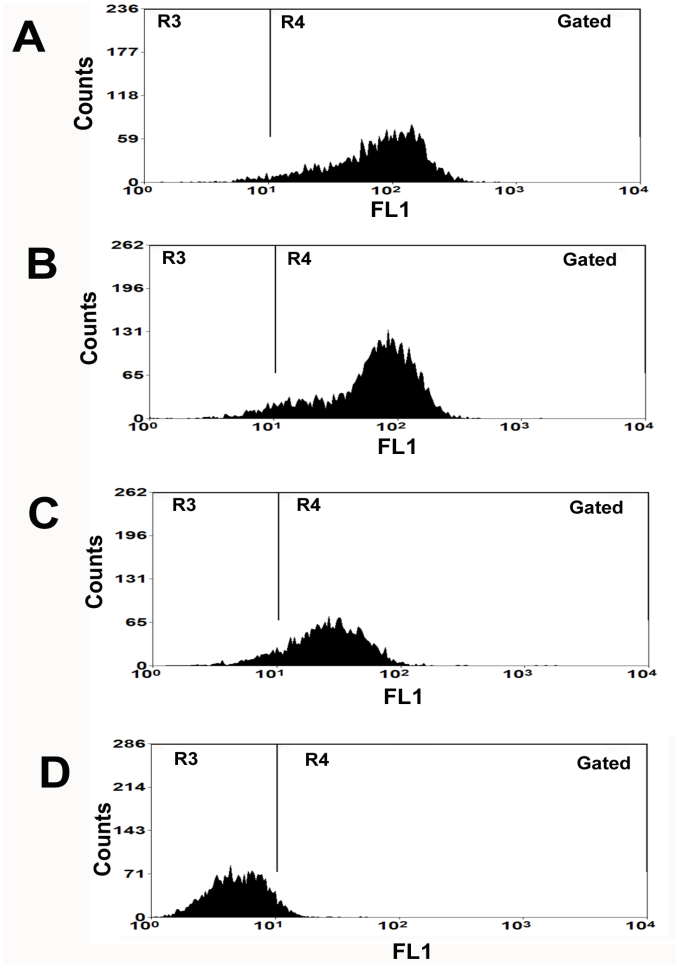

Fig. (2). Time course analysis of IgG1-N2A mAb binding to CD48 expressing-Raji cells using flow cytometry. Cells were incubated with IgG1-N2A mAb in growth medium for 0.5 h (**A**), 2.5 h (**B**) and 24 h (**C**) at 37 °C. The negative control (**D**) was a culture with no antibody added. Following the primary antibody incubation, cells were then incubated with FITC conjugated-goat anti-human IgG (Fc specific) and placed on ice for 0.5 h prior to flow cytometry analysis. The X-axis, labeled as FL1 is level of fluorescent intensity and the Y-axis is a measure of cell number (Experiments were repeated 3 times with similar results).

Apoptosis Analysis

Another mechanism by which mAbs may exert their cytotoxic effect besides ADCC and CDC is induction of apoptosis on tumor cells. Several studies show that mAbs against CD20 [15,17], CD19 [18] and CD40 [19] could directly inhibit the growth of malignant B cells through induction of apoptosis. The ability of mAbs to interact with cell surface proteins, with subsequent activation of signaling pathways leading to apoptosis provides an additional mechanism for eliciting anti-tumour cytotoxic activity. It has been reported that in some instances, the signaling functions of mAbs may be more important than recruitment of effectors in the treatment of malignancies [20-22]. Cross-linking of cell surface antigens by mAbs on the surface of B cells may also be important in initiating apoptosis, but may not be essential [23].

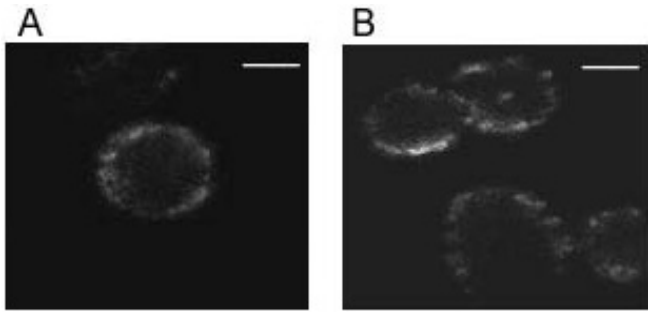

Fig. (3). Confocal laser scanning microscope imaging of Raji cells. Raji cells were incubated with IgG1-N2A mAb (10 μg/ml, **A**) or biotinylated Huly-m3 mAb (25 μg/ml, **B**) for 3 h, followed by incubation with secondary antibodies, FITC-conjugated anti-human Fc or FITC-Streptavidin conjugate, respectively. Scale bars indicate 10 μm.

Nuclear damage (DNA fragmentation) has long been used to distinguish apoptosis from necrosis and is among the most reliable methods for detection of apoptotic cells [24,25]. To evaluate the potential of anti-CD48 mAbs to induce apoptosis, Raji cells were used to assay for changes in cell membrane permeability in response to IgG1-N2A mAb and HuLy-m3 mAb binding at the Raji cell surface. Firstly, Raji cells were incubated separately with HuLy-m3, IgG1-N2A and isotype control mAbs for 72 h followed by staining with EB for 5 min. Cross-linking of cell surface membrane antigens by mAbs can alter membrane permeability or pore size, allowing entry of EB with subsequent binding to nuclear DNA. The orange fluorescence of EB can be detected using flow cytometry. Flow cytometry analyses showed that the population of EB positive (EB^+) cells was increased compared to that of the controls. The EB^+ cells incubated with IgG1-N2A mAb and HuLy-m3 mAb were approximately 18% and 24% of total cells respectively, indicating elevated membrane permeability (Fig. **5**).

For DNA fragmentation effects, Raji cells were treated with 5 μg/ml IgG1-N2A, HuLy-m3 and isotype control mAbs for 72 h, and the whole cell DNA was extracted and analysed by electrophoresis on a 0.75% agarose gel. DNA of Raji cells incubated with HuLy-m3 mAb, isotype control mAb and untreated control showed one large band (approximately 27 kb) and two small bands (1.3 kb and 2.0 kb). However, analysis of DNA extracted from Raji cells pre-incubated with the IgG1-N2A mAb produced a different pattern, whereby the larger DNA band appeared at a lower density and the two smaller bands were cleaved into fragments between 0.5 kb and 1 kb in size (Fig. **6**).

The typical endonucleolytic cleavage pattern of DNA with apoptosis induction by anti-CD48 mAbs was not found in this study. However the three band DNA patterns of control Raji cells was similar to the endonucleolytic cleavage pattern of DNA extracted from Jurkat cells reported by Micoud *et al*. [26]. Raji cells have been reported to be relatively resistant to nuclear apoptosis (DNA fragmentation), induced by various stimuli, when compared to other apoptotic-sensitive human lymphoma cell lines, such as HL-60 and Daudi cells [27]. However it has been shown that selected reagents (proteins and chemicals) and specific physical con-

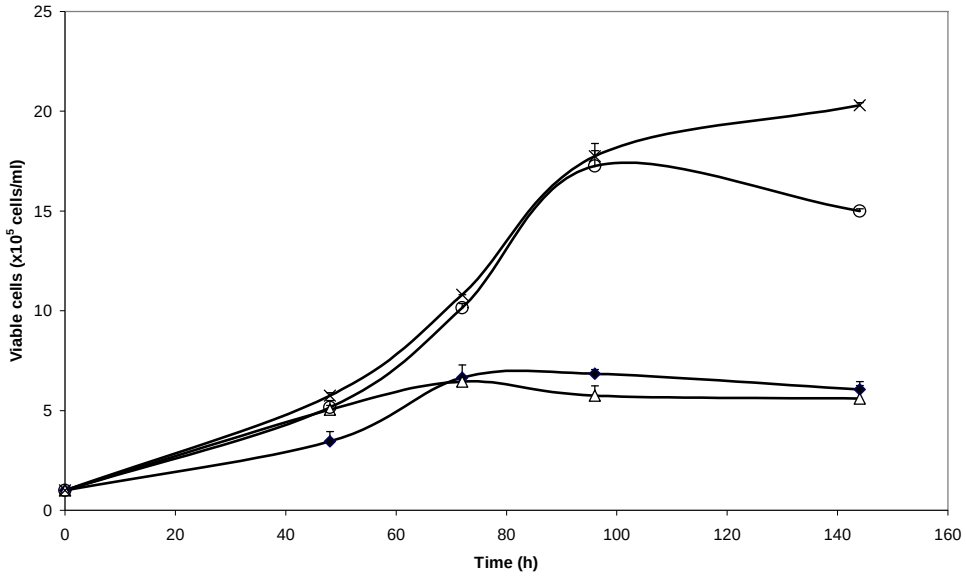

Fig. (4). Raji cell viability assay. IgG1-N2A (5 μg/mL; —◆—) or HuLy-m3 (20 μg/ml; —△—) mAbs were incubated with Raji cells respectively for up to 144 h. Growth inhibition over time was demonstrated. (—○—) isotype and (—X—) negative controls. Error bars show standard deviation of the mean.

ditions, such as heat shock used in this study (result not shown) can induce Raji cell apoptosis and DNA fragmentation [26,28]. The laddering of nuclear DNA fragments, when analysed by electrophoresis, is a typical hallmark of a cell

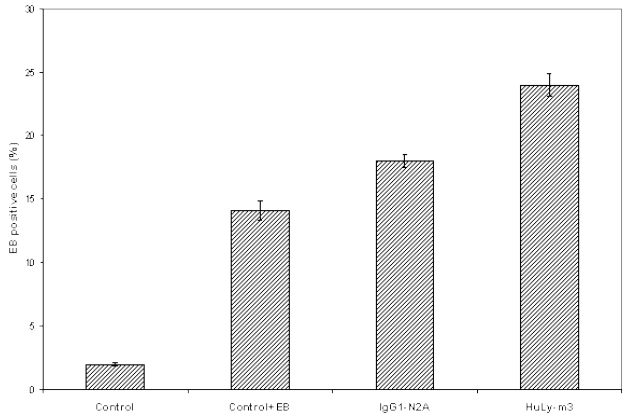

Fig. (5). Ethidium bromide uptake by Raji cells. Raji cells were treated with either IgG1-N2A or HuLy-m3 mAbs for 72 h, followed by a 5 min staining incubation with EB. The cells were analyzed using flow cytometry to determine the populations of EB^+ cells. Error bars indicate standard deviation from two separate cultures for each treatment (Experiment was performed three times with similar results).

undergoing apoptosis [29,30]. In contrast, some reports have shown that the typical ladder formations of nuclear DNA fragmentation are not always associated with apoptosis [24,31] and the DNA fragmentation pattern cannot be used as the sole criteria. Although the ladder pattern of DNA fragmentation was not observed in this study, cleavage of Raji cell DNA treated with human IgG1-N2A mAb did occur, and may indicate progression of apoptosis. The data suggests that IgG1-N2A mAb can block proliferation and promote apoptosis of lymphoma cells, and therefore has potential as a lead antibody candidate for the treatment of white blood cell malignancies.

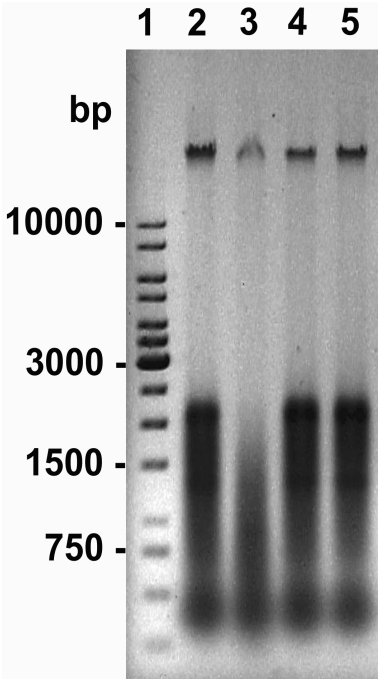

Fig. (6). DNA fragmentation assay. DNA of Raji cells incubated with either, IgG1-N2A, HuLy-m3 or isotype control mAbs was extracted and analysed by electrophoresis on a 0.75% agarose gel. Lane 1, 1 kb DNA ladder (Fermentas); Lane 2, Untreated Raji cells (negative control); Lane 3, Raji cells treated with IgG1-N2A mAb; Lane 4, Raji cells treated with HuLy-m3 mAb; Lane 5, Raji cells treated with isotype mAb. (bp, base pairs).

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