

**Research Article** 

# Differential Action of Anti-CD20 Monoclonal Antibodies: Role in Induction of Cell Death

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## Abstract

**Purpose:** To study and assess to the sensitivity of Burkitt's lymphoma cells harboring differential levels of cell surface CD20 for anti-CD20 monoclonal Antibodies.

**Material and methods:** Burkitt's lymphoma cell line 'Daudi' was used during present study. Cells were exposed to single dose of  $\gamma$ -radiation (0.5 Gy) and thereafter incubated with rituximab (Rtx) or tositumomab (Tst) (0.5  $\mu$ g/ml concentration each). The changes in expression of ReIA, Akt and Bax/Bcl-2 ratio was measured to assess sensitivity of cells in term of cell death.

**Results:** During the present investigation, we found that cells incubated with anti-CD20 mAbs at +20 hrs post radiation exposure showed higher levels of cell death in term of Bax/Bcl-2 ratio. In addition, we also found the significant changes in expression of pro-survival signaling proteins such as RelA and Akt pathways. Moreover, we found that tositumomab is a potent inducer of apoptotic cell death.

**Conclusion:** These findings suggested that the efficacy of anti-CD20 mAbs depends on the number of CD20 molecules expressed on cell surface and type of antibody used. It may provide new treatment options for selection of anti-CD20 mAbs even in aggressive B-cell lymphoma, which harbors low levels of CD20 or even resistant to current therapies *in vivo*.

Keywords: Ionizing radiation; CD20; ROS; Bax/Bcl-2; Cell death

**Abbreviations:** IR: Ionizing Radiation; BL: Burkitt's Lymphoma; Rtx: Rituximab; Tst: Tositumomab; mAbs: Monoclonal Antibodies

## Introduction

Burkitt's lymphoma (BL) is a highly aggressive form of B-cell associated cancer with the majority of patients younger than 40 years and one-third are at adolescent and young adult age. In modern days chemotherapy protocols achieve overall events free survival rates of nearly 90%; however, they are facing with the challenge of relapse. In addition, the use of anti-CD20 monoclonal antibodies holds great promise for improving long-term curative outcomes while diminishing acute and long-term toxicities. However, in clinical applications, the efficacy of therapy using CD20 mAbs creates resistance, affecting the long-term survival for patients with relapsed or refractory disease. The explanation for this therapeutic resistance is not clear. Although, some investigators reported that decreased CD20 expression and/ or low levels of CD20 on the surface of malignant B-cells is one of the major contributing factors and respond proportionally less well to rituximab and other mAbs [1-5]. CD20, an non-glycosylated transmembrane protein exclusively expressed on B-cells, appears during the pre-B cell stage, however its absent during the earlier or later stages of B-cell differentiation such as antibody-secreting plasma cells. The binding of anti-CD20 mAbs with CD20 cause intracellular oxidative stress, simultaneously inducing activation of members of the Src family of tyrosine kinases, elevation in intracellular Ca2+, phospholipase Cy activation, collectively leading to apoptosis [6-8]. Apoptosis is a tightly regulated process of cell death, which triggered by caspasedependent or independent manner and controlled by the interplay of a number of positive and negative regulatory proteins such as Bax and Bcl-2 family members [9-13]. Moreover, anti-CD20 mAbs have an ability to modulate major pro-survival pathways such as ERK1/2 MAPK, NF-KB and Akt, which directly or indirectly controlled proapoptotic and anti-apoptotic machinery [14-16]. Thus, the changes in CD20 levels on malignant B-cells would be a new treatment option for cancer prevention. In our previous study, we determined that the cells exposed to sub-lethal dose of  $\gamma$ -radiation (0.5 Gy) could induce ~3 ± 0.5-fold changes CD20 levels in Burkitt's lymphoma cell line 'Daudi. It was associated with changes in oxidative condition in the intracellular milieu and determined anti-CD20 mAbs mediated cell death [6,17]. Based on our previous findings, we hypothesized that cells treated with anti-CD20 mAbs just after exposure to a sub-lethal dose of  $\gamma$ -radiation might improve antibody-mediated cell death. During present investigation, we found higher level of induction of cell death in association with differential levels of CD20 expression in term of Bax/ Bcl-2 ratio and simultaneously significant reduce expression of prosurvival pathways such as RelA and Akt, which directly or indirectly control Bax/Bcl-2.

## Materials and Methods

RPMI-1640, Penicillin G potassium salt, streptomycin, N-2-Hydroxyethyl piperazine N-2-Ethane sulphonic acid sodium (HEPES sodium), Dimethyl sulfoxide (DMSO), sodium pyruvate, Bradford reagent, Protease and Phosphatase Inhibitor Cocktail were obtained

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from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (USA). Anti-CD20 chimeric antibodies, such as rituximab (Rtx) were obtained from Genentech (Genentech, Inc., South San Francisco, CA), and tositumomab (Tst) from Corixa (Corixa Corporation Seattle, WA). All other chemical were used of AR grade and obtained from local manufacturers, SRL and Himedia India. Cell culture and biochemical purpose related plastic wares were obtained from BD Bioscience (USA), Corning (USA) and Tarsons, India.

# Cell culture

Daudi cells were obtained from the American type culture collection; (USA) and culture was maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 10 mM HEPES, 50 units/ ml penicillin, 50  $\mu$ g/ml streptomycin and 1% non-essential amino acids at 37°C in a humid atmosphere with 95% air and 5% CO<sub>2</sub>.

# Irradiation and treatment of cells

Exponentially growing cells were exposed to  $\gamma$ -radiation, 0.5 Gy and 1.5 Gy separately using the <sup>60</sup>Co-teletherapy unit (Bhabhatron-II Telecobalt unit; obtained from Bhabha Atomic Research Center, Mumbai, India) at a dose rate of 1.67 Gy/min. Further, cells were incubated with Rtx or Tst (5 µg/ml) at +20 hrs post-radiation exposure. Control groups were treated similarly except for irradiation.

# Protein isolation and estimation

Briefly, cells were collected using centrifugation at 1000 rpm in ice-cold condition and washed with phosphate buffer saline (pH 7.4). The cell palate were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 1% Nonidat-P40, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, protease and phosphatase inhibitor cocktails) using vigorous vortexing as previously described method [6]. Samples were centrifuged at 10,000 rpm at 4°C and carefully supernatant containing soluble proteins were transferred in fresh vials. Protein concentration was measured of each sample by Bradford method [18] using bovine serum albumin (BSA) as a standard. The samples were prepared with 4x SDS sample buffer (1x equals 31.25 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, and 0.005% bromophenol blue) and heated for 5 min at 95°C.

# Western blotting

To determine the changes in expression of Bax/Bcl-2 protein levels and pro-survival signaling pathway's protein (RelA and Akt) were measured as previously described method [6]. Equal amounts of protein (60 µg/sample) from each sample were loaded in 12% SDS poly-acrylamide gel. A standard molecular weight marker (Bio-Rad, USA) was loaded in a separate well parallel to the samples. Initially, 40 volts of current was supplied for the formation of protein stack. Thereafter, it was constant run at 100 volts and proteins were resolved in the denaturating gel. After complete resolution, proteins were transferred from SDS poly-acrylamide gel on to a nitrocellulose membrane. The membranes were blocked in blocking buffer (3% BSA prepared in tris buffer saline with 0.1% Tween-20) for 1 hr at room temperature. Thereafter, membranes were incubated overnight at 4°C with anti-RelA; 1:1500, anti-Akt; 1:1000, anti-Bcl-2; 1:2000 and anti-Bax; 1:1500. After incubation, membrane was washed and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Sigma, St. Louis, MO, USA) for 3 hrs at room temperature. Thereafter, membranes were washed, and protein bands were visualized using enhanced ECL chemiluminescence Pico. β-actin was used to ensure equal loading of protein samples. The intensity of bands was determined using GS-900<sup>™</sup> Calibrated Densitometer software. The results were expressed as fold changes from mean of densitometer data  $\pm$  SD of three independent experiments.

# Data Analysis and Statistical Evaluations

Assays were set up in triplicates and the results were expressed as the mean  $\pm$  SD. The significant changes were analyzed by one-way ANOVA and p values were shown at different levels of significance (p<0.05, p<0.01, p<0.001).

# Results

# Preparation of protein samples

Equal amount of protein samples were used for immune-blotting to determine precise changes in expression of genes or changes in protein levels. The preparation of the sample for western blotting, protein concentration in each sample was measured using Bradford method. The proteins concentration in each sample were determined





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**Figure 2:** Measurement the anti-CD20 mAbs induced changes the expression of ReIA and Akt proteins using western blotting. (A) ReIA is a widely accepted pro-survival signaling protein. The percent changes in ReIA (p65) expression was measured from densitometry mean  $\pm$  SD and statistical analysis was performed using ONE-way ANOVA. Significant values are represented as; #p<0.05 for control vs 1.5 Gy, \$p<0.001 for control vs Rtx or Tst, \*p<0.05 for Rtx vs 0.5 Gy+Rtx, \*p<0.05 for Tst vs0.5 Gy+Tst. (B) Akt is a prominent anti-apoptotic signaling pathway. The percent changes in Akt expression was measured from densitometry mean  $\pm$  SD and statistical analysis was performed using ONE-way ANOVA. Significant values are represented as; #p<0.05 for Rtx vs 0.5 Gy+Rtx, \*p<0.05 for Tst vs0.5 Gy+Tst. (B) Akt is a prominent anti-apoptotic signaling pathway. The percent changes in Akt expression was measured from densitometry mean  $\pm$  SD and statistical analysis was performed using ONE-way ANOVA. Significant values are represented as; #p<0.05 for control vs 0.5 Gy or 1.5 Gy, \$p<0.001 for control vs Rtx or Tst, \*p<0.05 for Rtx vs 0.5 Gy or 1.5 Gy, \$p<0.001 for control vs Rtx or Tst, \*p<0.05 for Rtx vs 1.5Gy+Rtx.





using bovine serum albumin (BSA) standard curve as 1 OD considered equivalent to  $8.5 \ \mu g$  protein.

# Western blotting

Here, we measured the anti-CD20 mAbs induced attenuated expression of pro-apoptotic and anti-apoptotic gene products such as Bax and Bcl-2 respectively in association with changes in CD20 levels and also measured changes in the expression of RelA and Akt levels. As shown in Figure 1A, significant changes in expression of Bax was observed in cells exposed to 0.5 Gy or 1.5 Gy and in cells treated either Rtx or Tst with or without radiation. The expression of Bax in control sample was considered as zero percent change in expression (Figure 1A). The up-regulation of Bax expression was observed 14  $\pm$  2.2 percent in cells exposed with 0.5 Gy and 29  $\pm$  2.4 percent in 1.5 Gy irradiated cells with respect to sham-irradiated control. In addition,

cell incubated with anti-CD20 mAbs, Bax expression was also found to be significantly higher, 58 ± 3.5 percent changes in Bax expression was determined in Rtx treated cells and 70  $\pm$  3.3 percent changes in Tst treated cells. Moreover, the significant changes Bax expression was also observed in cells treated with Rtx or Tst at +20 hrs post radiation exposure (0.5 Gy, 1.5 Gy). Approximately 64 ± 1.5 percent was observed in the 0.5 Gy+Rtx group and 82 ± 3.7 percent in 1.5 Gy+Rtx treated groups as compared to Rtx treated alone group. Moreover, we also observed 81  $\pm$  2.3 percent changes in 0.5 Gy+Tst and 92  $\pm$  3.7 percent in 1.5 Gy+Tst treated groups as compared to Tst treated alone group. Besides measuring the up-regulation of Bax expression, the down-regulation of Bcl-2 expression was also observed. The changes in Bcl-2 expression were found to be significantly higher as dosedependent manner and also in the treatment of cells with anti-CD20 mAbs alone or in a combination with anti-CD20 mAbs + IR (Figure 1B). The cells were exposed to 0.5 Gy or 1.5 Gy, the changes in Bcl-2 expression was found to be as  $14 \pm 4.1$  percent and  $29 \pm 2.5$  percent with respect to sham-irradiated control respectively. In addition, cells treated with Rtx or Tst alone, Bcl-2 expression was found to be 58  $\pm$ 3.3 percent changes in Rtx and 70  $\pm$  4.2 percent changes in Tst treated group with respect to untreated control. Moreover, cells treated with Rtx at 20 hrs post radiation exposure (0.5 Gy or 1.5 Gy) were also found to be significant as  $64 \pm 2.4$  percent changes in 0.5 Gy+Rtx and  $82 \pm 3.8$  percent changes in the 1.5 Gy+Rtx group as compared to Rtx treated alone. Cells treated with Tst at +20 hrs post 0.5 Gy or 1.5 Gy radiation exposure also showed 81 ± 3.1 percent changes in 0.5 Gy+Tst treated group and 92 ± 2.8 percent changes in 1.5 Gy+Tst treated group as compared to Tst treated alone group. We, further investigated Rtx or Tst mediated activation of pro-survival signaling pathways that regulate these gene products. During present study we found that Rtx and Tst have ability to down-regulate RelA (NF-kB family's protein) and Akt pro-survival signaling pathways which participate in regulation of Bax and Bcl-2 expression (Figures 2A and 2B). During present investigation the changes in expression of RelA (NF-kB family's protein) were determined followed by Rtx and Tst treatment or in combination with IR (Figure 2A). Our observation revealed that cells treated with Rtx and Tst separately showed significant changes in expression of RelA such as  $65 \pm 3.3$  percent changes in Rtx and  $58 \pm 4.1$ percent changes in Tst treated cells with respect to un-treated control. Interestingly, cells treated with Rtx or Tst at +20 hrs post radiation exposure (0.5 Gy or 1.5 Gy) showed 83  $\pm$  5.4 percent changes in 0.5

Gy+Rtx and 77  $\pm$  3.1 percent changes in 0.5 Gy+Tst treated group as compared to control. Moreover, cells treated with Rtx or Tst at +20 hrs post 1.5 Gy radiation exposure showed 92  $\pm$  3.8 percent and 80  $\pm$  4.9 percent changes in RelA expression respectively. Beside the changes in expression of RelA, the changes in expression of Akt were also found to be significant with respect to untreated control (Figure 2B). As shown in Figure 2B, cells exposed to 0.5 Gy or 1.5 Gy showed  $40 \pm 4.1$  percent and  $55 \pm 3.5$  percent changes in Akt expression with respect to control. Interestingly, Cells treated with Rtx at +20 hrs post radiation exposure (0.5 Gy or 1.5 Gy) showed  $81 \pm 4.4$  percent changes in 0.5 Gy+Rtx and  $92 \pm 3.8$  percent changes in 1.5 Gy+Rtx treated group with respect to untreated control. Moreover, Cells treated with Tst at +20 hrs post 0.5 Gy or 1.5 Gy radiation exposure showed 85  $\pm$  3.1 percent and 90  $\pm$ 3.9 percent changes in Akt expression respectively. In addition, cells treated with Rtx or Tst alone showed 75  $\pm$  3.3 percent changes and 78  $\pm$ 3.9 percent changes respectively.

# Discussion

Modulation in levels of CD20 on the cell surface and antibody engineering are current trends to enhance the efficacy of cancer therapy [3,19]. In our previous study, we investigated radiation-induced changes of CD20 surface levels which may play a crucial and central role in determining the relative efficacy of Rtx and Tst mAbs in treating Burkitt's lymphoma disease. We have examined the y-radiationinduced modulation of CD20 levels on the cell surface of the Burkitt's lymphoma cell line 'Daudi' [6]. Cells exposed with a sub-lethal dose of y-radiation (0.5 Gy) showed significant increas in CD20 expression as time-dependent manner and it was associated with changes in redox status in intracellular milieu [6,20]. In correlation to previous findings, here we determined the relative efficacy of anti-CD20 mAbs at differential levels of CD20 expression, Cells treated with Rtx and Tst mAbs, at +20 hrs post radiation exposure showed significant induced cell death in combination with radiation and mAbs as compared to antibodies treated alone. Interestingly, Tst was found to be a potent inducer of cell death as compared to Rtx.

During present investigations, we studied the changes in expression of the pro-apoptotic member such as Bax and the antiapoptotic member such as Bcl-2 induced by anti-CD20 mAbs mediated at differential levels of CD20 (Figures 1A and 1B). The members of the Bcl-2 family are a group of crucial regulatory factors in apoptosis. The permeabilization of mitochondrial outer membrane is regulated by Bcl-2 family proteins and it can bind selectively to the active conformation of Bax to prevent it from inserting into the mitochondrial outer membrane to maintain the normal permeability of membrane permeable transition pores and prevent the release of mitochondrial pro-apoptotic factors, such as cytochrome c, AIF and Smac/DIABLO [21-24]. In our previous study, we found significant changes in p38 and p53 activation in combination of IR+ mAbs and it was associated with DNA strand breaks [6]. In association with activation of p38 and p53, Here, we found significant reduce in the expression of Bcl-2 and up-regulation of Bax expression in radiation alone or in combinations (IR+mAbs) as compared to un-irradiated or untreated control (Figures 1A and 1B). Interestingly, Bax/Bcl-2 ratio was found to be higher (\*\*p<0.01) in cells treated at +20 hrs post 1.5 Gy radiation exposure as compared to antibodies treated alone. In our previous study, we were found significant activation of caspase-3 and higher levels of cleaved PARP in association with deferential levels of CD20 expression [6].

In addition, anti-CD20 mAbs also had the ability to change expression of major pro-survival pathways such as ERK1/2 MAPK, NF- $\kappa$ B and Akt [14,15]. These directly or indirectly play important

role in regulation of pro-apoptotic and anti-apoptotic machinery. Thus, we also investigated whether Rtx or Tst mediated changes the expression of RelA and Akt expression. Cells treated with both Rtx and Tst separately showed significant changes (p<0.001) in the expression of two major cell survival pathways *viz* RelA and Akt (Figures 2A and 2B). Moreover, cells treated with Rtx or Tst at +20 hrs post 0.5 Gy or 1.5 Gy radiation exposure showed significant changes (p<0.05) in the expression of both RelA and Akt level, which participate in down-regulation of Bcl-2 in Burkitt's lymphoma cell line 'Daudi'.

Our above observations revealed that treatment of cells with either Rtx or Tst at differential levels of CD20 on cell surface, preferentially reduce the expression of the anti-apoptotic gene product Bcl-2 and beside up-regulate the expression of pro-apoptotic gene product Bax. Simultaneously, we found anti-CD20 mAbs mediated changes in expression of pro-survival signaling protein RelA and Akt, which directly or indirectly controlled Bax/Bcl-2 ratio as shown in schematic Figure 3.

# Conclusion

Present studies suggested that the efficacy of anti-CD20 mAbs is associated with CD20 levels on the cell surface. In conclusion, this report provides evidence that CD20 expression can be induced using low dose  $\gamma$ -radiation and improves antibody-mediated cell death during cancer immunotherapy. Therefore, use of low dose of radiation just prior to immunotherapy may be beneficial for the eradication of B-cell malignancy. These results may be useful to establish a theoretical basis to improve the efficacy of immunotherapy/radio-immunotherapy in case of cells expressing low surface levels of CD20.

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### Author Contributions

Conceived and designed the experiments: Vijay Singh and Damodar Gupta. Performed the experiments: Vijay Singh. Analyzed the data: Vijay Singh, Damodar Gupta. Contributed reagents/materials/analysis tools: Damodar Gupta, Wrote the paper: Vijay Singh and Damodar Gupta.

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