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Brief Communication

Hexamerization-enhanced CD20 antibody mediates complement-dependent cytotoxicity in serum genetically deficient in C9

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ABSTRACT

We examined complement-dependent cytotoxicity (CDC) by hexamer formation-enhanced CD20 mAb Hx-7D8 of patient-derived chronic lymphocytic leukemia (CLL) cells that are relatively resistant to CDC. CDC was analyzed in normal human serum (NHS) and serum from an individual genetically deficient for C9. Hx-7D8 was able to kill up to 80% of CLL cells in complete absence of C9. We conclude that the narrow C5b-8 pores formed without C9 are sufficient for CDC due to efficient antibody-mediated hexamer formation. In the absence of C9, we observed transient intracellular increases of Ca^{2+} during CDC (as assessed with FLUO-4) that were extended in time. This suggests that small C5b-8 pores allow Ca^{2+} to enter the cell, while dissipation of the fluorescent signal accompanying cell disintegration is delayed. The Ca^{2+} signal is retained concomitantly with TOPRO-3 (viability dye) staining, thereby confirming that Ca^{2+} influx represents the most proximate mediator of cell death by CDC.

1. Introduction

Recently we reported that IgG antibodies bound to cognate cell surface antigens can assemble into ordered hexamers which efficiently interact with C1q to induce complement activation and complement-dependent cytotoxicity (CDC) [1]. Hexamer formation is mediated by non-covalent Fc-Fc contacts between IgG molecules which can be enhanced by specific point mutations in the Fc domain. 7D8 is an IgG1 Type I CD20 mAb that is almost identical to Type I CD20 mAb ofatumumab in primary amino acid sequence [2]. Hx-7D8 was generated by a glutamic acid to glycine (E430G) mutation in mAb 7D8, and represents an example of such a hexamerization-enhanced IgG (Hexabody molecule) [3]. We previously demonstrated that B-cells, including primary CLL cells opsonized with mAb Hx-7D8 are rapidly (<2 min) killed by CDC at high levels (>80%) in NHS as well as in C9-depleted human sera containing only trace amounts of C9 [4]. We

Abbreviations: CDC, complement-dependent cytotoxicity; CLL, chronic lymphocytic leukemia; C9-D, serum genetically deficient in C9; MAC, membrane attack complex containing C5b,C6,C7,C8 and multiple copies of C9; NHS, normal human serum; RTX, rituximab.

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also found that Hx-7D8 opsonized CLL cells but not Z138 cells could even be killed by CDC in the presence of a neutralizing anti-C9 mAb. This raised the important question as to whether an incomplete membrane attack complex (C5b-8) *alone* could promote CDC of CLL cells

reacted with Hx-7D8.

CDC	complement-dependent cytotoxicity
CLL	chronic lymphocytic leukemia
C9-D	serum genetically deficient in C9
MAC	membrane attack complex containing C5b,C6,C7,C8 and multiple copies of C9
NHS	normal human serum
RTX	rituximab.

2. Materials and methods

2.1. Characterization of C9-deficient serum

Serum (2.5 ml) was obtained under informed consent from normal donors and from an individual genetically deficient in C9 (C9-deficient serum; homozygous for Arg95-stop mutation) seen at Nagoya University Medical School. To confirm absence of C9, NHS and C9-deficient (C9-D) serum were diluted in PBS, run on 10% SDS-PAGE (reducing conditions), blotted onto nitrocellulose and probed with an anti-C9 monoclonal antibody (B7). The B7 mAb was generated in mice immunized with pure human C9 and was confirmed to be C9-specific by ELISA, western blotting and by capacity to immunoaffinity purify C9 from human serum in a specific manner [5]. To test effects of C9 deficiency on complement hemolytic activity, antibody-sensitized sheep erythrocytes (2% in veronal-buffered saline containing Ca^{2+} and Mg^{2+}; VBS^{++}) were incubated (37 °C; 15 min) with equal volumes of a dilution series (in VBS^{++}) of NHS or C9-D serum in triplicate in 96-well microtiter plates. Plates were centrifuged, supernatant harvested and absorbance read (540 nm). Controls (100% and 0% lysis) were included and used to calculate % hemolysis. In some hemolysis experiments, purified C9 was added to neat C9-D serum to restore approximate physiological concentration (100 μg/mL) prior to testing.

2.2. Tumor cell killing and evaluation of membrane attack complex (MAC) binding

We followed our published protocols for CDC assays and for labeling cells with Ca^{2+}-sensitive fluorescent indicator FLUO-4 [3,4]. FITC-labeled mAb aE11 (Hycult) is specific for a neoepitope on C9 in the MAC [6]. Fluorescence intensities are quantitated based on molecules of equivalent soluble fluorophore (MESF units) as we have described previously [3,4]. CLL cells were obtained as reported [3,4]. CDC assays were conducted at 37 °C in 25% serum at 5×10^6 cells/ml and 10 μg/ml CD20 mAb. All experiments were performed in duplicate or triplicate, and means and SD are displayed.

3. Results and discussion

3.1. Analyses of C9-D serum and CDC

We obtained serum from a patient devoid of C9 due to an Arg95-stop mutation. Using immunoblot (Fig. 1A) and a classical pathway hemolytic assay (Fig. 1B), we confirmed the absence of C9 and hemolysis. Addition of 100 μg/ml C9 restored hemolytic activity to levels comparable with NHS.

In 25% C9-D serum, CLL cells from six patients reacted with mAb Hx-7D8 were killed, with CDC varying between 36 and 85%, while CDC of CLL cells from the same patients in 25% NHS was ≥82% (Fig. 1C). Addition of 18 μg/ml purified C9 to 25% C9-D serum measurably increased CLL cell killing ($p < 0.01$); on average, CDC increased ~10%. In order to define background CDC, and to test for cell killing in the *absence of complement*, mAb Hx-7D8 was reacted with cells from three patients in media, and CDC averaged <20%, which was slightly less than the average CDC of cells incubated with the isotype control mAb Hx-b12 in 25% NHS (Fig. 1C). CDC of Z138 cells was low in C9-D serum, and remained low after C9 addition, but substantial CDC of Z138 cells was observed in 25% NHS (Fig. 1D). These results might be due to high levels of CD59 on these cells. Finally, we reacted CLL cells from two patients with rituximab (RTX), in NHS, or in C9-D serum ± C9 (Fig. 1C). CDC was 16% or less, even in NHS, in agreement with reports that RTX generally does not mediate CDC of CLL cells [7–9].

3.2. Binding of the MAC to cells

Based on probing with FITC mAb aE11, specific for the neoepitope on activated C9, there was no MAC assembly on CLL or Z138 cells reacted with mAb Hx-7D8 in C9-D serum (Fig. 1 EF). However, addition of C9 to C9-D serum led to substantial binding of MACs to CLL cells, with modest but statistically significant increases in binding to Z138 cells. Under comparable conditions in intact NHS, more MAC binding was demonstrable. There is a considerable range of binding of FITC mAb aE11 to CLL cells reacted with Hx-7D8 in NHS, and binding correlates with expression levels of CD20. That is, cells with more CD20 bind more mAb Hx-7D8 mAb and activate complement more efficiently, leading to deposition of more MACs (Fig. 1G). For CLL cells of 3 patients with lower CD20 levels, we observed a 10-fold increase in the FITC mAb aE11 signal (MESF units) for C9-D serum with C9 added. However, there was a 100-fold increase for CLL cells from patients with higher CD20 levels (Fig. 1E). The increase in the FITC mAb aE11 signal on addition of C9 was only 3-fold for Z138 cells (Fig. 1F), which might be explained by their high CD59 expression. In summary, these results demonstrate that there is no MAC generated in the C9-D serum, but the CLL cells can nevertheless be killed at moderate to high levels.

3.3. Analyses of the role of Ca^{2+} in CDC

Detailed CDC studies in NHS previously demonstrated that influx of large amounts of Ca^{2+} into cells, mediated by the membrane attack complex (C5b-9), is the most proximate mediator of cell death [3,4,10]. Whether Ca^{2+} influx mediated by C5b-8 plays a similar role in CDC of CLL cells incubated with mAb Hx-7D8 in sera lacking C9, is therefore a key issue.

We examined the kinetics of Ca^{2+} flux in CLL cells loaded with fluorescent Ca^{2+} indicator FLUO-4. In C9-D serum, FLUO-4 loaded cells opsonized with mAb Hx-7D8 were killed, but CDC was slower and less complete than observed in intact NHS: 71% dead at 2 min vs 89% dead at 1 min, respectively (Fig. 2A). Prior to onset of CDC, FLUO-4 labeled cells exhibited dim green fluorescence. Early in the CDC reaction, a bright fluorescent signal was generated in live cells as the cell membrane was first permeabilized [4]. This bright transition state was short lived; later, as the cell membrane was more completely permeated and the cell was killed, FLUO-4 leaked out and a smaller FLUO-4 signal remained in dead cells, due to Ca^{2+}-chelated FLUO-4 localized to mitochondria. However, in C9-D serum the transition state intermediate *was considerably more stable*, as reflected in the long time (>1.5 min) the cells had a bright FLUO-4 signal (Fig. 2B). It is likely that damage to CLL cell plasma membranes by C5b-8 allows influx of sufficient Ca^{2+} to kill cells, but egress of FLUO-4 is slower due to less efficient permeabilization of plasma membranes (i.e., smaller pores) of the CLL cells by C5b-8 relative to C5b-9 [11]. Due to the small amount of C9-D serum available we were not able to directly measure pore sizes in these experiments. We can also differentiate the action of C5b-8 on CLL cells compared to that of C5b-9 based on a slower killing reaction. The results in both Figs. 1 and 2 indicate that less killing is observed in C9-D serum compared to intact NHS, and although there are several possible explanations for this finding, including the relative