Short communication

Ocrelizumab and ofatumumab, but not rituximab, trigger complement induction in vitro

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ABSTRACT

The clinical and adverse effects of the therapeutic monoclonal antibodies (mAb) ocrelizumab, ofatumumab and rituximab in multiple sclerosis (MS) are presently subject to extensive study. While the two former are approved for MS, the older and less costly rituximab is used off label, and adverse effect profiles are important in their evaluation. The three mAbs all induce B cell depletion, with complement-dependent cytotoxicity (CDC) as one of several mechanisms of action. Complement activation is also postulated to underlie adverse reactions related to infusion/injection. Such administration-related reactions are associated with all three mAbs, but comparisons have so far been indirect, resting on incidence reports from separate clinical trials. The objective of this study was to perform head-to-head comparison of complement activation by ofatumumab, ocrelizumab and rituximab. In vitro experiments were performed in whole blood from healthy donors. The complement-activating potential of the three mAbs was analyzed after 30 min of exposure to 0.3 mg/mL or 0.9 mg/mL of each drug, and compared with those of the well-known TNF inhibitory mAbs adalimumab and infliximab, the latter with recognized potential for infusion reactions. Ofatumumab, ocrelizumab, and infliximab, but not rituximab and adalimumab, triggered statistically significant complement activation measured as increased levels of terminal C5b-9 complement complex (TCC), a sensitive marker of such activation. While results demand careful interpretation, they provide an indication of distinct complement-inducing potential among anti-CD20 mAbs currently used to treat MS.

1. Introduction

The therapeutic monoclonal antibodies (mAbs) ocrelizumab, ofatumumab and rituximab are all in use in the treatment of multiple sclerosis (MS); ocrelizumab and ofatumumab as approved therapeutic agents, and rituximab as off-patent and off-label treatment [1]. The three mAbs exert their therapeutic effects by binding to CD20 epitopes to induce B cell depletion [1]. B cell depletion is the end result of activation of several molecular pathways, including antibody-dependent cell mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) (Fig. 1).

CDC is induced through activation of the classical complement pathway through complement 1q (C1q), with consequent target cell lysis [2]. Owing to its binding to both extracellular loops of CD20, ofatumumab activates CDC more avidly than rituximab [3], with ocrelizumab possessing an even less pronounced, but not negligible, CDC-

inducing potential [4,5]. Complement activation by mAbs may also have other downstream consequences than CDC, such as complement-dependent phagocytosis [6].

Clinical trials have pointed towards pronounced and comparable clinical efficacy of ocrelizumab, ofatumumab and rituximab [7], but recent evidence suggests that there could nevertheless be relevant clinical differences in treatment efficacy [8]. In the continuous clinical evaluation of these therapeutics, both risk/benefit considerations and treatment efficacy are important [7]. Although generally well tolerated, adverse events in the form of infusion/injection reactions and infections are common with all three mAbs [7,9]. Reactions related to administration – intravenous infusion for rituximab and ocrelizumab, subcutaneous injection for ofatumumab – frequently resolve spontaneously, but nevertheless represent a challenge, commonly demanding prophylactic or symptomatic co-medication [7,10]. Infusion/injection-related effects are mediated by complex mechanisms which may be closely intertwined with therapeutic effects, and complement activation is likely an important mediator [10,11]. In theory, therefore, differences in complement-inducing potency may be associated with differences in efficacy or adverse event profile between mAbs. While infusion/injection reactions occur with all anti-CD20 mAbs compared in this study, ocrelizumab has been suggested to be less prone to induce such adverse events, but head-to-head comparisons have not been performed [7].

The classical, lectin and alternative complement pathways converge on complement 3 (C3) with release of C3 activation products, while terminal C5b-9 complement complex (TCC) is the end result of the terminal pathway activation; assaying these components constitutes a sensitive marker of complement activation [12]. Acute effects of mAbs on these pathways are less studied than effects involving CDC. To directly compare complement-activating properties of ocrelizumab, ofatumumab and rituximab, we quantitated levels of the complement activation products C3bc and TCC after a 30-minute exposure in an established whole blood model. The complement-activating potential of the three mAbs was compared to those of adalimumab and infliximab, where the latter is associated with infusion reactions [13]. Rather than binding to CD20, infliximab and adalimumab exert anti-inflammatory effects by inhibiting tumor necrosis factor (TNF) (Fig. 1) [14]. Findings (primarily from transfected cells) indicate that both drugs are

nevertheless able to activate the complement system, i.e. in a CD20-independent manner [15].

2. Material and methods

2.1. Standard protocol approvals, registrations, and patient consents

The study was approved by the Regional committee for medical and health research ethics (REK SØR S-04114), and written consent was acquired from all volunteer blood donors.

2.2. Therapeutic monoclonal antibodies

For spiking of whole blood samples, minute surplus volumes available after preparation/administration of the following drugs were used: ocrelizumab, rituximab (Ocrevus, MabThera; both Roche, Basel, Switzerland), adalimumab biosimilar (Hyrimoz; Sandos, Basel, Switzerland) and infliximab biosimilar (Flixabi; Samsung Bioepis, Seoul, South Korea). Ofatumumab pre-filled pen (Novartis, Basel, Switzerland) was purchased from Haukeland University Hospital Pharmacy.

2.3. Preparation of whole blood samples

Blood samples were collected from healthy volunteers in tubes prepared with 80 μl 2.5 mg/mL (50 μg/mL final concentration) of the specific thrombin inhibitor lepirudin (Refludan, Pharmion, Copenhagen, Denmark) as anticoagulant. Four milliliters of whole blood were drawn, without the use of stasis, from four to six healthy volunteers (for detailed experimental setup, see below) not prescribed with any medication. The blood was carefully mixed by turning the tube 10 times, and immediately placed on a heating plate at 37 °C.

2.4. In vitro treatment with mAb

Ocrelizumab, ofatumumab, rituximab, adalimumab and infliximab were diluted in phosphate-buffered saline (PBS) with calcium (Ca) and magnesium (Mg) to a concentration of 1.8 mg/mL and 5.4 mg/mL, and 60 μl added to 300 μl blood, leading to a final concentration of 0.3 mg/
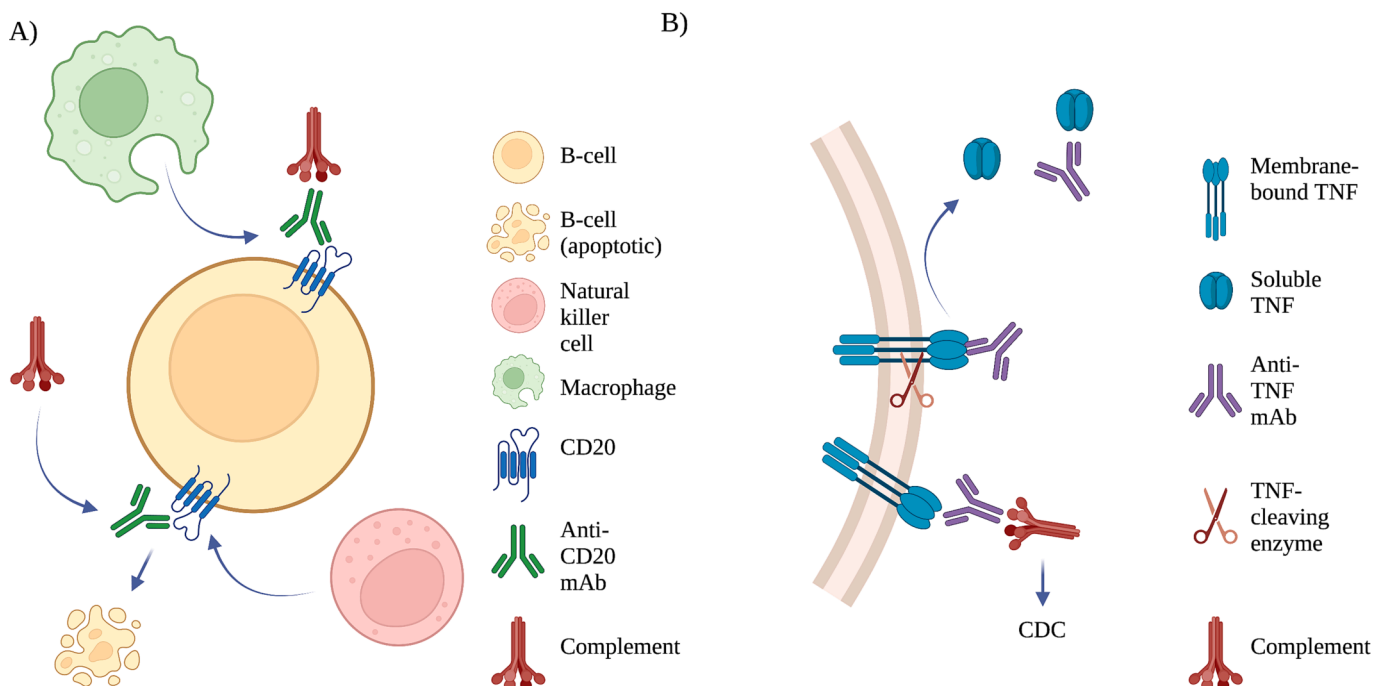

Fig. 1. Mechanisms of action for A) anti-CD20 and B) anti-TNF therapeutic monoclonal antibodies. CDC: complement-dependent cytotoxicity.

mL and 0.9 mg/mL, respectively. mAb was added to whole blood from four (adalimumab, infliximab, ofatumuab) or six (rituximab, ocrelizumab) donors. The selection of concentrations was based on our published or measured serum maximum concentrations (cMax) of rituximab (unpublished data) and ocrelizumab (0.3 mg/mL) [16], which are administered intravenously (IV) in samples from patients with MS [16]. In MS, ofatumumab is administered as subcutaneous (SC) injections, and at the presently approved dose of 20 mg yields much lower cMax (~0.001 mg/mL) [17] than IV infusion (cMax 0.15 mg/mL) [18]. For infliximab and adalimumab, a corresponding situation with IV infliximab yielding a Cmax of ~0.13 mg/mL [19] and SC adalimumab a cMax of ~0.004 mg/mL [20]. For the sake of direct comparison, the rituximab and ocrelizumab cMax dictated mAb concentrations in the experiments. Two control samples contained 60 µl PBS /w Ca and Mg, with one sample undergoing incubation and one sample being halted immediately after mixing, as well as a positive control with 60 µl 1 mg/mL zymosan, with each control sample being added to 300 µl blood. Each sample was incubated for exactly 30 min following the point of mixing. To halt the reaction, 7.2 µl of 0.51 M EDTA was added to each sample to a final concentration of 10 mM, and samples immediately placed on ice. Samples were centrifuged at 3000 rcf for 20 min at 4 °C, and the supernatant frozen at −80 °C until analysis.

2.5. Complement activation measurements

Plasma samples were thawed on ice. C3bc and TCC plasma levels were measured using in-house enzyme-linked immunosorbent assays (ELISA) as described previously [21]. In brief, ELISA plates (Nunc, Immunoplate II, Copenhagen, Denmark) were coated with monoclonal antibodies reacting with neoepitopes exposed only after activation (mAb bH6 reacts with an epitope exposed in C3b and C3c, and mA aE11 is specific for a neoepitope exposed in C9 after activation and incorporation into TCC). After plasma incubation and washing, detection antibodies were added to the respective activation products and after the final substrate step, optical density was measured at 405 nm using a Dynatech MR580 reader. Results are provided in complement arbitrary units (CAU)/mL, as described elsewhere [21].

2.6. Statistical analysis

A power analysis using α of 0.05 showed that n = 3 in each group gave a power of 97%. Spearman rank correlation was performed to assess the quality of data by correlating the C3bc and TCC activation products. A one-way ANOVA was performed to determine whether the different mAbs caused activation of the complement system as determined by TCC, compared to samples treated with PBS, followed by Dunnett's post hoc test. To compare effects of the two examined doses for each mAb, Levene's test for equality of variances was applied, followed by *t*-test for equality of means when Levene's test showed P

values > 0.05. The effect of group size was also analyzed using a post-hoc power analysis, which showed that our data produced a power of 99.8, despite differences in sample size. All statistical analyses were performed in SPSS (Version 29, IBM, NY).

2.7. Data availability

All data are available from the corresponding author upon request.

3. Results

Zymosan, the positive control, triggered pronounced activation, with expected levels of C3bc and TCC at 30 min (in the range of 200–400 CAU/mL; data not shown). Correlation between C3bc and TCC was significant (Spearman: r = 0.84, p < 0.0003; Fig. 2A), supporting the validity of the complement data. Subsequent interpretation focused on TCC, since this is a sensitive marker and directly reflects the formation of the membranolytic C5b-9 (Fig. 2B). Compared to vehicle (PBS), TCC was significantly higher after exposure to ocrelizumab, ofatumumab and infliximab at both 0.3 mg/mL and 0.9 mg/mL. With the exception of ocrelizumab, where 0.3 mg/mL yielded more pronounced TCC generation than 0.9 mg/mL (t score: 3.92; p = 0.002), no dose–response effects were observed. Rituximab and adalimumab did not result in significant complement activation.

4. Discussion

Complement activation by mAbs used in the treatment of MS could potentially impact both therapeutic and adverse event profiles. In a whole blood model, we performed a head-to-head comparison of acute complement-activating effects of the anti-CD20 mAbs ocrelizumab, ofatumumab and rituximab with those of TNF-blocking adalimumab and infliximab. Results indicated that ocrelizumab and ofatumumab had a complement-activating comparable to that of infliximab. Rituximab and adalimumab exposure did not result in activation of the complement cascade. Nevertheless, effects of all mAbs, including ocrelizumab, occurred at a much lower scale than the positive control zymosan, representing moderate to low-grade activation.

To our knowledge, the complement-activating potency of anti-CD 20 mAbs has not been extensively studied in similar in vitro settings, but mostly in transfected cells [5,6,10]. Humanization of antibodies reduces, but does not eliminate, immunogenicity in terms of anti-drug antibody prevalence [14]. Ocrelizumab is humanized, ofatumumab and adalimumab fully human, whereas rituximab and infliximab are chimeric, containing murine and human sequences [1,14]. Thus, the pattern observed by us seemingly does not correlate with the presence of non-human sequences in the respective mAbs, and neither with the CDC-inducing hierarchy described in the introduction, with ofatumumab as the more potent mAb followed by rituximab and ocrelizumab. With
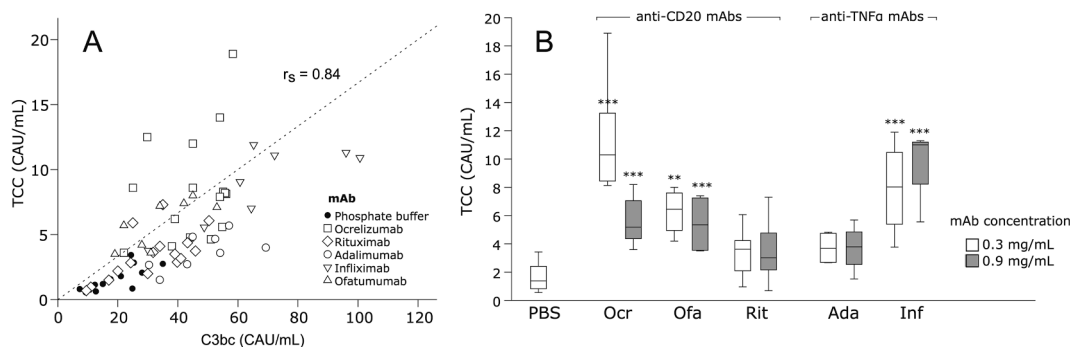

Fig. 2. A) Spearman's rank correlation for C3bc and TCC B) TCC, measured as complement arbitrary units (CAU)/mL, after 30 min of exposure. Abbreviations: Ocr, ocrelizumab, Ofa, ofatumumab, Rit, rituximab, Ada, adalimumab, Inf, infliximab. *** p < 0.005, ** p < 0.01, * p < 0.05, compared to PBS.

regard to the occurrence of infusion and injection reactions, comparison between results from clinical trials examining drugs in isolation is complicated by the lack of direct comparison, use of premedication to prevent such reactions, as well as dose and administration considerations [7]. Therefore, the real-life incidence of administration-related reactions remains to be determined, albeit with several clinical studies directly comparing rituximab and ocrelizumab underway, e.g. OVERLORD-MS [22], DanNORMS [23] and Noisy Rebels [24]. Results from these trials may indicate whether our findings of more pronounced complement activity have any clinical correlate. Ofatumumab is a more recent drug, and head-to-head trials have not yet been initiated [25].

Certain limitations of the study deserve mentioning. Although inter-individual variation in donors was relatively low, intrinsic differences in the complement system as well as vulnerable technical aspects of the experimental setup represent potential sources of error. Care was taken to diminish variation due to medication prescribed to blood donors as well as sampling procedures, with all sampling performed by an experienced biotechnician. Further, based on published and self-generated Cmax data for ocrelizumab and rituximab, we opted for standardized concentrations in order to allow head-to-head comparison of mAbs. In patients, Cmax shows relatively high interindividual variation, and different administration and dosing schemes are employed. Thus, the tested doses of ofatumumab and adalimumab exceeded Cmax values achieved at presently used doses and modes of administration (SC injections), whereas for rituximab, ocrelizumab and infliximab, administered as IV infusions, the range was directly clinically relevant [17,19,20]. Lastly, while the experimental setup offers opportunities for standardization and head-to-head comparisons of pharmaceutical agents, in vitro experiments represent an over-simplification of complex *in vivo* systems – particularly as blood from healthy donors were used, whereas in MS, background activation of inflammatory pathways is expected.

In summary, 30 min of in vitro exposure to ocrelizumab or ofatumumab, but not rituximab, led to activation of the complement cascade in whole blood from healthy donors. While several precautions should be taken when interpreting data, this finding suggests a somewhat surprising degree of complement activation by ocrelizumab, comparable to the expected activation seen with ofatumumab. The findings could have implications for further scrutiny of treatment efficacy and side effects of these three commonly used MS therapeutics.

CRediT authorship contribution statement

Jan-Lukas Førde: Conceptualization, Methodology, Investigation, Writing – review & editing. **Lars Herfindal:** Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Supervision, Writing – review & editing. **Kjell-Morten Myhr:** Conceptualization, Writing – review & editing. **Øivind Torkildsen:** Conceptualization, Writing – review & editing. **Tom Eirik Mollnes:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Silje Skrede:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Visualization, Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: J. L. Førde reports no disclosures relevant to the manuscript; L. Herfindal reports no disclosures relevant to the manuscript; K.M. Myhr has received unrestricted research grants to his institution: scientific advisory board, and speaker honoraria from Almirall, Biogen, Genzyme, Merck, Novartis, Roche and Teva, and has participated in clinical trials organized by Biogen, Merck, Novartis, and Roche; Ø. Torkildsen has received research grants and speaker honoraria from Biogen, Roche, Novartis, Merck and Sanofi; T.E. Mollnes reports no disclosures relevant to the manuscript, S. Skrede reports no disclosures relevant to the manuscript.

Data availability

Data will be made available on request.

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