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Original Research Article

Quantitative Monitoring of Rituximab Therapy in Autoimmune Diseases in a Tertiary Care Hospital, Kolkata, Eastern India

Abir Pattanayak[1], Mitali Chatterjee[2], Parasar Ghosh[3], Sukanta Sen[4]

[1]Assistant Professor, Department of Pharmacology, ICARE Institute of Medical Sciences & Research, Haldia, Purba Medinipur, West Bengal, India

[2]Ex-Professor & HOD, Department of Pharmacology, Institute of Postgraduate Medical Education & Research, 244, A. J. C. Bose Road, Kolkata, West Bengal

[3]Professor & HOD, Department of Rheumatology, Institute of Postgraduate Medical Education & Research, 244, A. J. C. Bose Road, Kolkata, West Bengal

[4]Professor and HOD, Department of Pharmacology, ICARE Institute of Medical Sciences & Research, Haldia, Purba Medinipur, West Bengal, India

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Corresponding author: Dr. Abir Pattanayak

Conflict of interest: Nil

Abstract

Background: There is qualitative monitoring of the effectiveness of rituximab therapy in autoimmune diseases by measuring IgG, IgM and IgA. Due to limited resources, measurement of B cell count is difficult. Thus, the study will provide a comparison between the quantitative and qualitative monitoring which may help to prevent immunosuppression due to excessive treatment with rituximab.

Materials and Methods: Patients with autoimmune diseases admitted in Rheumatology department of IPGMER and SSKM Hospital, Kolkata, receiving Rituximab (RTx) therapy were included as study population (n=48). The B cell count was performed in the Flow Cytometer of the Institute's 'Multidisciplinary Research Unit' and the serum IgG, IgM and IgA was measured in the Dept. of Rheumatology of peripheral venous blood drawn from antecubital vein. Level of immunoglobulins (IgG, IgA, and IgM) were measured in the Department of Rheumatology by nephelometry and B cell count (CD19% & CD20%) was done by flow cytometry in the Department of Pharmacology.

Results: An overwhelming majority of patients who had either normal or depleted %CD19 had normal levels of serum IgG. This result reinforces the fact that mature long-lived antibody-secreting plasma cells are apparently not depleted by RTX.

Conclusion: The purpose of our study was to compare CD19% and CD20% with serum Ig levels in patients receiving Rituximab in order to assess whether clinicians can use serum Ig levels as predictors for long term immunosuppression, thereby allowing more tailored monitoring of such patients especially when receiving long-term treatment with RTX. An overwhelming majority of patients who had either normal or depleted %CD19 had normal levels of serum IgG. This result reinforces the fact that mature long-lived antibody-secreting plasma cells are apparently not depleted by RTX.

Keywords: Autoimmune Diseases, Monoclonal Antibody, B Cell Marker, Rituximab, Flow Cytometry, Serum IgG, IgM, IgA, B cell count, CD19, CD20, Immunophenotyping.

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Introduction

Rituximab, a mouse–human chimeric monoclonal antibody targeting the B cell marker CD20, was initially approved in 1997 by the United States Food and Drug Administration (FDA) for the treatment of relapsed or refractory, low-grade or follicular, CD20 antigen-positive, B-cell non-Hodgkin's lymphoma (NHL). Central drug standard control organizations (CDSCO) approved Rituximab in India in 2000 as an antineoplastic agent. Current studies have shown that B cells play a role in autoimmunity and disease expression through several functions. These include autoantibody production, cytokine secretion, antigen presentation, and co-stimulatory effect. Thus, therapies targeting B cells could be utilized for treating connective tissue disorders [1, 2]. Rituximab is also FDA-approved and CDSCO approved (2012) for rheumatoid arthritis and vasculitis, such as granulomatosis with polyangiitis and microscopic polyangiitis. Owing to the well-

established role of B-lymphocytes in the pathogenesis of Sjogren Syndrome (SS), growing evidence suggests that B-cell depletion by rituximab (RTX) is also effective in SS. Of interest besides clinical efficacy, RTX also showed biologic effects, consistently affecting the inflammation and the lymphoid organization that occur in target tissues [3].

Systemic lupus erythematosus is a multisystem autoimmune disease characterized by the formation of autoantibodies that target a variety of self-antigens. B cells are fundamental to the development of these antibodies and are a target for intervention in the disease [4]. Rituximab therapy targets B cells by inducing B-cell depletion, reduction in B-cell proliferation and differentiation, or modulation of B-cell function.

Rituximab can be an effective treatment for non-renal lupus and lupus nephritis [5]. The treatment of systemic sclerosis (SSc) represents a great clinical challenge because of the complex disease pathogenesis including vascular, fibrotic, and immune T- and B-lymphocyte-mediated alterations. Rituximab (RTX) may down regulate the B-cell over expression and correlated with immunological abnormalities [6, 7].

Rituximab has been used off-label in the treatment of numerous autoimmune diseases Viz. undifferentiated connective tissue disease (systemic autoimmune disease), Sjogren syndrome (SS with serositis), systemic lupus erythematosus (SLE), systemic sclerosis, dermatomyositis, systemic sclerosis with interstitial lung disease, systemic lupus nephritis [8]. While we know that rituximab works by removing B lymphocytes from the circulation, exactly how this leads to clinical improvement in many of the conditions that rituximab is used to treat is still to be determined. Although the loss of B cells from the circulation is transient (usually for about six months), the duration of depletion can be highly variable among individuals.

Currently, rituximab therapy is quantitatively monitored in tertiary care hospitals by B cell count by flow cytometry. In spite of being an expensive procedure with high sensitivity and specificity, the Flow cytometric analysis fails to adequately account for this adverse effect [9]. On the other hand, analysis of serum immunoglobulins can act as markers for both the prognosis of autoimmune diseases and the associated side effects of rituximab therapy like immunosuppression, thereby saving costly interventions to manage any post-therapeutic infections. Therefore, instead of going for the costly B-cell count by Flow Cytometry, Ig analysis (serum IgG, IgM, IgA) by nephelometry can pave the way for a better cost-effective monitoring of the progression of autoimmune diseases [10] while sparing the patients from the immunosuppressive actions of the drug in developing countries like India.

Presently, there is qualitative monitoring of the effectiveness of rituximab therapy in autoimmune diseases by measuring IgG, IgM and IgA. Due to limited resources, measurement of B cell count is difficult. Thus, the study will provide a comparison between the quantitative and qualitative monitoring which may help to prevent immunosuppression due to excessive treatment with rituximab.

Materials and Methods

Study Design: The study was a prospective, observational study.

Study Setting: All patients admitted in the Rheumatology department of IPGME&R, SSKM Hospital, Kolkata, eligible for rituximab therapy were enrolled for the study as per the eligibility criteria. Level of immunoglobulins (IgG, IgA, and IgM) were measured in the Department of Rheumatology by nephelometry and B cell count (CD19% & CD20%) was done by flow cytometry in the Department of Pharmacology.

Chart 1:

Study population: Potential study subjects were screened as per the following criteria:

Inclusion criteria

- Autoimmune disease patients who were current candidates for rituximab therapy.
- Willing to provide written informed consent.

Exclusion criteria

- Patients of HIV, Hepatitis B, Hepatitis C or current infection
- Pregnant mothers
- Heart failure patients (New York Heart Association class IV)
- Inadequate past medical record data availability.

Study Duration: The study was conducted for a period of eighteen months from December 2017 to May 2019 during which information about clinical, haematological and B cell profile was collected in a predesigned case report form.

Sample Size: Since the study was planned as a time bound observational study, no formal sample size calculation has been undertaken. We recruited 48 subjects with 4 overlaps during this time period.

Ethics: The study commenced after receiving approval from IPGMER Ethics Committee (Memo No. Inst/IEC/2018/106). Written informed consent was obtained from all subjects prior to inclusion into the study abiding by the Declaration of Helsinki and ICMR Ethical Guideline for Biomedical Research on human subjects.

Confidentiality was maintained throughout the study and the identity of the subjects was not revealed under any circumstances. All aspects of privacy and confidentiality were complied with.

Laboratory: The B cell count was performed in the Flow Cytometer of the Institute's 'Multidisciplinary Research Unit' and the serum IgG, IgM and IgA was measured in the Dept. of Rheumatology of peripheral venous blood drawn from antecubital vein through aseptic procedure (5ml of blood was drawn from antecubital vein in heparinised/EDTA vial).

Study Technique: Biological Sample Collection: From each enrolled subject 5 ml of venous blood was drawn aseptically in anticlot vial. 2.5 ml for immunoglobulin count and 2.5 ml for B cell count.

Parameters evaluated: Age and sex; history and general physical examination; dose of rituximab and other treatment received; B cell count (CD 19% & CD 20%) and serum immunoglobulins level (IgG, IgA, IgM)

List of drugs and chemicals used: Rituximab: Two 1000/500 mg intravenous infusions separated by two weeks (one course) followed by maintenance dose of 1000/500 mg every 6 monthly is indicated.

Chemicals: CD20-FITC (fluorescein isothiocyanate) antibody cocktail, CD19-APC (allophycocyanin) antibody cocktail, 1X BD FACS lysing solution, Phosphate buffered saline (PBS, 1X)

Immunophenotyping: Peripheral blood (100 µl) collected in EDTA was stained with monoclonal anti-human CD20-FITC/CD19-APC antibody cocktail and incubated at room temperature for 30 minutes in dark; 1X BD FACS lysing solution (2 ml) was added and incubated in the dark for 15 minutes at room temperature. Cells were be centrifuged (1800rpm X 5 min), the cell pellet resuspended in 1X PBS (1 ml) and centrifuged at 1800 rpm for 5 min; supernatant is discarded and 400 ml 1X PBS is added; then analysed on a flow cytometer (FACS Verse, BD Biosciences, San Jose, CA, USA). Lymphocytes were gated on their characteristic forward versus side scatter and 20,000 events were acquired per tube and subsequent gating was done based on the surface markers. The frequency of different B-cell subsets was analysed using the FAC Suite software (BD Biosciences, San Jose, CA, USA) [11].

Results: Patients with autoimmune diseases admitted in Rheumatology department of Institute of Post Graduate Medical Education and Research and SSKM Hospital, Kolkata, receiving Rituximab (RTx) therapy were included as study population (n=48).

Table 1: The different diseases among participants were as follows

Autoimmune Diseases	No. of Subjects (%)
SLE with lupus nephritis	11(22.92)
Diffuse Systemic sclerosis with/without interstitial lung disease	18(37.5)
Rheumatoid arthritis	4((8.33)
Granulomatosis polyangiitis (Wegener's granulomatosis)	4(8.33)
Mixed Connective Tissue Disease (MCTD)	4(8.33)
Dermatomyositis	2(4.17)
Sjogren Syndrome with/without serositis	2(4.17)
Polyarticular juvenile idiopathic arthritis	1((2.08)
Neuromelitis optica	1(2.08)
RPGN (IgA nephropathy)	1(2.08)

They were sub grouped as follows-

- Before 1st dose of rituximab (pre D1)- 18 subjects
- Before 2nd dose of rituximab (pre D2)- 16 subjects (2 overlap)
- Before 3rd dose of rituximab (pre D3)- 10 subjects
- Before 4th dose of rituximab (pre D4)- 2 subjects (1 overlap)
- Before 5th dose of rituximab (pre D5)- 5 subjects
- Before 6th dose of rituximab (pre D6)- 1 subject (1 overlap)

Subject Code	Pre D1 CD19%	Pre D1 CD20%	pre D1 IgG(mg/dl)	pre D1 IgA(mg/dl)	pre D1 IgM(mg/dl)
R01	22	21.9	1390	286	130
R02	8.28	7.89	1020	182	196
R13	**1.95**	**2.05**	**1120**	**576**	**152**
R15	**5.61**	**5.19**	**1790**	**360**	**131**
R17	**4.12**	**3.89**	**1670**	**211**	**65.8**
R18	**5.62**	**5.09**	**206**	**84.3**	**<17.3**
R22	**4.16**	**4.57**	**1870**	**447**	**155**
R25	44.75	44.33	1660	181	115
R30	34.23	33.12	1670	264	132
R33	37.25	34.21	2100	362	236
R34	6.15	**5.89**	1580	152	158.2
R38	29.31	25.3	1740	318	216
R39	29.16	28.23	1400	221	141
R52	16.47	19.14	1320	203	89
R54	14.93	15.97	1650	218	140
R60	11.59	13.21	2640	267	93.2
R65	**2.16**	**2.84**	**1070**	**147**	**53.1**
R66	**3.68**	**4.1**	**242**	**186**	**38.4**

Table 2: Levels of CD19/20 and immunoglobulin prior to 1st dose of rituximab therapy (pre D1)

	CD19 (%)	CD20 (%)	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)
Mean ± SEM (%)	15.63 ± 3.24	15.38 ± 3.09	1452.11 ± 138.63	259.18 ± 28.08	125.50 ± 13.90
SD	13.74	13.06	588.14	119.15	58.98
Range (%)					
(i) <6	7/18	8/18	<700=2/18	<70=0/18	<50=2/18
(ii) 6-23	6/18	5/18	700-1700=11/18	70-350=14/18	50-300=16/18
(iii) >23	5/18	5/18	>1700=5/18	>350=4/18	>300=0/18

Before starting rituximab, blood was provided for measuring CD19/20 and IgG/IgA/IgM levels. Of the 18 patients, the mean ± SEM of CD19% (whose normal range 6-23%) was 15.63 ± 3.24. This included 7/18 patients who had a CD19 count <6, mean ± SEM= 3.9 ± 0.55; 6/18 patients who had a count 6-23, mean ±SEM =13.24 ± 2.36 and 5/18 patients who had a count >23, mean ± SEM=34.94 ± 2.89. For CD20% (normal range being 6-23%), in the 18 patients the mean ± SEM was 15.38 ± 3.09. This included 8/18 patients who had a CD20 count<6, mean ± SEM of 4.20 ± 0.45; 5/18 patients who had a CD20 count 6-23, mean ±SEM= 15.62 ± 2.42; 5/18 patients who had a count >23, mean ± SEM=33.04 ± 3.25 (Figure 1). For IgG level (normal range being 700-1700 mg/dl), of the 18 patients the mean ± SEM was 1452.11 ± 138.63. This included 2/18 patients who had a level <700, mean ± SEM=224 ± 18; 11/18 patients who had a level 700-1700, mean ± SEM= 1413.64 ± 76.54; 5/18 patients who had a level >1700, mean ± SEM=2028 ± 164.97. For IgA level (normal range being 70-350 mg/dl), of the 18 patients the mean ± SEM was 259.18 ± 28.08. This included 0/18 patients who had a level <70; 14/18 patients who had a level 70-350, mean ± SEM=208.59 ± 16.43; 4/18 patients who had a level >350, mean ±SEM=436.25 ± 50.80. For IgM level (normal range being 50-300 mg/dl), of the 18 patients the mean ± SEM was 125.50 ± 13.90. This included 2/18 patients who had a level <50, mean ±SEM=27.85 ± 10.55; 16/18 patients who had a level 50-300, mean ± SEM=137.71 ± 12.49 and no patient had a level >300 (Figure 1).

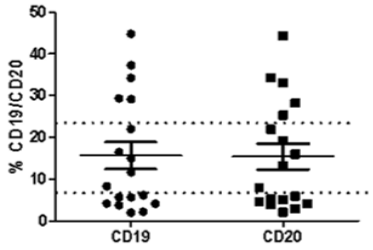

Figure 1A:

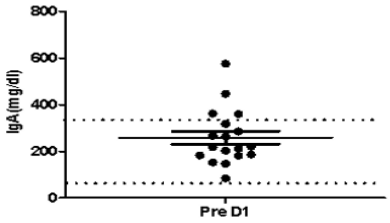

Figure 1B:

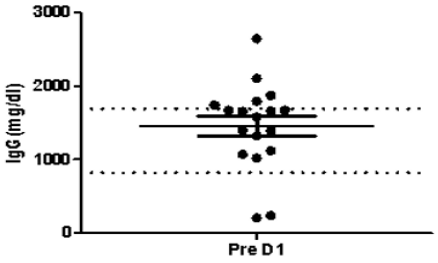

Figure 1C:

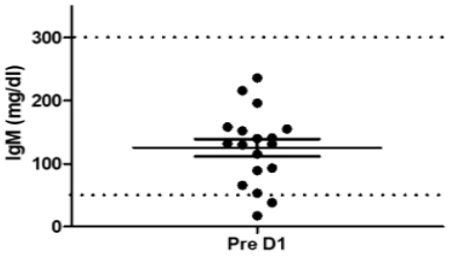

Figure 1D:

Figure 1: Scatter plots showing the %CD19 and %CD20 status prior to initiating treatment (Pre D1, n = 18). A: %CD19 and CD20; B: IgG; C: IgA and D: IgM levels

Subject Code	Pre D2 CD19 %	Pre D2 CD20%	pre D2 IgG(mg/dl)	pre D2 IgA(mg/dl)	pre D2 IgM(mg/dl)
R13A	**1.29**	**1.46**	**1030**	**63.4**	**145**
R52A	0.18	0.14	1200	286	73.2
R09	**4.82**	**1.06**	**1260**	**199**	**161**
R19	**2.16**	**2.01**	**1610**	**194**	**103**
R20	**4.05**	**3.15**	**1380**	**330**	**158**
R24	**1.54**	**1.21**	**1710**	**402**	**194**
R29	9.6	9.16	1286	172	165
R36	**2.91**	**2.65**	**525**	**70.4**	**21.6**
R42	**1.76**	**1.48**	**1029**	**190**	**209**
R43	37.28	36.19	1870	231	187
R45	**5.52**	**5.16**	**1320**	**202**	**104**
R46	**2.37**	**2.14**	**978**	**106**	**131**
R49	25.69	22.6	174	100	319
R55	14.8	15.44	1240	168	35.5
R57	7.7	7.67	1758	179	101
R58	**0.2**	**0.18**	**1620**	**329**	**143**

The horizontal bars represent mean ± SEM and the dotted line represent the normal range.

Table 3: Level of CD19/20 and immunoglobulin prior to 2nd dose of Rituximab therapy (pre D2)

Mean±SEM	7.62±2.57	6.98±2.48	1249.38±111.82	201.36±24.10	140.64±17.91
SD	10.29	9.93	447.29	96.39	71.65
Range (%)					
(i)<6	11/16	11/16	<700=2/16	<70=1/16	<50=2/16
(ii)6-23	3/16	4/16	700-1700=11/16	70-350=14/16	50-300=13/16
(i) >23	2/16	1/16	>1700=3/16	>350=1/16	>300=1/16

After the 1st dose of Rituximab, blood was provided for measuring CD19/20 and IgG/IgA/IgM levels. Of the 16 patients the CD19% (normal range 6-23%) mean ± SEM was 7.62±2.57. This included 11/16 patients who had a CD19 count <6, mean ± SEM= 2.44 ± 0.53; 3/16 patients who had a count 6-23, mean ± SEM =10.7 ± 2.12; 2/16 patients who had a count >23, mean ± SEM=31.49 ± 5.80. For CD20% (normal range being 6-23%), in the 16 patients the mean ± SEM was 6.98 ± 2.48. This included 11/16 patients who had a CD20 count<6, mean ± SEM= 1.88 ± 0.43; 4/16 patients who had a

CD20 count 6-23, mean ±SEM = 13.72 ± 3.40; 1/16 patient had a count >23, 36.19 (Figure 2). For IgG level (normal range being 700-1700mg/dl), of the 16 patients the mean ±SEM was 1249.38 ± 111.82.

This included 2/16 patients who had a level <700, mean ± SEM=349.50 ± 175.50; 11/16 patients who had a level 700-1700, mean ± SEM= 1268.45 ± 64.65; 3/16 patients who had a level >1700, mean ± SEM=1779.33 ± 47.40. For IgA level (normal range being 70-350 mg/dl), of the 16 patients the mean ± SEM was 201.36±24.10. This included 1 patient who had a level <70, =63.40; 14/16 patients who had a level 70-350, mean ± SEM=196.89 ± 20.94; 1/16 patient had a level >350 which was 402. For IgM level (normal range being 50-300 mg/dl), of the 16 patients the mean ± SEM was 140.64±17.91.

This included 2/16 patients who had a level <50, mean ± SEM=28.55 ± 6.95; 13/16 patients who had a level 50-300, mean ± SEM=144.17±11.27; 1/16 patient had a level >300 which was 319 (Figure 2).

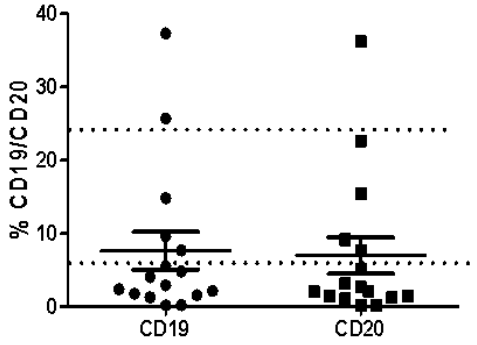

Fig-2A

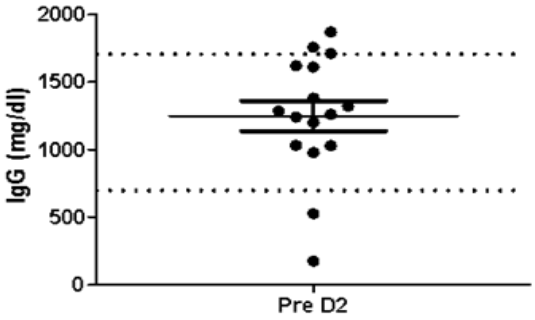

Fig. 2B

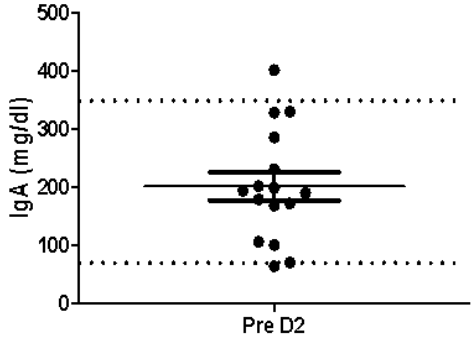

Fig- 2C

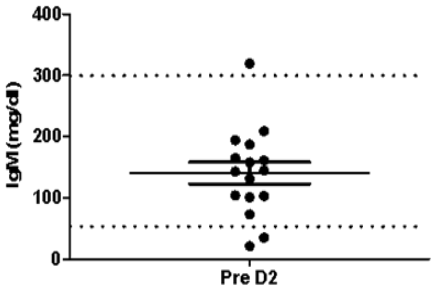

Fig-2D

Figure 2: Scatter plots showing the %CD19 and %CD20 status prior to initiating treatment (Pre D2, n = 16). A: %CD19 & CD20; B: IgG; C: IgA and D: IgM levels

Subject Code	pre D3 CD19%	pre D3 CD 20%	pre D3 IgG(mg/dl)	pre D3 IgA(mg/dl)	pre D3 IgM(mg/dl)
R03	9.02	9.21	2120	381	285
R06	6.9	6.81	1720	292	140
R08	**4.12**	**4.26**	**1240**	**209**	**190**
R10	**3.19**	**3.18**	**1130**	**180**	**180**
R23	**2.01**	**1.94**	**1870**	**202**	**186**
R26	31.24	30.24	1240	210	186
R35	25.19	23.98	1340	371	294
R50	29.19	28.2	1280	188	108
R64	14.78	15.4	1770	431	81.5
BN1	**0.69**	**0.73**	**437**	**110**	**31.5**

The horizontal bars represent mean ± SEM and the dotted line represent the normal range.

Table 4: Level of CD19/20 and immunoglobulin prior to 3rd dose of rituximab therapy (pre D3)

Mean±SEM	7.96±3.72	8.01±3.57	1414.70±150.77	257.40±33.28	168.20±26.11
SD	11.76	11.30	476.78	105.26	82.56
Range (%)					
(i) <6	4/10	4/10	<700=1/10	<70=0/10	<50=1/10
(ii) 6-23	3/10	3/10	700-1700=5/10	70-350=7/10	50-300=9/10
(iii) >23	3/10	3/10	>1700=4/10	>350=3/10	>300=0/10

After 2nd dose of Rituximab, blood was provided for measuring CD19/20 and IgG/IgA/IgM levels. Of the 10 patients the CD19% (normal range 6-23%) mean ± SEM was 7.96 ± 3.72. This included 4/10 patients who had a CD19 count <6, mean ± SEM= 2.50±0.74; 3/10 patients who had a count 6-23, mean ± SEM =10.23 ± 2.35; 3/10 patients who had a count >23, mean ± SEM=28.54 ± 1.78.

For CD20% (normal range being 6-23%), in the 10 patients the mean ± SEM was 8.01 ± 3.57.

This included 4/10 patients who had a CD20 count<6, mean±SEM= 2.53 ± 0.76; 3/10 patients who had a CD20 count 6-23, mean ± SEM= 10.47 ± 2.56; 3/10 patients who had a count >23, mean ± SEM=27.47 ± 1.84. For IgG level (normal range being 700-1700mg/dl), of the 10 patients the mean ±SEM was 1414.70 ± 150.77. This included 1/10 patients who had a level <700 =437; 5/10 patients who had a level 700-1700, mean ± SEM=1246 ± 34.29; 4/10 patients who had a level >1700, mean±SEM=1870 ± 88.98. For IgA level (normal range being 70-350 mg/dl), of the 10 patients the mean ± SEM was 257.40 ± 33.28.This included no patient who had a level <70; 7/10 patients who had a level 70-350, mean ± SEM=198.71 ± 20.28; 3/10 patients who had a level >350, mean ± SEM=394.33 ± 18.56. For IgM level (normal range being 50-300 mg/dl), of the 10 patients the mean±SEM was 168.20 ± 26.11. This included 1 patient who had a level <50, 31.50±0.00; 9/10 patients who had a level 50-300, mean ± SEM=183.39±23.74; no patient had a level >300 (Figure 3).

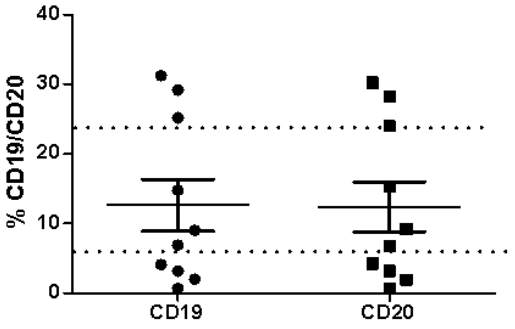

Fig 3A

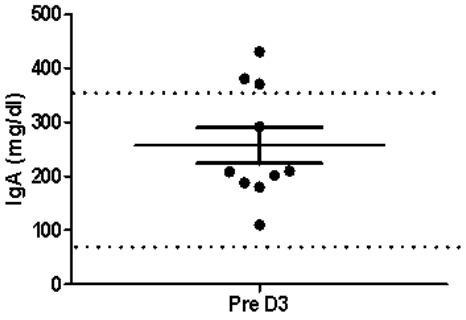

Fig 3B

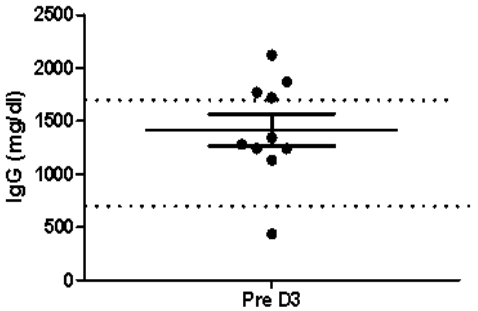

Fig 3C

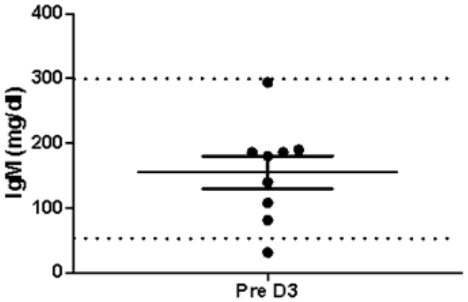

Fig 3D

Figure 3: Scatter plots showing the %CD19 and %CD20 status prior to initiating treatment (Pre D2, n = 10). A: %CD19 & CD20; B: IgG; C: IgA and D: IgM levels

Subject Code	pre D4 CD 19%	pre D4 CD 20%	pre D4 IgG(mg/dl)	pre D4 IgA(mg/dl)	pre D4 IgM(mg/dl)
R 6A	0.9	0.43	610	254	173
R21	2.67	2.51	1280	180	228

The horizontal bars represents mean ± SEM and the dotted line represents the normal range.

Table 5: Level of CD19/20 and immunoglobulin prior to 4th dose of Rituximab therapy (pre D4)

Mean±SEM	1.79±0.89	1.47±1.04	945±335	217±37	200.50±27.50
SD	1.25	1.47	473.76	52.33	38.89
Range (%)					
(i) <6	2/2	2/2	<700=1/2	<70=0/2	<50=0/2
(ii) 6-23	0/2	0/2	700-1700=1/2	70-350=2/2	50-300=2/2
(iii) >23	0/2	0/2	>1700=0/2	>350=0/2	>300=0/2

After 3rd dose of Rituximab, blood was provided for measuring CD19/20 and IgG/IgA/IgM levels. Of the 2 patients the CD19% (normal range 6-23%) mean ± SEM was 1.79±0.89.

This included 2/2 patients who had a CD19 count <6, mean ± SEM= 1.79 ± 0.89; no patient had a count 6-23 or >23. For CD20% (normal range being 6-23%), of the 2 patients the mean ± SEM was 1.47±1.04. This included 2/2 patients who had a CD20 count<6, mean±SEM=1.47 ± 1.04; no patient had a CD20 count 6-23 or >23 (Figure 4). For IgG level (normal range being 700-1700mg/dl), of the 2 patients the mean ± SEM was 945 ± 335. This included 1/2 patient who had a level <700, mean ± SEM=610±0.00; 1/2 patient who had a level 700-1700, mean ± SEM= 1280±0.00; no patients had a level >1700. For IgA level (normal range being 70-350 mg/dl), of the 2 patients the mean ± SEM was 217 ± 37.

This included no patient who had a level <70; 2/2 patients who had a level 70-350, mean ± SEM=217±37; no patient who had a level >350. For IgM levels (normal range being 50-300 mg/dl), of the 2 patients the mean ± SEM was 200.50±27.50. This included no patient who had a level <50; 2/2 patients who had a level 50-300, mean±SEM=22.50±27.50; no patient had a level >300 (Figure 4).

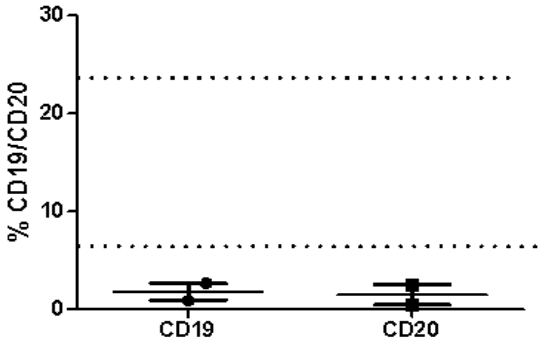

Fig 4A

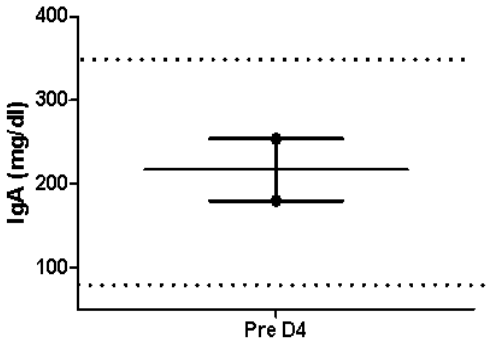

Fig 4B

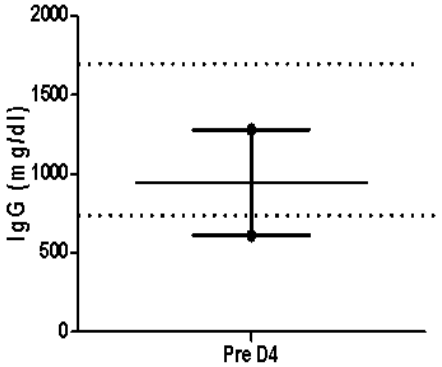

Fig 4C

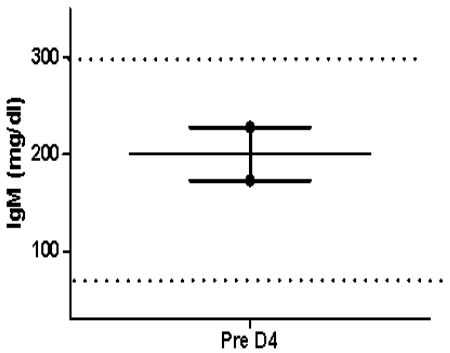

Fig 4D

Figure 4: Scatter plots showing the %CD19 and %CD20 status prior to initiating treatment (Pre D4, n = 2). A: %CD19 & CD20; B: IgG; C: IgA and D: IgM levels

Subject Code	pre D5 CD 19%	pre D5 CD 20%	pre D5 IgG(mg/dl)	pre D5 IgA(mg/dl)	pre D5 IgM(mg/dl)
R04	**1.56**	**1.45**	**1340**	**371**	**116**
R16	**2.96**	**3.01**	**1140**	**142**	**159**
R32	**2.01**	**1.97**	**919**	**304**	**178**
R56	22.18	20.93	1590	186	20
R62	12.35	10.36	1590	424	85

The horizontal bars represent mean ± SEM and the dotted line represent the normal range.

Table 6: Level of CD19/20 and immunoglobulin prior to 5th dose of Rituximab therapy (pre D5)

Mean±SEM	8.21±4.02	7.54±3.71	1315.80±130.25	285.40±53.54	111.60±28.08
SD	8.98	8.30	291.25	119.72	62.80
Range (%)					
(i) <6	3/5	3/5	<700=0/5	<70=0/5	<50=1/5
(ii) 6-23	2/5	2/5	700-1700=5/5	70-350=3/5	50-300=4/5
(iii) >23	0/5	0/5	>1700=0/5	>350=2/5	>300=0/5

After 4th dose of Rituximab, blood was provided for measuring CD19/20 and IgG/IgA/IgM levels. Of the 5 patients the CD19% (normal range 6-23%) mean ± SEM was 8.21±4.02. This included 3/5 patients who had a CD19 count <6, mean ± SEM= 2.18 ± 0.41; 2/5 patients who had a count 6-23, mean ± SEM =17.27 ± 4.92; no patient had a count >23. For CD20% (normal range being 6-23%), of the 5 patients, the mean ± SEM was 7.54 ± 3.71. This included 3/5 patients who had a CD20 count<6, mean ± SEM=2.14 ± 0.46; 2/5 patients who had a CD20 count 6-23, mean ± SEM= 15.65 ± 5.29; no patient had a count >23 (Figure 5). For IgG level (normal range being 700-1700 mg/dl), of the 5 patients the mean ± SEM was 1315.80 ± 130.25. This included no patients who had a level <700; 5/5 patients who had a level 700-1700, mean±SEM= 1315.80±130.25; no patient had a level >1700.For IgA level (normal range being 70-350 mg/dl), of the 5 patients the mean ± SEM was 285.40±53.54.This included no patient who had a level <70; 3/5 patients who had a level 70-350, mean ± SEM=210.67±48.36; 2/5 patients who had a level >350, mean ±SEM=397.50 ± 26.50. For IgM level (normal range being 50-300 mg/dl), of the 5 patients the mean ± SEM was 111.60 ± 28.08. This included 1/5 patient who had a level <50 which was 20; 4/5 patients who had a level 50-300, mean ± SEM=134.50 ± 20.99; no patient had a level >300 (Figure 5).

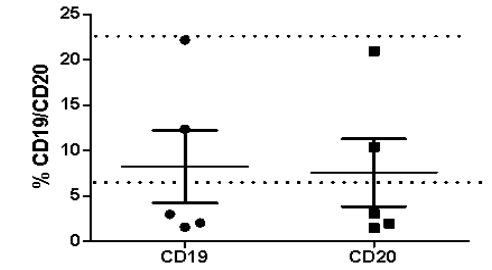

Fig-5A

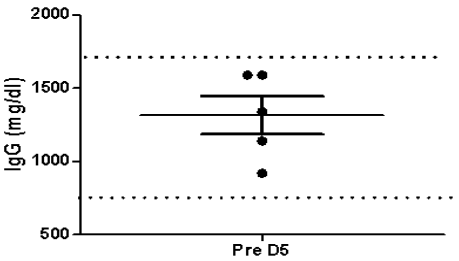

Fig 5B

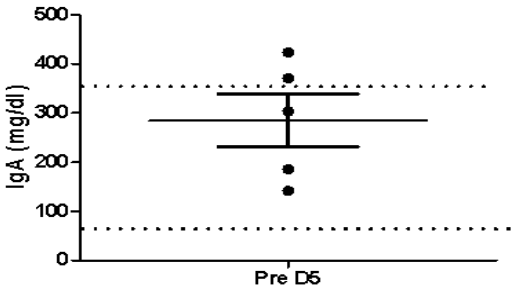

Fig 5C

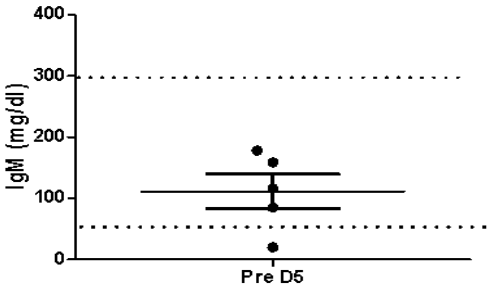

Fig 5D

Figure 5: Scatter plots showing the %CD19 and %CD20 status prior to initiating treatment (Pre D5, n = 5). A: %CD19 & CD20; B: IgG; C: IgA and D: IgM levels

Subject Code	pre D6 CD 19%	pre D6 CD 20%	pre D6 IgG(mg/dl)	pre D6 IgA(mg/dl)	pre D6 IgM(mg/dl)
R 4A	1.98	1.69	1020	137	91

The horizontal bars represent mean ± SEM and the dotted line represent the normal range.

Table 7: Levels of CD19/20 and immunoglobulin prior to 6th dose of Rituximab therapy (pre D6)

Mean±SEM	1.98	1.69	1020	137	91
Range (%)					
(i) <6	1/1	1/1	<700=0/1	<70=0/1	<50=0/1
(ii) 6-23	0/1	0/1	700-1700=1/1	70-350=1/1	50-300=1/1
(iii) >23	0/1	0/1	>1700=0/1	>350=0/1	>300=0/1

After 5th dose of Rituximab, blood was provided for measuring CD19/20 and IgG/IgA/IgM levels. Of the 1 patient the CD19% (normal range 6-23%), 1.98.

This included 1/1 patient who had a CD19 count <6 of 1.98 and patient had a count 6-23 or >23. For CD20% (normal range being 6-23%), 1 patient had a count <6, being 1.69; no patient had a CD20 count 6-23 or >23. For IgG level (normal range being 700-1700mg/dl), of the 1 patient the mean ±SEM was 102. For IgA level (normal range being 70-350 mg/dl), of the 1 patient the value was 137. For IgM level (normal range being 50-300 mg/dl), of the 1 patient the value was 91 (Figure 6).

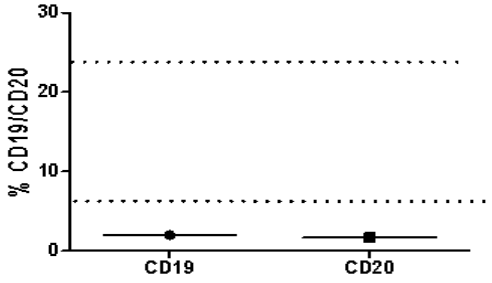

Fig 6A:

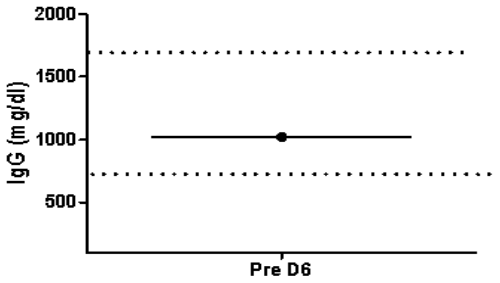

Fig 6 B:

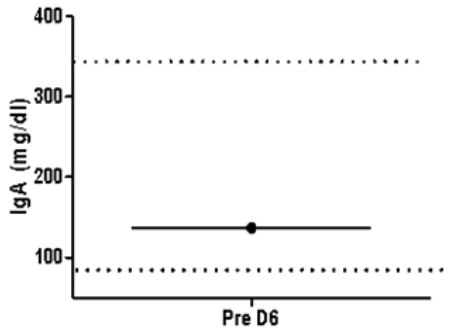

Fig 6C:

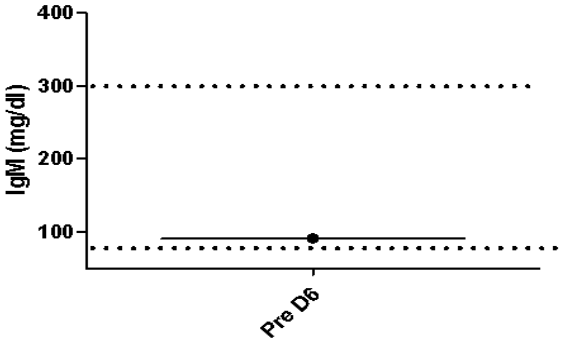

Fig 6D:

Figure 6: Scatter plots showing the %CD19 and %CD20 status prior to initiating treatment (Pre D6, n = 1). A: %CD19 and CD20; B: IgG; C: IgA and D: IgM levels

The horizontal bars represent mean and the dotted line represent the normal range.

Discussion

Rituximab (Mabthera, Rituxan) is a chimeric human/murine monoclonal antibody against CD-20 surface antigen expressed on B-cells. Rituximab, by causing B-cell depletion, appears to be effective in several autoimmune disorders; it has been approved for rheumatoid arthritis and is a promising new agent in the treatment of several autoimmune neurological disorders. B-cells have been demonstrated to play a key role in the pathogenesis of auto immune diseases beyond their polyclonal activation and the production of auto-antibodies against self-antigens. Targeting the B-cell compartment is therefore an attractive alternative to current available therapies [12]. However, one of the drawbacks of a prolonged peripheral B-cell depletion is associated suppression of protective antibodies and an increased risk for infectious events. However, few long-term data are available on predictors for the development of low levels of serum

immunoglobulins in patients receiving repeated courses of RTX. Low IgG levels (<600 mg/dL) before RTX treatment have been strongly associated with an increased risk of severe infection in the 12 months following the first cycle of RTX, emphasizing the need for monitoring the extent of B-cell depletion [13, 14].

The purpose of our study was to compare CD19% and CD20% with serum Ig levels in patients receiving Rituximab in order to assess whether clinicians can use serum Ig levels as predictors for long term immunosuppression, thereby allowing more tailored monitoring of such patients especially when receiving long-term treatment with RTX. An overwhelming majority of patients who had either normal or depleted %CD19 had normal levels of serum IgG. This result reinforces the fact that mature long-lived antibody-secreting plasma cells are apparently not depleted by RTX. The regeneration of B cells is from naive B cell populations produced in the bone marrow. It is these naïve B cells which go on to produce the long-lasting memory B cells which is responsible for the normal levels of serum IgG even after repeated doses of RTX [15].

In patients with CNS demyelinating disorders (n = 15), their median (interquartile range [IQR]) %CD20 and EDSS at baseline was 9.8 (5.6–18.8)% and 8.0 (7.5–8.0)%, respectively. In Group 1 (n = 7, %CD20 ≥ 1.5), there was a gradual decrease of %CD20 and EDSS, whereas in Group 2 (n = 8, %CD20 < 1.5), despite withholding RTX, patients remained asymptomatic, and their %CD20 remained <1.5 and EDSS showed a gradual decrease. 87% of patients experienced at least one ADR, the median (IQR) of ADRs per patient was 3 (0–3), and all 31 ADRs were infusion-related, with 100% recovery. RTX was relatively safe to use in these disorders, and monitoring its efficacy was adequately achieved using EDSS, with no additional benefits accrued by measuring %CD20 counts. Our data has suggested that the CD19% does not correlate with serum IgG levels during treatment and hence, the use of serum IgG as a predictive biologic marker for long-term immunosuppression on prolonged RTX therapy has limited merit. These findings are of importance since our study population comprised consecutive patients starting RTX treatment in clinical practice.

We acknowledge the methodological limitations generated by the non-randomized, open nature of this study. The power of the study was limited due to the small sample size. Due to paucity of time and loss of follow-up in a government setting, we were unable to monitor the clinical effectiveness of RTX after successive doses. The decision to start treatment or select dose was based solely on clinical practice and available guidelines.

Conclusion

Our findings suggest that the CD19% is poorly related to the serum IgG levels during treatment. The marked depletion of the B cell count is not accompanied by a similar fall in the serum IgG levels, which remain at normal levels. Irrespective of the dosing schedule, IgG levels do not correlate with the B-cell count. Hence, the use of serum IgG as a predictive biologic marker for long-term immunosuppression on prolonged RTX therapy has no merit. These findings are of importance since our study population comprised consecutive patients starting RTX treatment in clinical practice. Therefore, tertiary hospitals should have B cell monitoring by Flow Cytometry in order to ensure the safety of the drug so that immunosuppression is detected well in advance. Tertiary care hospitals should be equipped with a Flow Cytometer to assess the safety of the subsequent doses of the drug by analyzing the B cell count. Therefore, our study suggested that serum Ig levels should not be used as a prognostic marker for monitoring long-term immunosuppression in patients with autoimmune diseases undergoing RTX therapy.

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