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Development of a Murine model to dissect the CpG-oligonucleotide-enhancement of the killing of human B Cells by rituximab

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ABSTRACT

As a model to dissect the effects of CpG-oligonucleotides (CpG) on rituximab (RTX)-mediated therapeutic killing of autoimmune or malignant B lymphocytes, nude mice were grafted with Daudi human B cells. These mice were then injected with RTX alone or together with CpG. The human B cell aggregate was measured, and the reactive infiltrate analyzed after selective depletion of murine circulating cells. Macrophages (MØ) were identified in infiltrates, but not polymorphonuclear neutrophils (PMN), as confirmed by the failure of quantitative polymerase chain reaction to detect transcripts for PMN-specific myeloperoxidase in graft extracts. Evidence that MØ predominate over PMN in the anti-B cell RTX-induced immune mechanisms, include the presence of MØ-derived cytokines, and the lack of consequences of depletion of NK cells or B lymphocytes on the CpG-mediated effects on RTX. Interestingly however, removal of circulating PMN reduced the number of MØ attracted by the Daudi B cells. Our interpretation that CpG-induced complement activation is required for PMN to influence MØ was first based on overproduction of C5a in treated mice. This excess was due to the binding of the inhibitor of the alternative pathway of complement to CpG, as demonstrated by the elution of factor H from CpG-affinity-chromatography columns. Thus MØ are recruited to the tissue in the presence of C5a, and exploited locally by RTX.

1. Introduction

The early approval of rituximab (RTX) for the treatment of non-Hodgkin lymphoma [1], coupled with the recent revival for B cells in autoimmunity [2], invited to consider CD20-specific antibodies (Ab) for a variety of immune disorders [3]. This biotherapy shows promises in autoimmune diseases, but some patients resist while others eventually relapse [4]. The most efficient way to decipher the reasons of such diverging responses is to gain insights into how RTX kills the cells [5,6].

Lessons have been derived [7,8] from clinical trials in systemic lupus erythematosus (SLE) where the monoclonal Ab (mAb) operates *via* complement-mediated cytotoxicity (CMC), Ab-dependent cell cytotoxicity (ADCC), and apoptosis [9]. The question thus arises as to which mechanism prevails. The approach to this issue was to neutralize the candidates one by one, *i.e.*, remove macrophages (MØ) or inhibit complement components. It appeared that the former cells were required to destroy B lymphocytes, whilst the latter proteins were not, suggesting that ADCC predominates over CMC [10]. Further support for ADCC stems from the lack of correlation between sensitivity of B cells to RTX, and their load in the complement regulatory proteins CD46, CD55 and CD59 [11]. A third evidence is that an anti-CD20 mAb which recognizes the same epitope as RTX, but with a different isotype, is less efficient than RTX [12]. The implication of these results is that the anti-B cell activity of RTX relies on the cogency of insertion of the Fcγ portion into its receptors (FcγR). This is reflected by the poor efficacy of their low-affinity variants in SLE [13] but not in Sjögren's syndrome [14], and in B cell lymphoma [15] but not in chronic lymphocytic leukaemia [16]. In theory, MØ and polymorphonuclear neutrophils (PMN) share the potential to mediate ADCC [17].

RTX benefits from phosphorothioate (PS) CpG-oligonucleotides (ODN), and their phosphodiester counterparts [18]. These ODN (thereafter referred to as CpG for the sake of simplicity) alter NK cells, B and T lymphocytes [19], dendritic cells, and MØ, depending on their sequence [20]. In short, A-class CpG induce interferon

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(IFN)-α secretion by dendritic cells, enhance the activity of NK cells, but exert little impact on B lymphocytes, whilst linear B-class CpG favor the proliferation on the latter cells, depending on single nucleotide polymorphisms [21] of Toll-like receptor (TLR)9. In turn, B lymphocytes supply IFN-γ, tumor-necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, and IL-12 to effector cells. PMN would indeed be influenced by cytokines induced in response to CpG, rather than by the CpG themselves [22]. Given their relative resistance to RTX, Daudi human B cells are ideally suited to identify any therapeutic improvement, and thereby to dissect mechanisms involved in this effect. Preliminary results obtained using this model [18] suggest that CpG synergize with RTX to destroy B cells, whereas either agent alone causes moderate anti-B cell effects.

CpG may thus restrict their help to the promotion of ADCC. In line with other investigators [23], we believe that, at least under the present conditions, the role of NK cells remains modest, whereas that of MØ becomes central to anti-B cell responses. Furthermore, PMN may influence MØ, but have themselves been influenced by complement, as suggested by high levels of the C5a split product upon treatment with RTX. These increases might be indirectly facilitated by a defect in complement inhibitory factors. Such consumption of factor H in the alternative pathway has been ascribed to a DNA-binding protein [24] and to CpG [25]. Factor H might be diverted by these compounds, and its consumption favors the production of PMN-activating C5a.

2. Materials and methods

2.1. Synthetic oligonucleotides

PS and PO B-class ODN 2006 which contain four CpG motifs were custom-synthesized by Sigma (St-Louis, MO). Their sequences were 5′-TCGTCG-TTTTGTCGTTTTGTCGTT-3′. All preparations were free of endotoxin, as determined using the *Limulus* assay (Bio-Wittaker, Walkesville, MO).

2.2. Human B-cell lines and primary B cells

Daudi B cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics (Gibco, Paisley, Scotland). They were analyzed by fluorescence-activated cell sorter (FACS), after a 60-h incubation of 10^6 cells with 2 μM CpG, permeabilization of the cells with 0.5% saponin, and staining with fluorescein-isothiocyanate (FITC)-conjugated anti-TLR9 mAb (Amgen, Seattle, WA). They were also stained with FITC-conjugated anti-CD20 mAb (BD Pharmingen, Mountain View, CA) before and after treatment with CpG.

Peripheral blood mononuclear cells (MNC) were isolated from three healthy volunteers from the staff, using density gradient centrifugation on Ficoll-Hypaque and T lymphocytes eliminated following the rosette procedure. A first aliquot of T cell-depleted MNC was treated with a mixture of unconjugated anti-human CD3, anti-CD4, anti-CD8, and anti-CD56 mAb (all from Beckman-Coulter, Fulterton, CA). Then, B cells were purified negatively using goat anti-mouse IgG Ab-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). A second aliquot of T cell-depleted MNC served to separate positively monocyte using the human CD14 selection kit (Stem Cell Technologies Inc, Vancouver, Canada). Purity of B cells and monocytes were 95 and 94%, respectively, as ascertained by FACS analysis.

2.3. Mice

Since we suspected a role for complement in B cell lysis, we chose BALB/c mice, based on their higher levels of complement activity, compared with C57Bl/6 mice. Accordingly, BALB/c *nu/nu*, SCID CB-17, and Swiss mice, aged 6–8 weeks, were selected and purchased from the Charles River Breeding Laboratories (Wilmington, MA), housed under pathogen-free conditions, and fed with irradiated food and water.

2.4. In vivo *studies*

All experiments were performed at least twice. For inoculation, Daudi human B cells were harvested in their log-phase, washed and re-suspended at 10^7 cells/0.1 ml in phosphate-buffered saline (PBS) before being injected s.c. into the mice. RTX was kindly supplied by Dr. N Mackenzie (Roche-France) at a stock concentration of 10 mg/mL, and diluted to 30 μg/100 μL in PBS to be injected *via* the tail vein. The volume of the Daudi B cell aggregates were measured every 3 days, according to $(Dxd^2)/2$ (where "D" is the long and "d" the short diameter). This volume took 35 days to reach 1 cm^3.

The mice were then distributed into four experimental groups of 14 individuals, and injected weekly four times. The first group received PBS only ("PBS mice"), the second 1 mg per kg of body weight of RTX alone ("RTX mice"), the third 200 μg of CpG alone ("CpG mice"), and the fourth 1 mg/kg of RTX plus 200 μg of CpG ("RTX plus CpG mice"). Before and 35 days after treatment, the Daudi cell aggregates were scored as complete response when all the cells disappeared, partial when it was reduced by 50%, absent when the reduction was under 25%, and progressive when there was a 25% increase in their number. Anti-CD25 (Novartis, Basel, Switzerland) and anti-Her/2neu (Genentech, Palo Alto, CA) mAb were chosen as negative controls, because their isotypes matched that of RTX.

2.5. In vitro *studies*

B lymphocytes from each of the six normal donors were distributed into two aliquots of 10^6 cells each. Both underwent a 4 h incubation with 10 μg/mL RTX, and CpG was absent in the first aliquot, but present at the dose of 5 μg/mL in the second. As described [26] in B cells from patients with chronic lymphocytic leukaemia (CLL), apoptosis was analyzed by FACS through a combination of propidium iodide (PI) and FITC-annexin V. Cells in the early stage of apoptosis were positive for FITC-annexin V and negative for PI, and those in the late stage or necrotic were positive for PI.

To determine whether CpG affects apoptosis elimination of apoptotic B cells by MØ-mediated phagocytosis, triplicates of 2×10^5 MØ were incubated with 10 μg/mL of CpG or left in the medium for 3 h MØ with CpG and control MØ were then incubated with 2×10^5 B lymphocytes, together with 10 μg/mL RTX. 1 h later, apoptosis was evaluated as above, and confocal microscopy (Leica, Benshein, Germany) utilized to analyze the cells, in search for Daudi B cells engulfed by MØ.

2.6. Transcripts for PMN-specific myeloperoxidase

Daudi cell aggregates were lysed on ice with 1 mL (TRIzol® Reagent) per 50 mg of tissue using the Ultraturax technology (Invitrogen, Carslbad, CA). Blood samples were collected from Balb/c mice, and PMN used as positive control for myeloperoxidase (MPO) measurement. Due to their high sensitivity to the isolation procedure, these PMN were assessed in whole blood. Samples were incubated for 5 min to allow nucleoprotein complexes dissociation before addition of 0.2 mL chloroform per 1 mL TRIzol®. The tubes were shaken for 15 s, incubated for 3 min, and centrifuged at 12,000 *g* for 15 min. Supernatants were collected, and RNA precipitated by mixing with 0.5 ml isopropylic alcohol per 1 mL TRIzol®.