High reproducible ADCC analysis revealed a competitive relation between ADCC and CDC and differences between FcγRIIIa polymorphism

Yuji Mishima¹, Yasuhito Terui¹,², Yuko Mishima¹,², Ryoko Kuniyoshi¹, Satoshi Matsusaka¹,², Mariko Mikuniya¹, Kiyotsugu Kojima³ and Kiyohiko Hatake¹,²

¹Clinical Chemotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-8-31, Ariake, Koto-ku, Tokyo 135-8550, Japan
²Division of Medical Oncology, Cancer Institute Hospital, 3-8-31, Ariake, Koto-ku, Tokyo 135-8550, Japan
³Life Science Business Division, OLYMPUS Corporation, 2951, Ishikawa-machi, Hachioji-shi, Tokyo 192-8507, Japan

Correspondence to K. Hatake, Clinical Chemotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-8-31, Ariake Koto-ku, Tokyo, Japan 135-8550; E-mail: khatake@jfcr.or.jp

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Abstract

The anti-CD20 chimeric monoclonal antibody rituximab mediates cytotoxicity in malignant B cells via multiple mechanisms, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and direct induction of apoptosis. To optimize treatment of non-Hodgkin’s lymphoma, a fuller understanding of these mechanisms and their relative contributions to clinical efficacy is required. Here, we report the characteristics of the mutual impact between ADCC and CDC, the two major effector functions through the Fc receptors. To compare ADCC induced under various conditions, we developed a highly reproducible method of estimating ADCC activity using immortalized effector cells. The set of the effector cells that we established was able to calculate net ADCC with high reproducibility by comparing the cytotoxicity of effector cells expressing exogeneous FcγRIIIa to those of mock effector cells. In addition, the different property of effector cells of two FcγRIIIa single-nucleotide polymorphisms (SNP) could be also evaluated in exactly identical background. ADCC assessment in the presence of human serum directly provided the evidence of the competitive interaction of ADCC and CDC. The inhibition of ADCC of effector cells having low affinity SNP of FcγRIIIa by active complement was more potent than those having high-affinity SNP at the rituximab-concentration comparable to the serum level obtained in patients. These findings could have a profound impact on optimization of the regimen of therapeutic antibodies and on the development of antibodies that will enhance effector function.

Keywords: Fc receptor polymorphism, timelapse-imaging

Introduction

It has been proposed that rituximab is associated with anti-body-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or direct apoptotic induction, and ADCC is, in particular, thought to be important for tumor regression in clinical practice (1–4). In a recent study, we have shown a correlation between CDC susceptibility and the therapeutic responses of the lymphoma cells in individual cases by performing a living cell imaging assay using a confocal microscope with lymphoma cells obtained from patients (5). Our data suggest that the cytotoxicity of the complement system may affect the treatment prognosis, directly or indirectly. The evaluation of ADCC activity requires effector cells derived from fresh blood, preferably from the identical patient that tumor cells were obtained from; cytotoxic activity of primary NK cells varies greatly in different donors and even in the same donor according to physical conditions (6). Therefore, it is not easy to compare inter-patient ADCC susceptibility. In this study, we aimed to design a way to evaluate the in vivo relationship of CDC and ADCC by keeping effector cells always available and having stable effector activity. Several studies suggested that NK cells have a major role in the ADCC activity induced by rituximab (7, 8). For this reason, we chose an aggressive NK leukemia cell line, KHYG-1, as the origin of the effector cell line, focusing on its strong cytotoxicity. KHYG-1 cells have lost the expression of CD16 (FcγRIIIa) during their establishment and do not induce antibody-dependent cytotoxicity by themselves. We introduced functional Fcγ-receptors into this cell
line and obtained immortal cells with antibody-dependent cytotoxicity. These cells exhibited ADCC activity that was dependent on rituximab concentration. The cytotoxic activity of this cell line was stable enough for us to measure rituximab-induced ADCC activity with high reproducibility. This rituximab-concentration dependency of established effector cells in vitro is almost the same as with primary NK cells and much lower than the rituximab blood level required for clinical efficacy. Then we analyzed ADCC activity in the presence of human serum to mimic a condition reflecting the environment in a living body. In the presence of human serum, ADCC activity was greatly suppressed, and a more detailed analysis determined that at least part of the suppression was caused by immunoglobulins. Furthermore, we found that activation of the complement system’s classical pathway markedly inhibited ADCC activity. As for the therapeutic administration of rituximab, it was reported that both the consumption of the complement and the decrease in blood immunoglobulin levels were induced by depletion of normal B cells, which was caused by CDC (9, 10). These phenomena suggest that the factors that have inhibited ADCC activity in vivo are decreased by CDC and that ADCC activity may be gradually enhanced.

Methods

Antibodies and cytokines

Rituximab was purchased from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan) and dialyzed three times into PBS. Recombinant human endotheial IL-2 (rhIL-2) was purchased from R&D Systems (Minneapolis, MN, USA). Purified human immunoglobulins were purchased from Sigma-Aldrich (St Louis, MO, USA).

Serum samples

Serum samples were obtained from healthy volunteers. Written informed consent was obtained from each subject. Component component-depleted sera were purchased from Complement Technology Inc. (Tyler, TX, USA).

Cell lines and cell culture

A human aggressive NK leukemia cell line, KHYG-1, was obtained from the Health Science Research Resources Bank (HSRRB; Osaka, Japan) and was maintained in RPMI1640 supplemented with 10% heat-inactivated FCS and 20 ng ml⁻¹ of rhIL-2 (11). The Burkitt’s lymphoma lines, Daudi and Ramos, were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in vitro in RPMI1640 medium supplemented with 2 mM L-glutamine, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10% FCS, all from Invitrogen (Carlsbad, CA, USA). A human myeloma cell line, KMS12PE, was obtained from HSRRB and maintained in vitro in RPMI1640 medium supplemented with 2 mM L-glutamine, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 10% FCS.

Plasmids

The region encoding human FcγRIIla protein was amplified by reverse transcription-PCR from total RNA extracted from human peripheral mononuclear cells and ligated into the mammalian pRetroX-IRES-ZsGreen1 vector (Clontech). The construct of FcγRIIla harboring the valine158-to-phenylalanine single-nucleotide polymorphism (SNP) was created by Pfu-based PCR using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). All sequences were confirmed with an Applied Biosystems 3130 Genetic Analyzer.

Transfection

The retroviral expression vectors and the envelope expression vector pVSV-G were cotransfected into GP2–293 cells with the Retro-X Universal Packaging system (Clontech). At 2 days after transfection, the culture supernatants were collected by low-speed centrifugation for 5 min at 4°C and then used to infect KHYG-1 cells. At 3 days after infection, ZsGreen-expressing KHYG-1 cells were sorted by FACS-Vantage (BD Biosystems).

Cytotoxicity assay

Cytotoxicity activity was assessed using a standard ⁵¹Cr release assay. Target cells were labeled with 100 µCi (3.7 MBq) Na₂¹⁵⁶CrO₄ for 1 h at 37°C, washed three times with culture medium and then plated at the indicated effector-to-target ratio in a 96-well round-bottom plate. For ADCC assays, the indicated concentration of rituximab was incubated with target cells. In some experiments, human serum was added ahead of the addition of rituximab. After a 4-h incubation at 37°C, 25 µl of supernatant were removed from each well and added to a 96-well formatted microplate containing a solid scintillator (LumaPlate; Perkin Elmer). The ⁵¹Cr activity was quantified with a scintillation counter (TopCount; Perkin Elmer). Each test was performed in triplicate. The results are expressed as the percentage of lysis, which is calculated according to the following equation:

\[
\text{Experimental release} = \text{Experimental release} - \text{Spontaneous release} / \text{Total c.p.m.} - \text{Spontaneous release} \\
\text{Spontaneous release} = \text{Spontaneous release for target cells incubated without effector cells}.
\]

where experimental release represents the mean counts per minute (c.p.m.) for the target cells in the presence of effector cells; spontaneous release represents the mean c.p.m. for target cells incubated without effector cells and maximal release represents the mean c.p.m. for target cells incubated with 2.5% Triton X 100.

Results

Establishment of effector cells and in vitro ADCC assay

We transduced functional FcγRIIla into the NK leukemia cell line KHYG-1 by using retroviral vectors and selected cells that express FcγRIIla stably by flow cytometry (Fig. 1A). Analysis of the surface antigen, after having cultured it for a long period after gene introduction, showed that the expression of FcγRIIla was quite stable (Fig. 1B). Next, we evaluated rituximab-dependent cytotoxic activity of our established effector cells against a Burkitt’s lymphoma cell line, Daudi, and confirmed that only the cells transduced a Fcγ receptor had ADCC activity in a rituximab dose-dependent manner. When compared with the SNP of FcγRIIla, effector cells having FcγRIIla of the 158V type showed
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Fig. 1. Establishment of immortal effector cells for the reproducible ADCC assay; (A) Construction of retrovirus vectors for bicistronic expression

of functional Fcγ-receptor and a fluorescent protein (ZsGreen) (B) FcγRIIIa and ZsGreen expression of effector cells. KHYG-1 cells were

transformed by using retrovirus vectors. Transduced protein expression was estimated after 10 passages (30 days), Results are representative of

at least three independent experiments for each panel. (C) Different cytotoxicity of effector cells carrying a SNP, ADCC activity of KHYG-1 cells

transduced FcγRIIIa with valine (V) or phenylalanine (F) at amino acid 158 against Daudi cells were analyzed using 51Cr release assay. For the

control experiment, a KHYG-1 cell-transduced empty vector was used. Data shown are the mean plus standard deviation of three independent

experiments.

significantly higher ADCC activity at rituximab levels lower

than 0.3 μg mL⁻¹ (Fig. 1C). In addition, observation by living

cell imaging using a confocal microscope revealed that rit-

uximab-coated target cells were rapidly damaged by direct

interaction of effector cells (Fig. 2 and Supplementary video,

available at International Immunology Online).

Analysis of the effect of the serum on ADCC

To mimic the activity of the effector cells in a living body, we

carried out an in vitro ADCC assay using a B-cell lymphoma

cell line, Daudi, as target cells under the condition that hu-

man serum was added. On this occasion, the complement

activity of human serum was inactivated by heat treatment

to avoid interference with evaluation of ADCC activity by

induction of CDC. Daudi cells were co-cultured with KHYG-

1/mock, KHYG-1/FcγRIIIA-158V or KHYG-1/FcγRIIIA-158F

in culture media containing heat-inactivated human serum

at 25% of final concentration. By adding the serum, the

ADCC activity of KHYG-1/FcγRIIIA-158V and KHYG-1/

FcγRIIIA-158F was inhibited to 36.3% [29.2–43.4% of 95% confidence interval (CI)] and 29.1% (23.8–34.4% of 95% CI)
of serum-free condition, respectively (Fig. 3A). No significant difference was found in the degree of suppression in either allotype. ADCC suppression was reduced to 64.3% (KHYG-1/FcγRIIIa-158V, 60.6–68.1% of 95% CI) and 57.2% (KHYG-1/FcγRIIIa-158F; 54.4–60.0% of 95% CI) by removal of IgG from serum. In contrast, ADCC activity was also inhibited when the IgG fraction collected from immobilized protein A was added in a volume equivalent to 25% of serum. These results suggested that ADCC activity was inhibited by the IgG in the human serum, as previously reported (12). In addition, mechanisms other than IgG may be participating because the suppression did not completely disappear with the removal of IgG from serum.

Fig. 2. Time-lapse images of rituximab-induced ADCC. Binding of Alexa-647-labeled rituximab (light blue pseudocolor) on the cell membrane of Daudi cells was observed. Meanwhile, the NK cells expressed a fluorescent protein in cytoplasm (ZsGreen, green pseudocolor) from the IRES reading frame as well as FcγRIIIa at the cell membrane. By the direct interaction of NK cells, the Daudi cells coated by rituximab had damaged plasma membranes, and intercalation of propidium iodide was observed within a few minutes from contact with the effector cells. Data shown is representative of at least five independent experiments. Scale bar is 20 μm.

Fig. 3. Evaluation of rituximab-induced ADCC activity in the presence of human serum. (A) Analysis of the effect on ADCC activity of the serum immunoglobulin. ADCC induced by rituximab in Daudi cells was analyzed in the presence of heat-inactivated human serum and its fractions of the protein A-affinity chromatography. Data shown are the mean plus standard deviation of three independent experiments. (B) Analysis of the participation of the complement system in ADCC activity. ADCC was analyzed in the presence of intact or heat-inactivated human serum (left; KHYG-1/FcγRIIIa-158V, right; KHYG-1/FcγRIIIa-158F). For these experiments, a myeloma cell line genetically engineered to express human CD20 protein was used as the target to avoid cytotoxicity of CDC. Data shown are the mean plus standard deviation of at least three independent experiments. Data are the mean plus-minus standard deviation of three independent experiments.

Participation of the complement system in ADCC

Next, we examined the effect of the complement system on ADCC activity. Because Daudi cells show strong susceptibility not only to ADCC but also to CDC, it is difficult to evaluate only the ADCC activity in the presence of intact serum. Therefore, we used a myeloma cell line KMS12PE that had been transformed with human CD20 gene (KMS12PE/hCD20) as the target cells of rituximab inducing ADCC. This cell line has little CDC susceptibility at rituximab levels lower than 100 μg ml⁻¹ (data not shown). In the serum-free condition, the ADCC induced in KMS12PE/hCD20 cells increased with rituximab levels and reached a plateau in 0.1 μg ml⁻¹ (KHYG-1/FcγRIIIa-158V) or 1 μg ml⁻¹ (KHYG-1/FcγRIIIa-158F), respectively (Fig. 3B). Meanwhile, in the condition that heat-inactivated serum was added at 25% final concentration, the ADCC level was inhibited by ~50% at every rituximab level we tested. Interestingly, by addition of intact serum, the ADCC activity was further inhibited as compared with the case of heat-inactivated serum at rituximab levels >0.1 μg ml⁻¹. Suppression of the ADCC was remarkable at higher rituximab levels (>1 μg ml⁻¹). Induction of cell death was completely lost, particularly when KHYG-1/FcγRIIIa-158F cells were used as the effector.
Using sera depleted of each component of complement (C1q-C9), we carried out a similar examination for the Daudi cells, which were sensitive in both ADCC and CDC. By adding serum from which either of the complement components had been removed, CDC by rituximab was at a markedly suppressed level (C2, C3 or C9) or was not observed (C1q, C4, C5, C6, C7 or C8) (Fig. 4A). Then we evaluated the cytotoxicity of rituximab in the presence of both effector cells and these sera. In the case that intact serum was added to the effector and target co-culture, strong cytotoxicity was observed even in the FcγRIIa null effector group. Besides, in the FcγRIIa-158V/158F groups, the additive cytotoxicity was observed only in the lower rituximab-doses where sufficient CDC was not induced. These results suggested that ADCC and CDC do not act additively when sufficient complement factors and rituximab are present. On the other hand, significant cytotoxicity was not observed in the FcγRIIa null effector group when serum depleted of one of the complement components was added. In addition, induction of ADCC was suppressed by adding complement-depleted serum, except C1q, C2 and C4, as compared with heat-inactivated serum, and suppression was remarkable at a rituximab level >10 μg ml⁻¹ (Fig. 4B). These results suggested that C1q, C2 and C4 are indispensable for ADCC suppression. ADCC was not observed in the C7-depleted serum at any rituximab concentration tested. This was presumed to be an unidentified problem derived from the C7 depletion process because addition of C7 did not affect ADCC induction (data not shown).

Discussion

Rituximab combination chemotherapy has remarkably improved the prognosis in B-cell non-Hodgkin’s lymphoma (13–15). Several studies based on clinical research have suggested that effector mechanism including ADCC and CDC play critical roles in anti-lymphoma mechanism in clinical setting (5, 16, 17). However, the relative contributions of these effector mechanisms in vivo are still a matter of debate.

Because conventional methods for evaluating ADCC have a disadvantage in reproducibility arising from effector cells being derived from peripheral blood on each use, we established immortal NK cells with stable effector activity. These cell lines proliferate IL-2 dependently and show stable expression of exogenous FcγRIIa, therefore exhibiting constant ADCC activity. By using these cells, we repeatedly evaluated ADCC activity under various conditions with high reproducibility. The in vitro study done by van Meerten et al. (18), using a series of CD20 transfected cell lines, suggested that CDC depends on CD20 expression level on target cells and that both CDC and ADCC act complementarily, probably because ADCC can occur in cells unresponsive to CDC due to low CD20 expression. In a living human body, CD20-positive lymphoma cells that are a target of rituximab are surrounded by various soluble factors, host cells or extracellular matrices. So the antitumor mechanisms of rituximab are influenced by many factors. In particular, it is estimated that components included in serum or extravascular fluid greatly affect CDC and ADCC. Therefore, we evaluated ADCC and CDC in various conditions in the presence of a serum component. It is challenging to evaluate the interaction of ADCC and CDC because both mechanisms can cause lysis of target cells. To address this issue, we used an experimental approach using a myeloma cell line that is less sensitive to CDC. The cell line used in this study had little sensitivity to rituximab-induced CDC. Though the exact mechanism underlying the insusceptibility to CDC was not determined, this cell line has very high CD59 expression, and it may contribute to this phenotype (19). CD59 is a potent inhibitor of the complement membrane attack complex, whereby it binds complement C8 and/or C9 during the assembly of this complex. This thereby inhibits the incorporation of multiple copies of C9 into the complex, which is necessary for osmolytic pore formation. Weiner et al. (20) demonstrated that C3b deposition induced by rituximab-coated target cells inhibits the interaction between Fc of rituximab and FcγRIIa of NK cells. They established this phenomenon mostly by examining the activation markers of NK cells co-cultured with rituximab-coated cells or plastic surface. In the present study, we have shown direct evidence that active complement activity inhibits cell death by rituximab-induced ADCC. Cell-death inhibition was not obvious at lower rituximab concentrations but becomes prominent as the rituximab concentration rises. These results were consistent with the data of NK cell inactivation that was shown by Weiner et al. (20). Although the C3-depleted serum that we used did not completely cancel the ADCC suppression, this may be because of insufficient depletion of C3 component as indicated by assessing remaining CDC inducibility.

To exclude the possibility of a direct effect of active complement on effector cells, rituximab-independent cytotoxicity against the cells lacking MHC class I molecules such as K562 cells was tested, and we confirmed that there was no such effect (data not shown). Thus our data demonstrated directly that an intact complement system indeed suppressed cell death induced by ADCC.

In the assay performed in the absence of serum in vitro, the difference of ADCC between the FcγRIIa allotypes was observed only at rituximab concentrations far lower than pharmacokinetics. On the other hand, at clinical rituximab concentrations, where CDC was fully induced in CDC sensitive cells, the effector cells expressing the FcγRIIa of the 158F allotype could no longer induce ADCC in the presence of complement active serum. These results may explain the prognostic difference observed in the clinical studies. When we are discussing the in vivo mechanisms of therapeutic antibodies, the suppression of ADCC by the complement system, demonstrated in the present study, offers informative evidence. Unfortunately, it is difficult to determine the concentration of each complement factor in lymph nodes of patients or the effective concentration of rituximab. However, ADCC could be so markedly inhibited in the environment that enough complement components and rituximab are present, such as in the peripheral blood. And, as complement protein is consumed and/or as rituximab levels decrease, conditions may become favorable for ADCC induction. To explore an optimal regimen as well as to develop more potent therapeutic antibodies, it is very important to understand the mutual relations of the in vivo effector
Fig. 4. Evaluation of the contribution of each complement factor on rituximab-induced ADCC activity. (A) CDC-inducibility of each complement-factor-depleted serum. CDC induced by 10 µg ml⁻¹ rituximab to Daudi cells was assessed in the presence of the serum that depleted each complement factor: C1q, C2, C3, C4, C5, C6, C7, C8 and C9 at final concentration of 20%. (B) Analysis of the ADCC in the presence of each complement-factor-depleted serum. Overall cytotoxicity of rituximab was measured in the presence of the serum that depleted each complement factor at a final concentration of 20% as well as the effector cell lines. Daudi cells were used as target cells at an effector/target ratio of 1:1. Results are representative of at least three independent experiments for each panel.
mechanisms. In this study, we offered a new screening strategy for therapeutic antibody development, proposing both novel and reproducible methods of ADCC evaluation that make it possible to elucidate the interaction of two major effector mechanisms, ADCC and CDC.

Supplementary data
Supplementary data are available at International Immunology Online.

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