ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
October 2015; 14(5):502-508.

Production and Characterization of New Anti-Human CD20 Monoclonal Antibody

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Received: 18 November 2014; Received in revised form: 7 March 2015; Accepted: 8 March 2015

ABSTRACT

The B-cell CD20 antigen is one of the most reliable surface targets in immunotherapy of B lymphoma. In this project, we studied the production and characterization of a new monoclonal antibody against chimeric human CD20 extra loops (hCD20 exl).

The results showed that clone C12H, IgG2/k isotype reacted with the antigen in ELISA and immunoblot. The Kd value was found to be 2×10^{-9}M and flow cytometry results showed that 99.9% and 99.7% of the Daudi and Raji cells respectively were stained with C12H monoclonal antibody (mab) but not with Jurkat cell lines. It also effectively competed with Rituximab, thus, the staining of the Daudi and Raji cell lines was reduced to 55.9% and 40.5% of cells respectively.

Based on the high affinity reaction of C12H mab and appropriate reactivity of C12H mab with the native antigen on the surface of Raji and Daudi cells in flow cytometry, it was concluded that development and evaluation of C12H mab could be a beneficial candidate for further application in genetically engineered monoclonal antibody.

Keywords: CD20; Immunotherapy; Lymphoma; Monoclonal antibody

INTRODUCTION

CD20 protein is one of the most reliable cell surface markers of human B cells and functions as part of complex receptor in the cell signaling.[1] Moreover, the CD20 antigen plays an important role in B-cell activation and proliferation[2] and is present and over expressed on most B-cell lymphomas.[3] Following antibody binding on target antigen on the cell membrane, CD20 is not shed or internalized, consequently this highly performance of the antibody makes CD20 a vital target in the field of cancer immunotherapy.[4] Non-Hodgkin's lymphoma (NHL) is a lymphoid tissue malignancy which has been known as the fifth most common type of cancer. Depending on the lymphocyte lineage, it is usually divided into two main categories including T-cell and B-cell lymphoma.

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

The chance of survival in this cancer also depends on the overall health, grade, stage of the disease, and response to treatment by the host.[5] However, in the last two decades, immunotherapy has been the first choice for treatment and Rituximab (Rituxan) is the first monoclonal antibody introduced by FDA in the treatment of B-cell lymphoma.[6] Indeed, Rituximab, is a chimeric anti-hCD20 antibody which has brought a new insight for treatment of relapsed/refractory low-grade B-cell non-Hodgkin's lymphoma in the last decade.[7] Presently, Rituximab is one of the main agents for the management of B cell lymphoma patients, as a single medicine or more often is given in combination with chemotherapy.[8] The anti hCD20 Monoclonal antibody (mabs), which target the CD20 cell surface antigen, have antitumor activity via various mechanisms, such as apoptosis, recruitment of natural killer cells, monocytes and macrophages, complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC).[9-12] Depending on the type of antibody, more than one of these mechanisms may play a role in the effectiveness of immunotherapy. In spite of all the beneficial effects of Rituximab, which so far has led to cure a considerable number of patients, it is believed that it is successful in half of the patients suffering from NHL.

Thus, the limitation of current anti hCD20 mabs in immunotherapy of NHL was the main reason for researchers in the present study to perform further investigation for development of another anti hCD20 monoclonal antibody. However, the effective therapeutic monoclonal antibody production has always been limited to a suitable immunogen. Regarding this problem, we have previously designed and expressed the new chimeric hCD20 extra-loops that duplicated with the appropriate linkers (GenBank KM076765). The new chimeric hCD20 protein consists of 135 amino acids which is less than that of native antigen. In this study, a new anti-hCD20 monoclonal antibody, C12H, was developed and characterized based on novel 14KDa chimeric hCD20 exl antigen for diagnostic and therapeutic purposes.

MATERIALS AND METHODS

Human and Animal Right

All protocols of the study were approved by institutional animal ethics committee of Baqiyatallah University of Medical Sciences which follows the NIH guidelines for care and use of animal.

Immunization of Mice

Two female Balb/c mice aged 8 weeks old (Pasture Institute, Tehran, Iran) were immunized five times with Daudi whole cells as described by Fasihi Ramandi et al.[13] One week after the last immunization, blood samples were collected by a vertical incision of the tail vein for determination of antibody titers by enzyme-linked immunosorbent assay (ELISA). The last injection of 20µg of chimeric hCD20 exl without any adjuvant was performed intravenously three days before the cell fusion. Then blood samples of mice were collected and their antibody titers were determined by ELISA using 96 wells ELISA plates coated with the new chimeric hCD20 exl molecule. The sera were stored as reference for the further experiments. The mouse with the highest antibody titer was selected for cell fusion and hybridoma cell generation.

Hybridoma Cell Generation

Briefly, splenocytes from the immunized mouse were mixed with murine myeloma Sp2/0 cell line (Pasture Institute, Tehran, Iran) at a ratio of 1:5 (1 Sp2/0 and 5 spleen cells). Cell fusion and selecting hybridoma cell was performed by a conventional procedure.[14] The reactivity of hybridoma supernatants were determined using 96-well ELISA plates coated with 100µl of phosphate buffered saline (PBS) containing 20µg/ml of chimeric hCD20 exl antigen. Finally, positive hybridoma cells were subcloned 4 times by limiting dilution to select stable hybridomas.[15] Ascetic fluid was collected and purified based on the method described by Brodeur et al.[16]

Antibody Characterization

The isotypes of monoclonal antibodies were determined by isotyping ELISA kit (Sigma). Isostrip isotyping kit (Roche Diagnostics, Mannheim, Germany) was also employed for isotyping confirmation. The produced anti-hCD20 antibody was confirmed by SDS-PAGE following western blot using chimeric hCD20 exl molecule as antigen.[17] The reaction of produced mab was also checked with EPANPSEK (the epitope recognized by Rituximab) pre-coated ELISA microplate. The affinity of antibody was measured based on the ELISA method described by Beatty, et al.[18] In this competition ELISA, a constant

concentration of the specific antichimeric hCD20 exl monoclonal antibody was incubated with different concentrations of antigen in solution. The Optical density (OD) value was read at 450 nm and Kd value was determined by linear regression for different concentrations of antigen.

Flow Cytometry

Daudi and Raji as CD20 positive and Jurkat as CD20 negative cell lines were purchased from Pasteur Institute of Iran. The cells as suspension cultures were grown in DMEM (Gibco), supplemented with 10% fetal bovine serum, plus penicillin (100 units/ml), streptomycin (100 µg/ml), and l-glutamine (2mM). After 48h, the cultured cells were harvested and washed in PBS. Single cell suspensions were treated with optimal concentrations of new anti hCD20 exl monoclonal antibody and incubated on ice for 60 minutes. The cells were washed and incubated with FITC conjugated goat anti-mouse IgG (Sigma) for 60 minutes on ice. The cells were then fixed and examined by FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA).

A competitive binding assay was also performed to evaluate the binding site of the new anti-hCD20 exl mab. The cells were incubated with anti- hCD20 exl mab in the presence or absence of Rituximab (produced in Switzerland by F.Hoffmann-la Roche ltd, Basel). Unbound mabs were removed by three washings of cells in PBS before adding FITC conjugated anti-mouse IgG. Each experiment was done with two sets of cells: (a) cells treated with a mixture of Rituximab and C12H anti-hCD20 exl. (b) Cells treated only with C12H mab. The FITC conjugated goat anti-mouse antibody was added to these cells. After three washings, the cells were analyzed by FACSCalibur flow cytometer. A minimum of 10,000 cells were analyzed in each experiment. Cells with green fluorescence above this background were counted. Flow cytometer data analysis was performed using CellQuest software (BD Cell Quest software).

RESULTS

Hybridoma Cell Generation and Characterization of Antichimeric CD20 Monoclonal Antibody

Analysis results of antibody titer from two immunized mice by ELISA revealed that one of antibodies had the highest antibody titer. Therefore, the spleen cells from this mouse were selected for cell fusion. The screening results indicated several clones producing monoclonal antibodies against chimeric hCD20 exl antigen. C12H clone produced specific IgG2a/k and some clones produced IgM isotype. The C12H showed a very high reactivity with the chimeric hCD20 exl and EPANPSEK peptide in ELISA assay, therefore, in this research we followed up our work based on clone C12H (Figure 1).

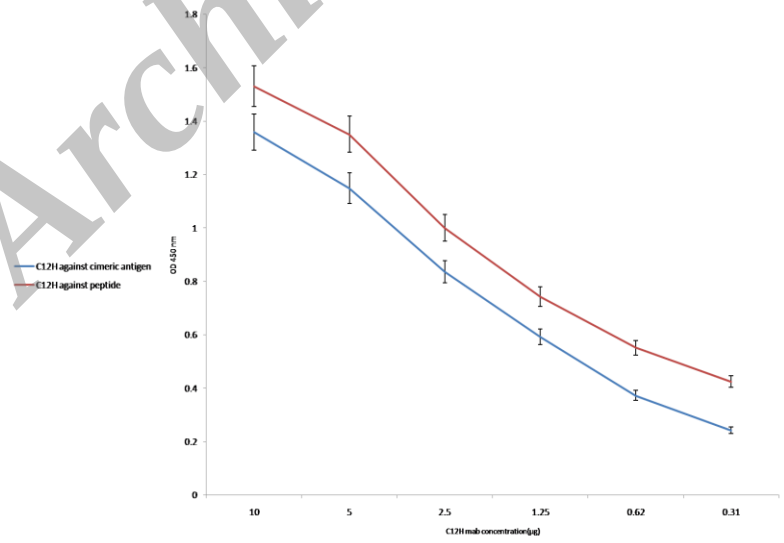


Figure 1. Titration of purified mouse monoclonal antibody against chimeric CD20 antigen and EPANPSEK peptide. A serial dilution of purified concentrated ascitic liquids among 10µg to 310 ng were added to chimeric CD20 antigen (blue) and EPANPSEK peptide (red) pre-coated 96 wells plates and titration of antibody was assayed by ELISA.

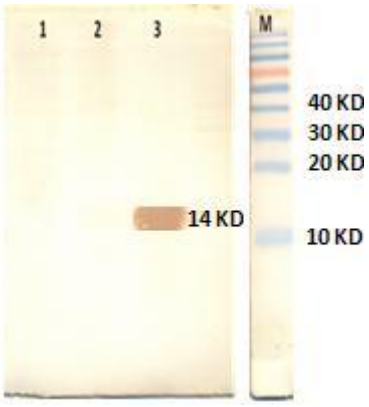


Figure 2. Immunoblot of monoclonal antibody against chimeric CD20 antigen. Lane 1, negative control (pET28a without rCD20 fragment); lane 2, pellet of uninduced bacteria; lane 3, pellet of isopropyl-beta-D-thiogalactopyranoside (IPTG) induced bacteria; lane M, pre-stained protein size marker (kDa). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:4000) and DAB were used.

The purified antibody showed a very high absorbance values in ELISA at concentrations between 10µg and 310 ng with new chimeric hCD20 exl antigen and eight amino acid EPANPSEK peptide (Figure 1). The results of isotype identification of the monoclonal antibody against antigen with isotyping ELISA kit and isostrip kit indicated that the antibody belongs to IgG2a/κ subclass.

The results of immunoblot showed that specific monoclonal antibody has recognized a single band corresponding to 14 KDa molecular weight protein (Figure 2). The ELISA results showed that C12H bind to EPANPSEK epitope. The straight line of slope in linear regression showed a Kd value of 2×10^{-9}M.

Flow Cytometry

The results clearly showed that our antibody reacts with Daudi and Raji (CD20 positive cell line) but not with Jurkat cell lines (CD20 negative cell line). Thus, 99.9% and 99.7% of Daudi and Raji cell lines, respectively, were stained with C12H mab (figure 3b and c), while treating the CD20 negative cell lines (Jurkat) with our mab showed that the cells have not been stained (Figure 3 d).

However, the competetive results revealed that the C12H mab has the ability to compete with Ritoximab for binding to CD20 molecule. Therefore, in the presence of Ritoximab, the number of stained Daudi cell lines has been reduced from 99.9% (figure 3b) to 55.9% (Figure 3e). It also showed that staining of Raji cells was reduced from 99.7% (figure 3c) to 40.5% (figure 3f).

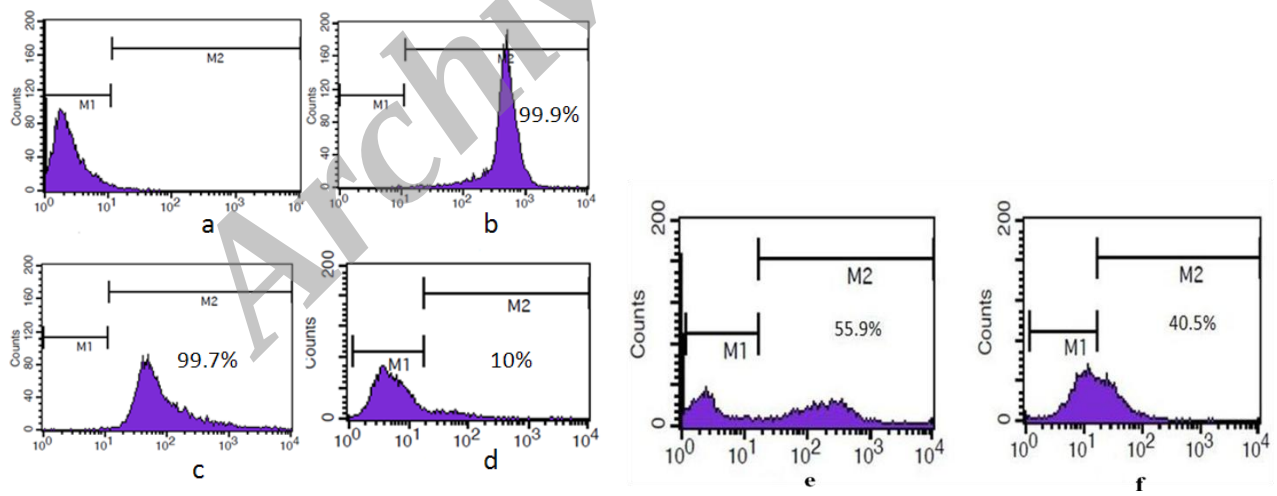
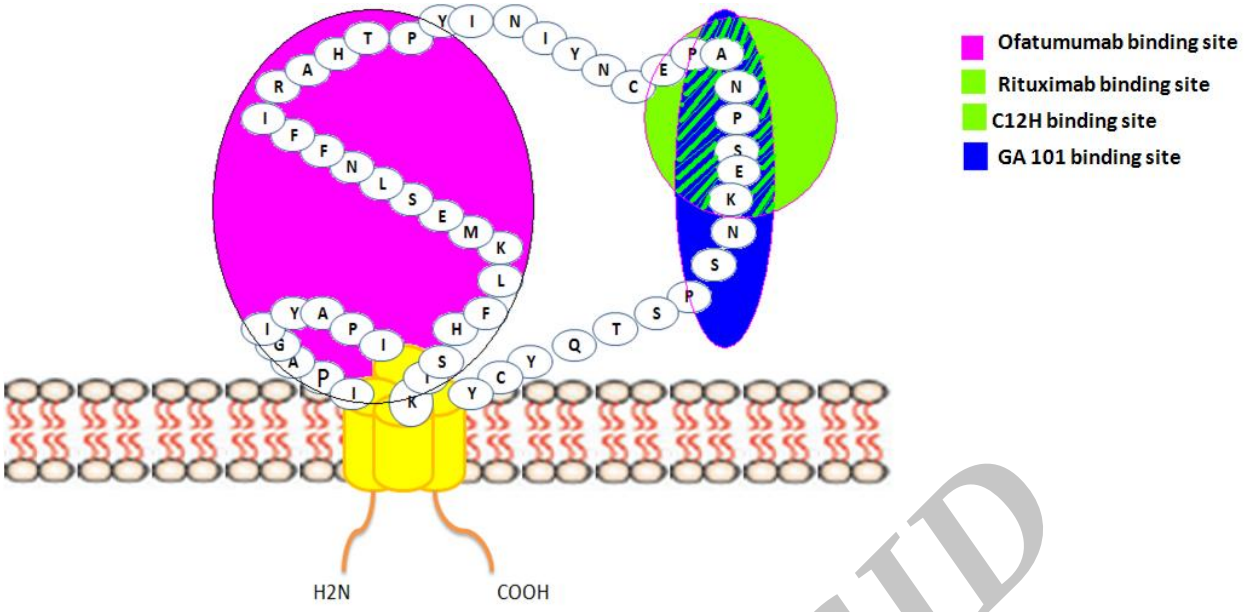


Figure 3. Flow cytometric analysis of Raji and Daudi cells using C12H anti CD20. a: isotype control; lymphocytes treated with goat serum (a), Daudi cells (b), Raji cells (c) and Jurkat cells (d) treated with the C12H mab followed by FITC conjugated goat anti mouse antibody. In the next step, competition of C12H mab and Rituximab for binding to Daudi cells (e) and Raji cells (f) was assayed. Daudi and Raji cells were treated simultaneously with C12H mab antibody and Rituximab. The presence of Rituximab significantly reduced the presence of stained cells compared to the controls.



Figures 4. The structure and topology of CD20 and the epitopes recognized by Rituximab, ofatumumab, GA101(31) and C12H.

DISCUSSION

All currently available anti-hCD20 monoclonal antibodies have been selected and produced against different epitopes of the whole extra loops of hCD20,[19,20] however, in this study, we produced a monoclonal antibody against EPANPSEK sequences on the part of the extra loops of hCD20 instead of its full length. This antigen is a novel chimeric 135 amino acids containing small and large extra loops of CD20 which are repeated twice (GenBank KM076765). In this study, it was confirmed by ELISA that a high level of monoclonal antibody against chimeric hCD20 exl was produced. The chimeric hCD20 exl antigen had appropriate immunogenic properties to stimulate immune system and produce suitable level of antibody. Western-blotting and flow cytometry also confirmed these results and demonstrated that C12H mab has been able to specifically recognize both chimeric CD20 exl peptides and also native hCD20 molecules on B lymphocytes. The high binding activity (figure 3) of C12H mab to Raji and Daudi cells in flow cytometry demonstrated the high affinity of produced mab. This was also confirmed by the measurement of the Kd value by competition ($Kd=2\times10^{-9}M$). These results also indicated the specificity of C12H mab that binds to all CD20 positive B-cells, but not to other cells such as Jurkat. In the competitive flow cytometry, C12H mab was shown to have binding activity similar to commercial anti CD20 mab, suggesting that the relative affinities of the two antibodies were almost equal. Due to the fact that both of these two cell lines (Daudi and Raji) are pure B cells with high expression of CD20 molecule, recognition of most Daudi and Raji cells by C12H mab in flow cytometry was expected. In contrast to this study, other researchers have worked with $CD20^{+}$ cells collected from whole blood instead of cell lines; this may bring about fewer B cells to bind to antibody.[21]

Since our mab is a mouse anti hCD20, yet it is comparable to other mabs. Rituximab, a type I chimeric IgG1 mab, is one of the first FDA approved mabs in the treatment of NHL.[22] However, the resistance to treatment by a minority of patients, indicates that it has not been fully successful.[23,24] Despite the similarity of binding site of the all mabs,[25,26] there is some resistance or recurrence of the disease after current available anti CD20 mab for therapy.[27] Therefore, producing new anti hCD20 antibody by the antibody engineering or other methods is a favorable idea. In this regard, the design and expression of a new chimeric hCD20 exl molecule created an opportunity to develop monoclonal antibody production. Both Rituximab and Obinutuzumab recognize the same CD20 epitope but bind to different sites of the molecule.[28] Nevertheless, Obinutuzumab covers a larger surface area of CD20 antigen that

results in a higher affinity than other mabs.[25,29] Moreover, the ELISA results showed that C12H mab in addition to hCD20 exl antigen with 135 amino acids also react to synthetic EPANPSEC peptide. This synthetic peptide is the epitope that is recognized by mabs such as Rituximab (Figure 4). Due to this fact that C12H mab reacts to both rhCD20 exl and EPANPSEK in ELISA, therefore, it is suggested that our mab may also recognize the same epitope as Rituximab did.

Among a wide spectrum of killing mechanisms which have been suggested for anti CD20 monoclonal antibodies; CDC, ADCC,[9] apoptosis,[30] $CD4^{+}FoxP3^{+}$ regulatory T cells (T regs) via IFN-γ and IL12,[12] are the most important known mechanisms. However, it should be mentioned that, at the same time, one or more mechanisms might be involved. In the case of mouse monoclonal antibody, its Fc is a mouse protein and does not bind to human Fc receptor, therefore ADCC and CDC could not be involved in cell killing. Using mouse monoclonal antibody in human therapy, other mechanisms such as apoptosis can be effective in immunotherapy.[11] Based on the high affinity of C12H mab and its efficacy in recognition of CD20 molecule together with its specific binding to hCD20 antigen, it could be concluded that C12H mab is a suitable choice for production of chimeric mab for any application.

CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

Thanks to Molecular Biology Research Center, Baqiyatallah University of Medical Sciences for supporting us during this project.

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