

Fulminant liver failure triggered by therapeutic antibody treatment in a mouse model

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Abstract. Monoclonal antibodies are finding ever increasing therapeutic applications. However, lethal liver damage has been reported following monoclonal antibody (mAb) treatment in combination with subtoxic doses of cytotoxic drugs. In this study, mice were intravenously injected with 200 μ g/mouse of anti-CD8 (anti-Lyt-2.2), anti-CD4 (GK1.5) or anti-B220 (RA3-6B2) mAb. Subsequently, mice were administered 15 mg azoxymethane (AOM) per kg body weight by subcutaneous injection. Unexpectedly, all mice pretreated with mAb died within 72 h of a single injection of AOM. The injection of mAb-coated spleen cells accelerated the induction and the severity of liver disease. We found that mAb treatment activates Kupffer cells to produce inflammatory cytokines such as TNF- α and IL-12, and induces the expression of FasL on Kupffer and NKT cells. The concomitant upregulation of Fas on hepatocytes increases the susceptibility of the liver to apoptotic signals, and subsequent treatment with AOM causing mitochondrial injury synergistically induces lethal liver damage. Consistently, the lethal liver damage was abrogated in mice which were deficient for Kupffer cells, NKT cells or Fas-antigen. In conclusion, we have demonstrated a potential risk of lethal fulminant liver damage in the concomitant use of therapeutic antibodies and cytotoxic drugs. A possible side effect of antibody therapy is mediated through activation of the immune system, the very mechanism of action on which

this treatment depends. In this context, the risk of combining therapeutic antibodies with other agents, particularly cytotoxic drugs, requires careful consideration.

Introduction

Therapeutic antibodies are now used to treat a whole range of diseases, such as autoimmunity, cancer, inflammation and infectious diseases (1-3). Antibodies are highly specific, naturally evolved molecules that recognize and eliminate pathogenic antigens. The development of monoclonal antibody (mAb) technology was rapidly followed by their application in basic research and diagnostics. The discovery that antibodies to tumor necrosis factor- α (TNF- α) can ameliorate the symptoms of Crohn's disease (4,5) and rheumatoid arthritis (6) accelerated further research into therapeutic applications of mAbs. In addition, mAbs are being used with increasing success for the treatment of malignancy, for example, rituximab and trastuzumab for the treatment of lymphoma and breast cancer, respectively (7). Supported by growing evidence of the promise of these versatile new therapeutic agents to fight cancer, autoimmune diseases and infection, the number of mAbs under development for diverse indications is growing rapidly, with more than 300 different antibodies now in clinical trials [URL:www.clinicaltrials.gov; (keyword: antibody)].

The initial purpose of the treatment in this case was to examine the role of cellular immune responses in colorectal carcinogenesis, which has become a major cause of cancer mortality in Japan (8). The aim of these experiments was to examine whether host immune responses contribute to suppressing colorectal carcinogenesis. *In vivo* depletion of CD4⁺ or CD8⁺ T cells was achieved by intraperitoneal or i.v. injection of anti-CD4 or anti-CD8 mAb into 6 week-old mice. This procedure has long been accepted as a reliable and safe manipulation to study the requirement for T cell subsets for *in vivo* immune responses. To this end, we employed azoxymethane (AOM), a very potent carcinogen which induces a high incidence of colorectal cancers in rats and mice (9). The susceptibility of CD4⁺ T cell- or CD8⁺ T cell-depleted mice to colon carcinogenesis following administration of AOM was investigated. Surprisingly, a

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Abbreviations: TNF- α , tumor necrosis factor- α ; IL-12, interleukin-12; FasL, Fas ligand; mAb, monoclonal antibody; AOM, azoxymethane; AST, aspartate aminotransferase; ALT, alanine aminotransferase

Key words: monoclonal antibody, therapeutic antibody, FasL, Kupffer cell, NKT cell

single injection of a subtoxic dose of AOM induced lethal hepatic failure in mAb-pretreated mice, all of which died within 72 h. This promptly reminded us of the risk of concomitant application of therapeutic antibodies with chemotherapy, since it is known that AOM is not only carcinogenic but also cytotoxic (10).

In cancer therapy, the purpose of antibody administration is to induce the direct or indirect destruction of cancer cells. The elimination of the target cells depends on the recruitment of the body's own effector mechanisms, namely complement activation and Fc-receptor-dependent response (2). A possible side effect of antibody therapy is the cytokine-release syndrome that is also mediated through recruitment of the immune effector cells and interactions with Fc receptors. Life-threatening and fatal cytokine release syndrome has been reported with the use of Rituximab for treatment of chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphomas (11,12). Therefore, it is possible that activation of immune effector cells mediated by Fc receptors render the host susceptible to simultaneous administration of cytotoxic drugs.

Today, specific antibody therapy might be used in combination with classical chemotherapy. While generally very well tolerated, we report here that fatal fulminant hepatic failure can be associated with mAb treatment when this is combined with a cytotoxic agent. With this in mind, we would therefore like to raise a note of caution indicating the necessity for careful management of antibody therapy.

Materials and methods

Animals. Specific pathogen free, male C57BL/6J mice were purchased from CLEA Japan (Shizuoka, Japan). $V\alpha 14$ NKT KO mice were kindly provided by Dr T. Nakayama and Dr M. Taniguchi (Chiba University) (13). B6.MRL-Tbfrsf6lpr (lpr/lpr), mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were appropriately maintained according to the institutional Animal Care and Use Committee guidelines. All animal experiments were approved by this Committee of Mie University.

Treatment of animals. Depletion of CD8⁺ or CD4⁺ T cells was performed by i.v. injection of 200 μ g/mouse of anti-CD8 mAb (anti-Lyt2.2: mouse IgG2a, κ) or anti-CD4 mAb (GK1.5: rat IgG2b, κ) (14). Both mAbs were purified from ascites using HiTrap Protein G HP (Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer's instructions. Similarly, anti-CD8 mAb (3.155: mouse IgM), anti-B220 mAb (RA3-6B2: ratIgG2a, κ), anti-mouse class I mAb (M1/42: rat IgG2a, κ) and anti-mouse class II mAb (25-9-17S: mouse IgG2a, κ) was prepared. Control animals were injected with phosphate-buffered saline. Azoxymethane (Sigma, St. Louis, MO) was injected subcutaneously (s.c.) at a dose of 15 mg/kg body weight. Control animals were injected s.c. with 100 μ l PBS.

Spleen cells were isolated by compressing the spleens of syngeneic animals against the bottom of a Petri dish with the plunger of a 1-ml syringe as described previously (15). Syngeneic spleen (5×10^7 cells/ml) cells were incubated with 5 μ g/ml of indicated mAb at 4°C for 30 min then washed 4

times with PBS. The indicated number of spleen cells was intravenously injected from the tail vein.

To deplete Kupffer cells, mice were given intraperitoneal injections of 2 mg/mouse gadolinium chloride (Sigma) 24 h before AOM treatment. Depletion of NK cells was achieved by i.v. injection of 100 μ l/mouse of rabbit anti-asialo GM1 polyclonal Ab solution (Wako Pure Chemical Industries, Osaka, Japan) 3 days before challenge.

Lethality, biochemical and histological analyses. Lethality was scored up to 96 h after treatment. The extent of hepatocellular injury was monitored by measuring serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity at indicated time-points using a standard clinical analyzer. For histological analysis, liver was fixed in 10% formalin, embedded in paraffin, sectioned (3 μ m), and stained with hematoxylin and eosin.

mRNA analysis. Whole liver RNA was isolated using TRIzol (Sigma). Sample (1 μ g) was reverse-transcribed into cDNA and Fas, FasL, TNF- α , IL-1 β , IL-6, IL-12 p40, and GAPDH expression determined in a thermocycler (MJ Research Inc., Watertown, MA) using primers described previously (16). PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.

Isolation of hepatocytes, hepatic lymphocytes and Kupffer cells. Mice were anesthetized and livers were perfused sequentially with 5 ml Hanks' balanced salt solution (HBSS), followed by 5 ml of HBSS containing 0.1% collagenase. Livers were dissected and incubated in HBSS containing 0.1% collagenase and 0.01% DNaseI at 37°C for 15 min, then passed through a 200-gauge stainless-steel mesh. The cell suspension was centrifuged at 50 x g for 5 min and pellets were further washed for hepatocyte preparation. Supernatants were centrifuged at 500 x g for 5 min, pellets were resuspended in 33% Percoll and centrifuged at 500 x g for 10 min at room temperature. The cell pellet containing mononuclear cells was harvested. Kupffer cells were further purified by plating and selective attachment.

Flow cytometry. Cells at 10^6 - 10^7 /ml in PBS containing 1% FCS and 0.1% NaN₃ were incubated at 4°C for 20 min with 100 μ l of appropriate mAbs. The following mAbs from eBioscience (San Diego, CA) were used: FITC-labeled anti-TCRB (clone H57-597), PE-labeled anti-NK1.1 (clone PK136), and biotin-labeled anti-FasL (clone MFL3) and anti-Fas (clone 15A7). PE-labeled anti-B220 (clone RA3-6B2) and PE-labeled anti-F4/80 (clone CI:A3-1) were purchased from CALTAG (Burlingame, CA). For the biotin-labeled mAb staining, Cy5-streptoavidin (BD Bioscience, San Jose, CA) was used as a secondary reagent for detection. Cells were analyzed on FACScan flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software.

Statistical analysis. Results were expressed as mean \pm SD. Statistical analyses between one control group and several treated groups were performed by using ANOVA, followed by Dunnett's multiple comparison test. Differences between two groups were assessed by Student's t-test.

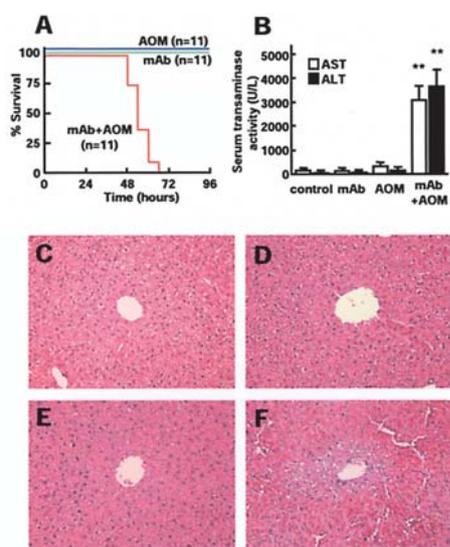


Figure 1. Induction of lethal liver damage by the combination of mAb and cytotoxic drug injection. (A) Survival of mice treated with AOM and/or mAb. Male C57BL/6 mice (11 per group) were injected intravenously with anti-CD8 mAb (clone 53-6.7; 200 μ g/mouse) to deplete CD8⁺ T cells. Subsequently, mice were given 15 mg AOM per kg body weight by subcutaneous injection. Blue, green, and red lines indicate survival curves of mice treated with AOM alone, mAb alone or mAb followed by AOM, respectively. (B) Serum transaminase values of AOM- and/or mAb-treated mice. Blood was collected 42 h after treatment. White bars and black bars represent sAST and sALT activity (U/L), respectively. Values represent means \pm SE. ** p <0.01. Histopathology of livers (x400) from mice treated with AOM and/or mAb. Livers were obtained 42 h after AOM and/or mAb injection and liver sections were stained with H&E. Representative results of untreated mice (C), AOM-treated (D), mAb-treated (E) or mAb followed by AOM (F) are shown. Severe hemorrhages and centrilobular hepatic necrosis are seen in the latter (F).

Results

Combined treatment with mAb and cytotoxic drugs can induce lethal liver damage. First, mice (11 mice per group) were intravenously injected with anti-CD8 mAb (anti-Lyt-2.2; 200 μ g/mouse) to deplete CD8⁺ T cells. Subsequently, mice were administered 15 mg AOM per kg body weight by subcutaneous injection. Unexpectedly, we found that all mice pretreated with mAb died within 72 h of a single injection of AOM (Fig. 1A, red line). Neither mAb treatment alone nor the AOM injection alone was lethal and in fact such mice maintained completely normal activity (Fig. 1A, green and blue line).

Because hepatotoxic effects of AOM have been reported (10), we monitored serum AST and ALT activity 42 h after AOM injection. It is of note that the amount of AOM that is necessary to induce hepatic failure is much larger (50-100 mg/kg) than for induction of colon cancer (10-15 mg/kg) and the dose used in this study. As expected, minimum or no liver damage was induced by separate injection of 15 mg/kg AOM or anti-CD8 mAb treatment, respectively (Fig. 1B). In contrast, serum AST and ALT levels were markedly elevated in mice receiving both mAb and AOM together (Fig. 1B).

To examine this hepatotoxicity in greater detail, the liver was evaluated histologically 42 h after AOM injection. Consistent with the biochemical findings, liver sections from mice treated with mAb and AOM displayed profound multifocal centrilobular necrosis with massive hemorrhage (Fig. 1F).

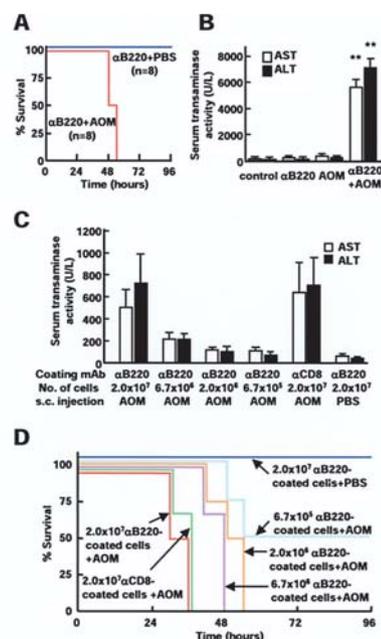


Figure 2. Early-onset of lethal liver damage by mAb-coated cells. (A) Survival of mice treated with anti-B220 mAb and AOM. Mice (8 per group) were injected intravenously with anti-B220 mAb (clone RA3-6B2; 200 μ g/mouse). Subsequently, mice were given 15 mg AOM per kg body weight by subcutaneous injection. Blue and red lines indicate survival curves of mice treated with AOM alone or mAb followed by AOM, respectively. (B) Serum transaminase values of AOM- and/or mAb-treated mice 42 h after treatment. Values represent means \pm SE. ** p <0.01. (C) Spleen cells were incubated with 5 μ g/ml of anti-B220 mAb or anti-CD8 mAb and antibody-coated spleen cells were i.v. injected into mice. Serum AST and ALT levels were elevated 24 h after AOM treatment. (D) Survival of mice treated with antibody-coated spleen cells. Male C57BL/6 mice (N per group) were injected intravenously with anti-CD8 mAb (clone 53-6.7; 200 μ g/mouse) to deplete CD8⁺ T cells. Subsequently, mice were given 15 mg AOM per kg body weight by subcutaneous injection. Red, purple, orange and skyblue lines indicate survival curves of mice treated with 2.0×10^7 , 6.7×10^6 , 2×10^6 and 6.7×10^5 anti-B220 coated cells followed by AOM, respectively. Green and blue lines indicate survival curves of mice treated with 2.0×10^7 anti-CD8 coated cells followed by AOM and 2.0×10^7 anti-B220 coated cells without AOM.

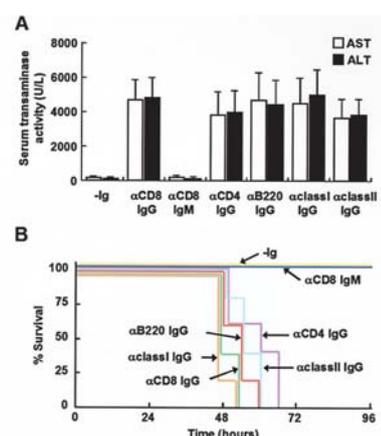


Figure 3. Pretreatment of IgG class mAbs induced lethal liver damage. (A) Mice were intravenously injected with 2×10^7 of indicated mAb-coated spleen cells and subsequently followed by subcutaneous AOM injection. Control mice received the same number of spleen cells without mAb treatment. Serum transaminase values of AOM-treated mice were examined 42 h after treatment. Values represent means \pm SE. (B) Survival of mice treated with mAb-coated spleen cells and AOM. Green, blue, purple, red, brown, and light blue lines indicate survival curves of mice treated with anti-CD8 IgG, anti-CD8 IgM, anti-CD4 IgG, anti-B220 IgG, anti-class I IgG, and anti-class II mAbs, respectively. Yellow line indicates survival curve of control mice.

Hepatocellular necrosis was not evident in tissues from mice treated with AOM alone (Fig. 1D) or mAb alone (Fig. 1E). These results suggested that concomitant use of mAb and cytotoxic drug resulted in lethal liver damage.

Liver damage was induced by antibody coated cells. To extend these observations, mice (8 mice per group) were treated with i.v. injection of anti-B220 mAb (clone RA3-6B2; 200 μ g) that targeted B cell surface as a murine model of rituximab treatment. Subsequently, mice were administered 15 mg AOM per kg body weight by subcutaneous injection. The combination of these two treatments represented a combination of therapeutic antibody treatment and chemotherapy for the treatment of cancer. Similar to anti-CD8 mAb pretreatment, all mice died within 72 h of a single injection of AOM (Fig. 2A) and serum AST and ALT levels were markedly elevated in mice receiving both mAb and AOM together (Fig. 2B). In contrast, minimum or no liver damage was induced by separate injection of 15 mg/kg AOM or anti-B220 mAb treatment, respectively (Fig. 2B).

Next, mice received i.v. injection of the mAb-coated spleen cells instead of free mAb treatment. Syngeneic spleen cells were incubated with 5 μ g/ml of anti-B220 mAb at 4°C for 30 min then washed 4 times with PBS. The indicated number of mAb-coated spleen cells was intravenously injected into mice from the tail vein. As shown in Fig. 2C, serum AST and ALT levels were elevated 24 h after AOM treatment according to the number of mAb-coated cells injected into mice. Serum AST and ALT levels were also elevated after AOM treatment in mice that pretreated with i.v. injection of anti-CD8 mAb coated spleen cells (Fig. 2C). Consistent with these results, all animals died within 50 h when mice received more than 2.0×10^6 mAb-coated spleen cells, 50% of animals died within 50 h when mice were injected with 6.7×10^5 antibody coated spleen cells (Fig. 2D). These results indicated that antigen-antibody complex, i.e. mAb-coated cell, were involved in the mechanisms of liver damage. Consistent with previous results, all mice without AOM treatment survived and no liver damage was observed even though they received 2×10^7 mAb-coated cells, indicating that both mAb or mAb-coated cells and cytotoxic drug (AOM) is necessary for the induction of severe liver damage.

IgM class mAb treatment did not induce lethal liver damage. Similar results were obtained when using an anti-CD8 mAb (anti-Lyt-2.2: mouse IgG2a, κ), anti-CD4 mAb (GK1.5: rat IgG2b, κ), anti-B220 mAb (RA3-6B2: rat IgG2a, κ), anti-mouse MHC class I mAb (M1/42: rat IgG2a, κ) and anti-mouse class II mAb (25-9-17S: mouse IgG2a, κ). As shown in Fig. 3, severe liver damage and mortality was observed in mice that received 2×10^7 of indicated mAb-coated spleen cells and AOM, demonstrating that this effect was not attendant on the use of one particular mAb. It is of note that lethal liver damage was induced when mice received spleen cells treated with mAbs of the IgG class. In contrast, no liver damage was observed and no mice died when spleen cells were coated with anti-CD8 mAb of the IgM class (3.155: rat IgM).

Intrahepatic expression of genes responsible for lethal liver damage. To investigate mechanisms leading to fulminant

hepatic failure in this study, mRNAs were extracted from the livers of mAb- and/or AOM-treated mice and the intrahepatic expression of genes related to liver injury was examined by RT-PCR. Fas and FasL mRNA expression was increased in mice treated with both mAb and AOM; however, the same was true for mice treated with mAb alone. In addition, mRNAs for inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-12p40 were also increased by treatment with mAb alone (Fig. 4). These results suggest that the liver is in some way 'preconditioned' or sensitized by mAb treatment and that subsequent exposure to AOM synergistically induces profound liver damage even though mAb treatment itself or the amount of AOM injected is not hepatotoxic.

Intrahepatic expression of Fas and FasL. Livers were harvested from mice 3 h after treatment in order to assess the expression of Fas and FasL. Hepatocytes and intrahepatic lymphocytes were isolated and their expressions of Fas and FasL were examined by flow cytometry. As shown in Fig. 5, strong induction of FasL expression was detected in Kupffer cells and NKT cells (Fig. 5A and B, respectively). Interestingly, mAb treatment alone upregulated FasL expression on Kupffer cells but not on NKT cells, while AOM alone induced strong expression of FasL on NKT cells, but not Kupffer cells. Furthermore, not only Fas message but also cell surface expression of Fas was increased on hepatocytes after either mAb or AOM treatment; after treatment with both, hepatocytes exhibited a 4.5-fold increase of Fas expression (Fig. 5C). These results suggest that after 'preconditioning' by mAb, interaction of Fas on the hepatocytes and FasL on Kupffer cells and/or NKT cells might be responsible for the liver damage.

Absence of either Kupffer cells or NKT cells prevents lethal liver damage induced by mAb and AOM. To examine whether Kupffer cells do play a role in this process, mice were intraperitoneally injected with GdCl₃ (2 mg/mouse) to deplete and/or suppress Kupffer cells 24 h prior to administration of mAb and 15 mg/kg AOM. All GdCl₃ treated mice now survived and the levels of serum AST and ALT activity were normal in the absence of Kupffer cells, whereas lethal hepatic damage was induced in control PBS-injected animals (Fig. 6A and B). This suggests that activation of Kupffer cells by mAb and/or AOM is responsible for the severe hepatotoxicity observed.

Next, we investigated the role of V α 14 NKT cells in the development of fulminant hepatic failure using V α 14 NKT-KO mice lacking only these V α 14 NKT cells. Again, neither liver damage nor lethality was observed in mice treated with mAb+AOM in V α 14 NKT-KO mice, suggesting that the activation of NKT cells also contributes to the liver damage (Fig. 6C and D).

Fas-dependent liver damage. To examine whether Fas-FasL interaction is responsible for the lethal liver damage in this system, Fas-deficient (*lpr*) mice were treated with anti-B220 mAb or anti-B220 mAb-coated spleen cells. Consistent with previous results, all C57BL/6 wild-type animals pretreated with mAb-coated cells died within 42 h after AOM treatment and all wild-type mice pretreated with i.v. mAb injection

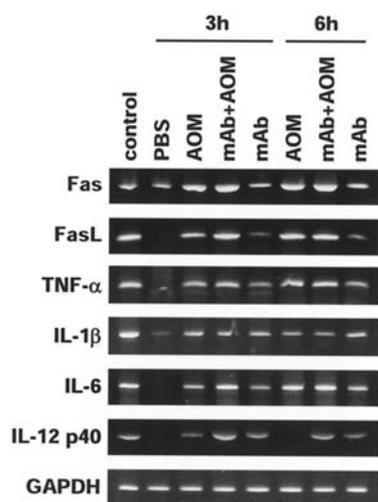


Figure 4. Induction of mRNA for Fas, FasL and cytokines in the livers of AOM- and/or mAb-treated mice. Livers were harvested from mice 3 or 6 h after treatment. RNA was extracted and analyzed by reverse-transcription PCR. Data shown are representative of 3 experiments with similar results.

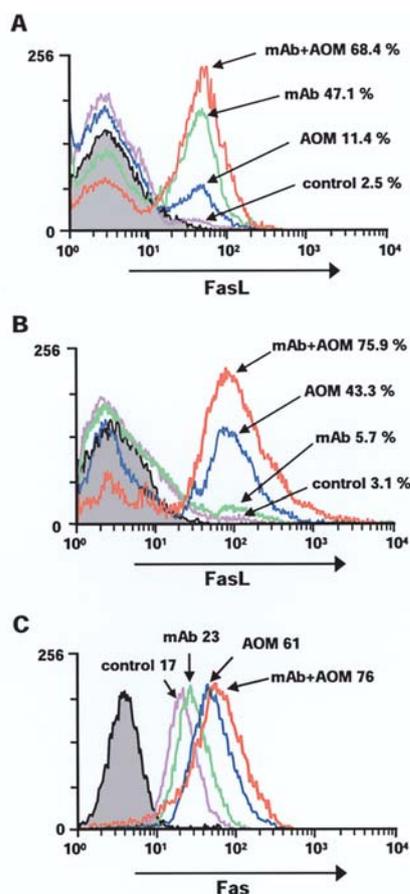


Figure 5. Expression of FasL on Kupffer cells (A) and NKT cells (B) and Fas on hepatocytes (C). Livers were harvested from mice 3 h after treatment. Hepatocytes and intrahepatic lymphocytes were isolated and their Fas and FasL expression examined by flow cytometry. Red lines, staining of cells from mice injected with mAb and AOM; green lines, mAb-injected mice; blue lines, AOM-injected mice; pink lines, PBS-injected mice (control); black lines, background staining with isotype-matched control IgG. (A) Percentages of FasL-positive Kupffer cells were 2.5% (PBS-injected), 11.4% (AOM-injected), 47.1% (mAb-injected), and 68.4% (mAb and AOM-injected). (B) Percentages of FasL-positive NKT cells were 3.1% (PBS-injected), 43.3% (AOM-injected), 5.7% (mAb-injected), 75.9% (mAb and AOM-injected). (C) Numbers indicate mean fluorescence intensities of Fas expression on hepatocytes. Similar results were obtained in three independent experiments.

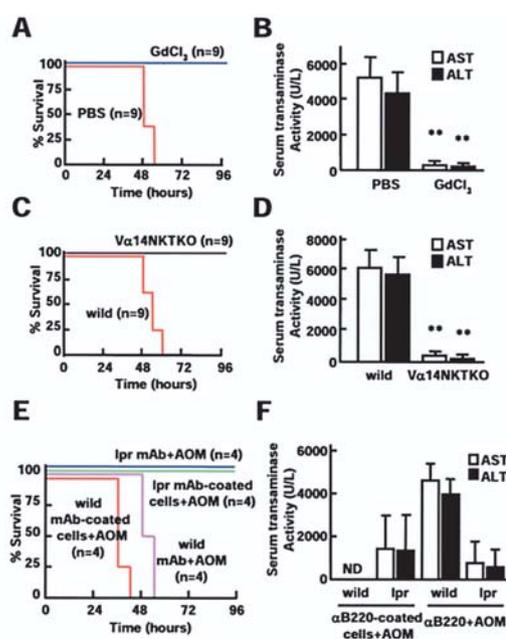


Figure 6. Absence of Kupffer cells, NKT cells or Fas-antigen abrogates lethal liver damage induced by mAb and AOM treatment. Survival rates after mAb and AOM treatment are shown for Kupffer cell-depleted (A), NKT cell-deficient mice (C) and Fas-deficient mice (E). Blood was collected 42 h (B, D and F) after treatment and serum transaminase activity was examined in these mice. ** $p < 0.01$. To deplete Kupffer cells, mice were i.p. injected with 2 mg of $GdCl_3$ 24 h prior to the administration of AOM and mAb. (A) Survival rates after mAb and AOM treatment are shown for Kupffer cell-depleted mice (blue line) and control PBS treated mice (red line). $V\alpha 14NKT$ KO mice and Fas-deficient mice (lpr/lpr) were also given AOM and mAb. Survival rates of $V\alpha 14NKT$ KO mice and control wild-type mice were shown in purple and red, respectively (C). Blue and green lines indicate survival rate of Fas-deficient mice (lpr/lpr) either treated with free anti-B220 mAb or anti-B220 mAb-coated spleen cells, while pink and red lines indicate that of wild-type mice treated with free anti-B220 mAb or anti-B220 mAb-coated spleen cells (E).

died within 50 h (Fig. 6E). In contrast, all lpr mice survived after AOM injection with pretreatment of mAb or mAb-coated cell injection (Fig. 6E). Furthermore, elevation of serum ALT activity observed in lpr mice was less severe than that of wild-type animals (Fig. 6F). These results indicate that the lethal liver damage after the combination of mAb and cytotoxic drug treatment is dependent on Fas-FasL pathway.

Discussion

In the present study, we demonstrate that mAb treatment 'preconditioned' the liver by activating Kupffer cells to produce inflammatory cytokines, e.g. $TNF-\alpha$ and $IL-12$, and induced FasL expression on Kupffer cells and NKT cells. Concurrent upregulation of Fas on the hepatocytes then rendered these cells sensitive to apoptosis. Thereafter, subsequent treatment with AOM, that causes mitochondrial injury, synergistically induced profound liver damage, resulting in fulminant hepatic failure.

Thus, sudden lethal liver damage was observed in experimental animals when two agents commonly used in the clinic were applied together, although they are usually perfectly safe and reliable if used separately. Here, depletion of $CD8^+$ cells was accomplished by i.v. injection of anti- $CD8$ mAb

and B cells were targeted by anti-B220 mAb, paralleling cancer treatment by therapeutic mAb such as rituximab. Subsequently, mice received a subtoxic dose of AOM. It has been reported that AOM acts as a mitochondrial toxin and causes dose-dependent centrilobular necrosis of the liver (10). Although the amount of AOM used in this study (15 mg/kg) was much less than necessary to induce hepatic failure by direct hepatotoxicity (50-100 mg/kg), mAb pretreated mice subsequently given low-dose AOM developed hepatocellular necrosis with elevated serum transaminase levels. Our results suggest a potential risk of using therapeutic antibodies together with chemotherapeutic drugs that might induce lethal hepatic damage, because many of the latter are as cytotoxic as AOM. The results presented here are therefore clinically relevant.

Regarding mechanisms, a number of points can be made. First, intrahepatic expression of inflammatory cytokines by and cell surface expression of FasL on Kupffer cells was observed within 3 h of treatment, suggesting that these cells were rapidly activated by mAb pretreatment (Figs. 4 and 5). Kupffer cells account for approximately 15% of the total number of liver cells and for 80-90% of resident macrophages in the entire body. They are located on the inside of the sinusoidal walls, from which they protrude, and are found predominantly in the periportal region. It is known that Kupffer cells take up and eliminate soluble circulating IgG immune complexes (17). It is also known that IgG immune complexes are capable of activating macrophages to produce inflammatory cytokines (18). Furthermore, it has been reported that Kupffer cells efficiently engulf apoptotic bodies and that this can result in production of inflammatory cytokines (19) and upregulation of FasL expression (20). Consistent with these previous reports, we found that Kupffer cells were indeed activated, produced TNF- α , IL-1 β , IL-6 and IL-12 mRNAs and expressed FasL after mAb treatment (Fig. 4). Importantly, antibody-coated spleen cells induced lethal liver damage more rapidly than free mAb treatment (Fig. 2A and D). Therefore, in this respect, the liver was 'preconditioned' probably as a consequence of Fc receptor-mediated uptake of IgG immune complexes and engulfment of apoptotic bodies by Kupffer cells. The fact that IgM class mAb did not induce lethal liver damage also supports the importance of Fc γ receptor-mediated activation of Kupffer cells (Fig. 3). Furthermore, depletion of Kupffer cells by GdCl₃ treatment prevented lethal liver damage, suggesting the pivotal role of these cells in its pathogenesis (Fig. 6A and B).

Not only Kupffer cells, but also NKT cells were involved in the pathogenesis of lethal liver damage. NKT cells constitute a distinct subpopulation of T cells with unique antigen specificity and rapidly inducible effector functions. It has been reported that these cells rapidly upregulate surface FasL expression and FasL-mediated cytotoxicity upon ConA administration (13,21). In the present study, we also detected FasL expression on NKT cells (Fig. 5B), and determined that lethal liver damage did not occur in the absence of these cells (Fig. 6C and D). This suggests that Fas-FasL interactions between hepatocytes and NKT cells may be responsible for mediating liver damage, following activation of Kupffer cells as the initiating event. In this scenario, the NKT cells were secondarily activated probably via IL-12 secreted by activated Kupffer cells, because mAb treatment alone did

not change FasL expression on NKT cells. For this to occur, subsequent AOM injection was required (Fig. 5B) (22).

Finally, hepatocytes were sensitized to Fas-FasL mediated cytotoxicity by increased surface Fas expression. These results were confirmed by the fact that all Fas-deficient mice survived after mAb and AOM injection (Fig. 6E and F). Furthermore, TNF- α and the other factors released by activated Kupffer cells might play additional roles in liver injury. Therefore, all of these events may have synergized to produce unanticipated lethal liver damage after mAb treatment and subsequent AOM injection.

A similar scenario might be operative in therapeutic antibody application in the clinic. Importantly, there are some cases where treatment of patients with antibody has indeed resulted in hepatotoxicity (23,24). Therapeutic antibodies can function primarily by three modes of action: by blocking the action of specific molecules, by targeting specific cells or by functioning as signaling molecules. Targeting involves directing antibodies towards specific populations of cells, as we did in this study. Both trastuzumab and rituximab, two mAb representative of this type, that have been approved for the treatment of cancer, do not have a radioisotope or toxin attached to them (7). The elimination of the target of these antibodies depends entirely on the effector mechanisms of the body, that is, complement activation and Fc-receptor-dependent responses. These Fc-receptors are located in the membrane of various effector cells, such as NK cells, neutrophils, monocyte/macrophages including Kupffer cells, dendritic cells and B cells (1,2,7). Therefore, Kupffer cells and NKT cells can be activated by this type of therapeutic mAb and consequently the liver could become preconditioned for hyperreactivity to cytotoxic drugs that are not commonly hepatotoxic.

In conclusion, we have demonstrated a potential risk of lethal hepatic failure in the concomitant use of therapeutic antibodies and cytotoxic drugs. A possible side effect of antibody therapy is mediated through intrahepatic activation of the immune system, the very mechanism of action on which this treatment depends. Attention needs to be paid to this issue for the concomitant use of any drugs during treatment with therapeutic antibodies.

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