Brief Definitive Report

A Monoclonal Antibody to the α2 Domain of Murine Major Histocompatibility Complex Class I that Specifically Kills Activated Lymphocytes and Blocks Liver Damage in the Concanavalin A Hepatitis Model

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Abstract

We earlier found that a rat monoclonal antibody (mAb) RE2 can induce rapid death of murine activated, but not resting, lymphocytes and lymphocyte cell lines, in a complement-independent manner, a cell death differing from typical apoptosis or necrosis. We here found that this cell death is independent of pathways involving Fas, caspase, and phosphoinositide–3 kinase. With the advantage of producing human B cell line transfectants with stable expression of human/mouse xeno-chimeric MHC class I genes, we found that RE2 epitope resides on the murine class I α2 domain. However, the α3 domain plays a key role in transducing the death signal, which mediates extensive aggregation of the MHC class I–integrin–actin filament system, giving rise to membrane blebs and pores. In mouse models with T/NKT cell activation–associated fulminant hepatitis, administration of mAb RE2 almost completely inhibited the development of liver cell injuries. Taken collectively, this form of cell death may be involved in homeostatic immune regulation, and induction of this form of cell death using the mAbs may be potentially therapeutic for subjects with immunological diseases mediated by activated lymphocytes.

Key words: adhesion molecule • cell death • cytoskeleton • immunotherapy • MHC class I

Introduction

Two types of cell death, apoptosis characterized by cellular shrinkage, membrane blebbing, and nuclear disruption, and necrosis characterized by cellular swelling, rupture of plasma membrane, and swelling of mitochondria, both participate in regulatory, protective, and pathogenic processes in the immune system (1–3). In earlier studies, we incidentally found that a rat mAb RE2, raised against MHC-associated cell surface components of a T cell clone, has the potential to specifically kill activated, but not resting, murine lymphocytes and lymphocyte cell lines in the ab-

sence of complement, irrespective of mouse strains (4). This pathway begins to occur rapidly and much faster than that seen in a complement-dependent cytolysis, i.e. within 5 min after target cells were exposed to mAb RE2. Electron microscopically, while dying cells formed gigantic pores on the cell surface, there was neither indication of DNA fragmentation nor swelling of mitochondria during the cytolysis; thus we considered it to be a novel form of cell death. Although mAb RE2 killed only activated lymphocytes and lymphocyte cell lines, it did immunoprecipitate 90, 60, and 44 kD molecules on the cell surface of virtually all organs, irrespective of mouse strains. These findings suggested that the target RE2 antigen resides on MHC class I molecules and that some lymphocyte-unique class I–associated molecules are also involved in this form of cell death.

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After this study, there were reports of human lymphocyte death induced by antibody-mediated ligation of HLA class I molecules (5–7). Skov et al. (6) reported that ligation of HLA class I molecules on human T cells induces cell death through phosphoinositide-3 kinase (PI-3) kinase–induced c-Jun NH2-terminal kinase activity, distinct from that induced by the Fas/Fas ligand pathway. Genestier et al. (8) demonstrated the anti-HLA class I–induced T cell apoptosis that was inhibited by okadaic acid, an inhibitor of phosphatases 1, 2A, and 2C. In the present studies, we investigated the RE2 epitope, and the mechanism of RE2-mediated cell death was examined.

Materials and Methods

Mice and Cells. C57BL/6 (B6) and MRL/*lpr* mice were obtained from Japan SLC Inc. Mouse strains deficient in β2-microglobulin and TAP-1 were provided by Prof. H. Ishikawa, Keio University School of Medicine (Tokyo, Japan). LFA-1–deficient mice originally generated by R. Schmits et al. (9) were donated by Dr. G. Matsumoto, Kanagawa Dental College (Kanagawa, Japan). IL-2–dependent T cell clone MS-S2 has been established from a C3H mouse, as described previously (10).

mAbs and Reagents. mAbs to murine CD3 (2C11), CD4 (GK1.5), CD8 (53–6.7), CD11a/LFA1 (M17/4), CD11b/Mac1 (M1/70), NK1.1 (PK136), and CD69 (H1.2F3) were purchased from BD Biosciences. The rat mAb RE2 was raised by immunizing a rat with cell lysate of a mouse T cell clone, as described (4), and purified using a protein G-Sepharose column (Pharmacia LKB, Biotechnology AB). Latrunculin B was purchased from Biomol Res. Lab., Z-VAD-fmk and Z-Asp-DCB from Peptide Institute, Inc., Concanavalin A (Con A) from Seikagaku Co. Other reagents used were purchased from Sigma-Aldrich.

Transfectants with Human/Mouse Chimeric MHC Class I Genes. C1R cells (10⁷ cells), a human EBV-transformed B cell line deficient in expressing HLA-A and -B genes (11), were transfected with 20 μg/ml of human HLA B7, mouse H-2K^b and their hybrid genes (12), in the presence of 2 μg/ml of pSV-neo, using electroporation method. Transfected cells were selected in geneticin (0.25 mg/ml) in in vitro culture over a 4-wk period. Antibiotic-resistant clones were isolated and expanded, and expression of the hybrid MHC class I molecules was confirmed, using flow cytometric analysis with FACStar^PLUS™ (Becton Dickinson).

Flow Cytometric Analysis and Cytotoxic Activity Assay. Expression of RE2 molecules was analyzed by incubating target cells with a rat mAb RE2, followed by FITC-conjugated mouse anti–rat immunoglobulins on ice, and subjected to the analysis using FACStar^PLUS™. Assay for cytotoxic activity of mAb RE2 was done by incubation of a mixture of target cells (10⁷ cells/ml) suspended in RPMI 1640 medium supplemented with 2% de-complemented fetal calf serum and the mAb (3 μg/ml) for 1 h at 37°C. Before incubation with mAb RE2, splenic cells were incubated with Con A (2 μg/ml) for 24 h at 37°C. Percentages of cell lysis were determined by trypan blue dye exclusion or by propidium iodide (PI) staining of cells using FACStar^PLUS™, in triplicate. As the results using PI staining were almost identical to those obtained by trypan blue dye exclusion, data of the latter test were shown in the present studies. Potential inhibitors for the cytotoxicity of mAb RE2 to MS-S2 cells, Z-VAD-fmk (13), Z-Asp-DCB (14), LY294002 (15), okadaic acid (8), cytochalasin D (16), and latrunculin B (17), were added in cell cultures 1 h before, and wortmannin (6) 2 h before addition of mAb RE2.

Amounts of reagents used were determined by preliminary tests for each inhibitor, referring to the methods described previously (6, 8, 13–17).

Fluorescence Microscopic Studies. MS-S2 cells were incubated with Cy5.5-conjugated mAb RE2 for 10 min on ice, followed by Alexa568-conjugated rat anti-LFA-1 mAb or anti-CD4 mAb and Oregon Green 488–conjugated phalloidin for staining actin filaments for 10 min on ice. After washing in cold PBS containing 2% FCS, cells were examined under an epi-fluorescence microscope BX-60 (Olympus). Color image of cells for each dye was acquired sequentially using cognate filter-set and cooled CCD camera (PXL 1400; Photometrics), and then assigned pseudo-color. Overlaid image was obtained by superposing pseudo-color-assigned images.

Con A–induced Hepatitis Model. Hepatitis was induced by giving an intravenous injection of 0.18 mg/mouse Con A dissolved in pyrogen-free saline into 9-wk-old C57BL/6 mice. A group of mice was given an intraperitoneal injection of mAb RE2 (0.8 mg/mouse) immediately after the Con A injection. As a control, a comparable amount of polyclonal rat IgG (Sigma-Aldrich) was injected. 24 h after the Con A injection, blood samples, liver tissues, and liver mononuclear cells from these mice were collected. Serum levels of glutamic oxaloacetic transaminase (GOT) and
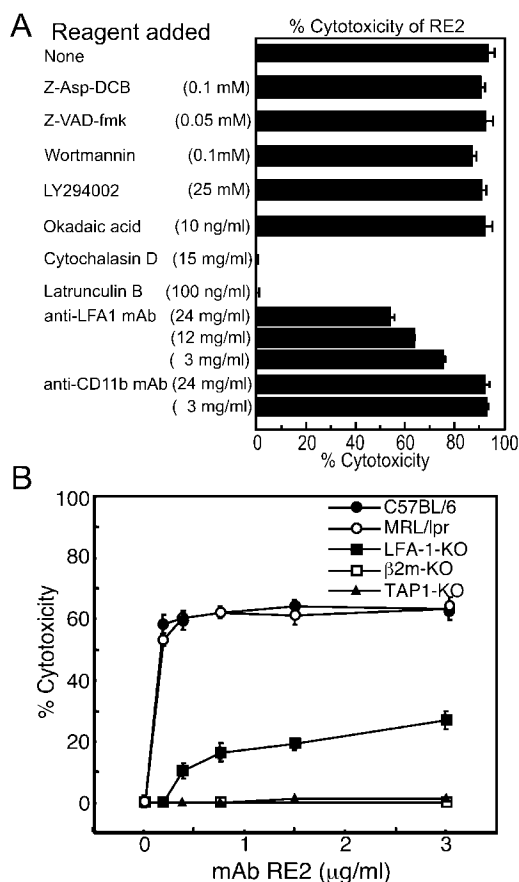

Figure 1. (A) Effects of potential inhibitors on cytotoxic activity of mAb RE2 to T cell clone MS-S2 cells. Inhibitors were added 1 or 2 h before the cytotoxicity assay. Percentages of dead cells were determined by trypan blue dye exclusion. (B) Cytotoxic sensitivity to mAb RE2 of Con A–activated splenic cells from various mouse strains. Splenic cells were first activated with 2 μg/ml of Con A for 24 h at 37°C, and then incubated with given concentrations of mAb RE2 for 1 h in the absence of complement.

glutamic pyruvic transaminase (GPT) were determined, using a standard clinical automatic analyser (Hitachi, Type 7350). Liver mononuclear cells were isolated as descried (18), double or triple stained with a combination of PE-conjugated anti-CD69 mAb, FITC-conjugated anti-NK1.1, anti-CD4, or anti-CD8 mAbs, and APC-conjugated anti-CD3 mAb, and were subjected to the analysis using FACStar[PLUS™]. For histopathological examinations, liver tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Results and Discussion

To precisely characterize the mechanism of mAb RE2-induced cell death, we first examined effects of several potential inhibitors on in vitro cytotoxic activity of mAb RE2 to the killing-susceptible murine T cell line MS-S2, in the absence of complement. The caspase inhibitors, Z-VAD-fmk (13) and Z-Asp-DCB (14); PI-3 kinase inhibitors, wortmannin (6) and LY294002 (15), and an inhibitor of phosphatase 1, 2A, 2C, okadaic acid (8) did not inhibit the cytotoxic activity (Fig. 1 A). As Con A–activated splenic cells from Fas-deficient MRL-*lpr/lpr* mice were also susceptible to the cytotoxic activity of mAb RE2 (Fig. 1 B), it was evident that this cell death differs from that involving the Fas/Fas ligand, caspase, phosphatase 1, 2A, 2C, and PI-3 kinase pathways.

To determine whether the cell death is mediated by MHC class I molecules, we examined the sensitivities to mAb RE2 of Con A–activated splenic cells from mutant mice deficient in TAP-1 (transporter associated with antigen processing-1) and those deficient in β2 microglobulin. Compared with the finding in normal healthy C57BL/6 mice, splenic cells from these two mutant strains were both resistant to the cytotoxic activity (Fig. 1 B). Based on these

data, we then investigated the RE2 epitope on mouse MHC class I molecules, taking advantage of producing transfectants of a HLA-A, -B negative human C1R cell line of B cell origin, expressing xeno-chimeric MHC class I molecules composed of either murine H-2K[b] or human HLA-B7 at α1, α2, and α3 domains (Fig. 2). In flow cytometric analysis of in vitro binding activities of mAb RE2, only transfectants expressing class I molecules carrying murine α2 domains were recognized by mAb RE2, while others with human α2 were negative, indicating that the epitope resides on murine class I α2 domain. Of note was the finding, however, that when we examined the cytotoxic sensitivity of these transfectants to mAb RE2, only those expressing class I with a combination of murine α2 and human α3 domains were killed. Thus, it is suggested that after α2 domain-mediated cross-linking of class I, α3 domain may play a critical role in transducing death signals to target cells, in a species-specific manner, i.e., human α3 domain-mediated signal transduction to human target cells and murine α3 domain to murine target cells. Our transfectants expressing α3 domain of human MHC class I were designed to carry the human cytoplasmic domain of class I molecules (12). However, because the class I–mediated signal transduction does not require the cytoplasmic domain (19), we assumed that some lymphocyte-unique α3 domain-associated molecules are involved in this form of cell death.

In this context, as shown in Fig. 1 A, we found that both cytochalasin D that depolymerizes cytoskeletal actin filaments to actin monomer (16) and latrunculin B that reduces the monomeric actin pool available for polymerization (17) completely inhibited the mAb RE2-mediated target MS-S2 cell lysis, while colchicine that depolymerizes
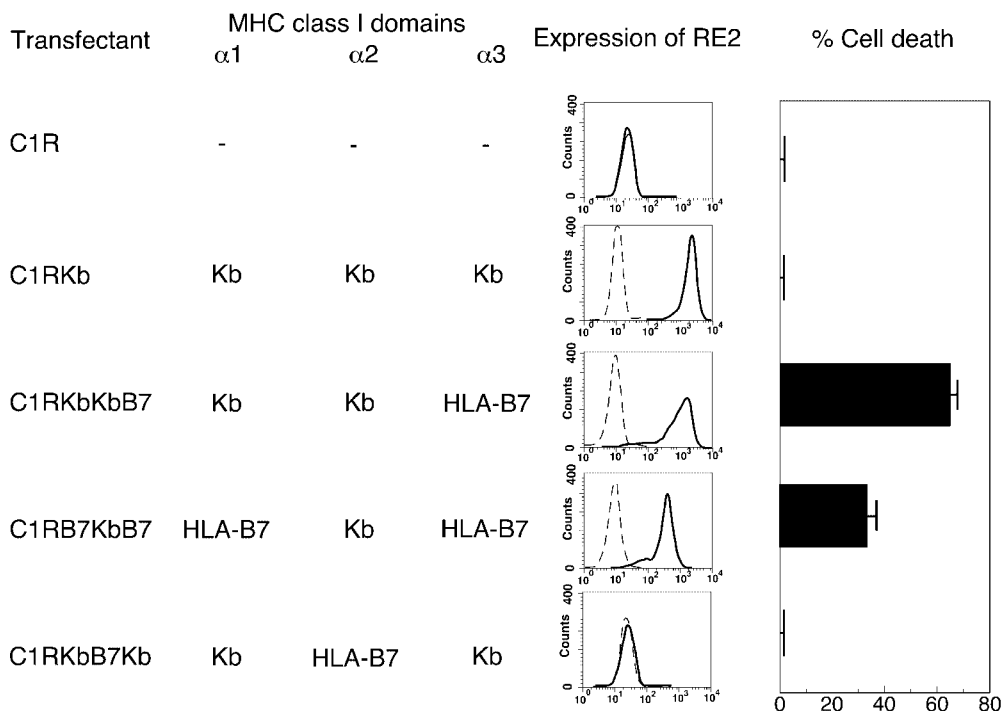
| Transfectant | MHC class I domains | | | Expression of RE2 | % Cell death |
	α1	α2	α3		
C1R	-	-	-		
C1RKb	Kb	Kb	Kb		
C1RKbKbB7	Kb	Kb	HLA-B7		
C1RB7KbB7	HLA-B7	Kb	HLA-B7		
C1RKbB7Kb	Kb	HLA-B7	Kb		

Figure 2. Expression of RE2 epitope and cytotoxic sensitivity to mAb RE2 of the human HLA-A, B-negative EBV-transformed B cell line C1R, transfected with xeno-chimeric MHC class I genes composed of either murine H-2K[b] or human HLA-B7 at α1, α2, and α3 domains. Expression of RE2 molecules was analyzed by incubating on ice target cells with mAb RE2, followed by FITC-conjugated mouse anti–rat immunoglobulins, and cells were subjected to the analysis using FACStar[PLUS™]. Cytotoxic sensitivity of transfectants to mAb RE2 was analyzed, as described in Materials and Methods (right column).

microtubule filaments had no effects (4). Therefore, we considered that the cell death is related to disorganization of the cytoskeletal actin filament system through signals of yet to be determined mediator molecules connected to class I α3 domain. There were reports indicating that both MHC class I molecules and integrins are connected to cytoskeletal actin filament networks (20) and that one of the antibodies to CD47, known as an integrin-associated protein, can induce rapid cell death resembling that induced by mAb RE2 (21). To determine if the cell death involves integrin molecules, we examined whether anti-integrin mAb may block the cytotoxic activity of mAb RE2 to MS-S2 cells. As shown in Fig. 1 A, an anti-integrin LFA-1 mAb blocked, though not completely, the RE2-induced killing of these cells, in a dose-dependent manner. To confirm this

finding, we then examined the cytotoxic sensitivity to mAb RE2 of Con A–activated splenic cells from mice deficient in LFA-1 (9). The results showed that these cells were resistant, though not completely, to the cytotoxicity of mAb RE2 (Fig. 1 B), thus indicating that LFA-1 plays an important role for this form of cell death, as a mediator, although other integrins and/or factors may also be involved in this process. This raised a question if the effect of integrin is ligand-dependent. Ligand binding by integrins is known to require divalent cations. However, as addition of EDTA in cell culture did not protect this mechanism of cell death (4), the role of LFA-1 was thought to be ligand-independent. Thus, it is suggested that the cell death is mediated by ligand-independent binding of integrins to MHC class I α3 domain, which eventually causes polymerization
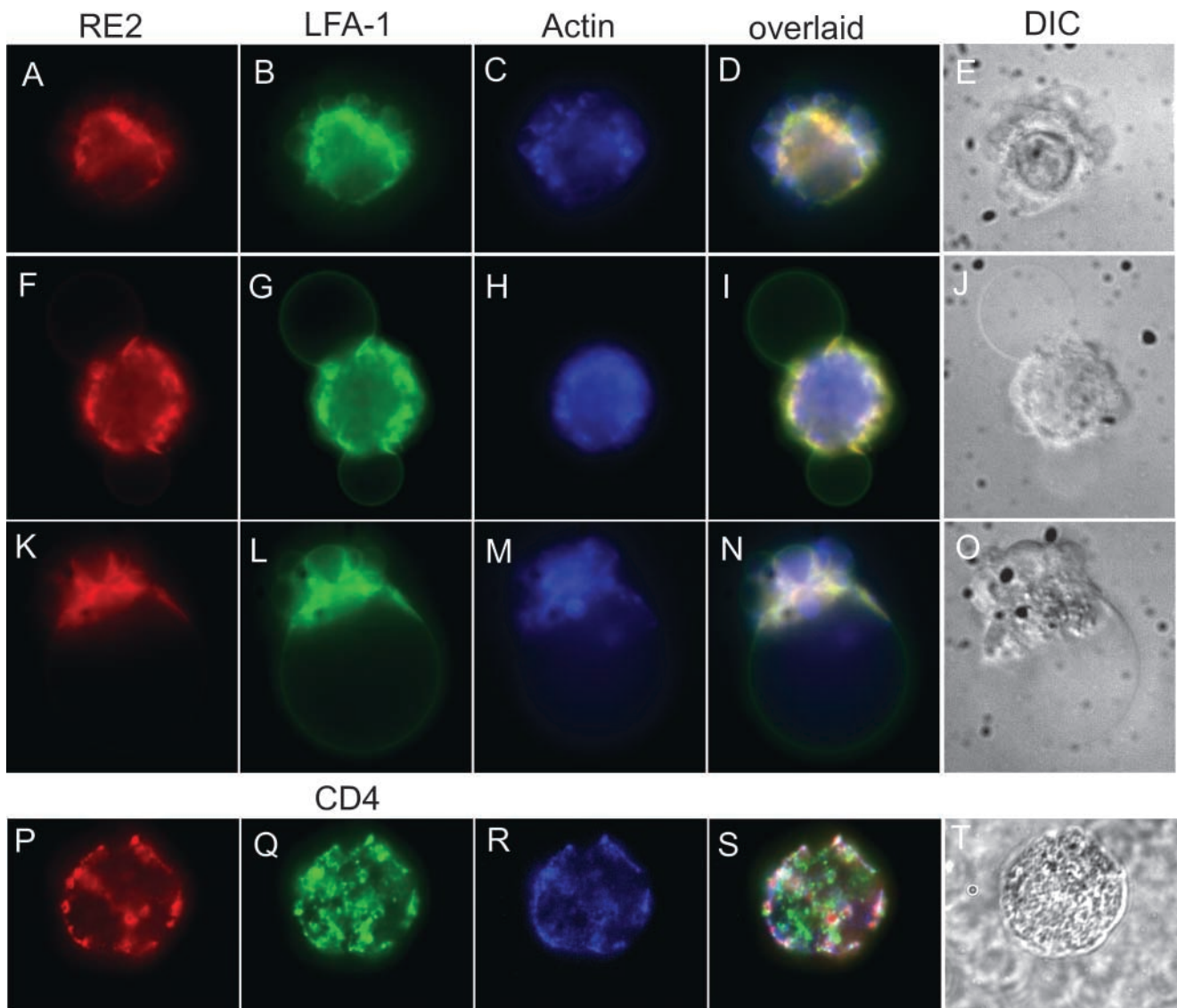
Figure 3. Coaggregation of MHC class I (RE2), LFA-1, and actin filament networks on dying cells with membrane blebs. MS-S2 cells were stained with mAb RE2 (red; A, F, K, and P), mAb LFA-1 (green; B, G, and L) and phalloidin for actin filaments (blue; C, H, M, and R). Coaggregation of these three molecules is represented by overlaid images in white (D, I, and N). Control CD4 molecules were not colocalized with RE2 (P–S). Differential interference contrast (DIC) images (E, J, O, and T) are also shown.

of actin filament networks, a hypothesis consistent with the finding of species specificity of class I α3–mediated signal transduction, as shown in Fig. 2.

Involvement of MHC class I, LFA-1, and actin filament system in this form of cell death was supported by triple fluorochrome staining of the dying cells. Target MS-S2 cells were incubated on ice with a mixture of fluorochrome-labeled mAb RE2, mAb LFA-1, and phalloidin for actin filaments. Under the epi-fluorescent microscopic examination, many membrane blebs formed all over the cell surface. At an early phase, sizes of blebs were relatively small and homogenous (Fig. 3, A–E). With time, there appeared gigantic blebs (Fig. 3, F–J) and cytoplasmic structures were disorganized and polarized (Fig. 3, K–O). On the cell membrane, RE2 (MHC class I), LFA-1, and actin filaments were colocalized in an aggregated form (Fig. 3, D, I, and N), and gigantic blebs were likely to have formed from sites of disrupted MHC class I–integrin–actin filament networks (Fig. 3 I). As a control, MS-S2 cells were triple stained with mAbs RE2, anti-CD4, and phalloidin. As shown in Fig. 3, P–S, CD4 molecules were not colocalized. Non-activated splenic cells or MS-S2 cells

treated with cytochalasin D were negative for these findings (unpublished data).

Taken collectively, the cell death seen in the present study appears to be initiated by extensive coligation of MHC class I molecules through cross-linking of α2 domains by mAb RE2. Such cross-linked class I molecules probably lead to aggregation of integrins, through class I α3 domains, eventually causing disorganization of the cytoskeletal actin filament system, giving rise to formation of large membrane blebs on the cell surface. Because an increase in the cell surface density of class I molecules occurs on activated lymphocytes (20), and because integrin like LFA-1 is preferentially expressed on lymphocytes, only activated lymphocytes are thought to be susceptible to the cytotoxic activity of mAb RE2. However, the mechanism of this type of cell death may be more complex, because our preliminary data showed that the treatment of lymphocytes with IFN-γ alone, which up-regulates MHC class I, but not LFA-1, expression, did not render cells susceptible to the cytotoxicity of mAb RE2. Further studies on this point are ongoing in our lab. The majority of murine anti-MHC class I antibodies do not have the potential to kill ac-
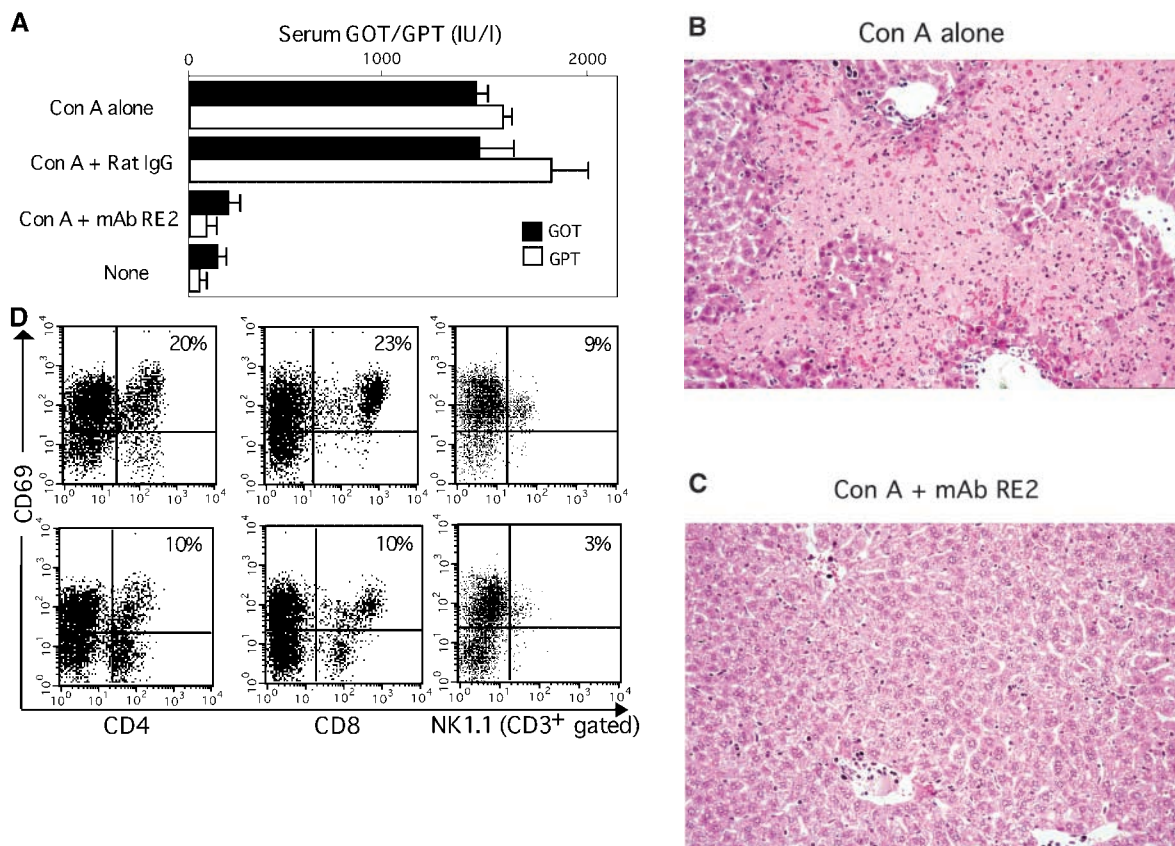

Figure 4. Therapeutic effects of mAb RE2 on Con A–induced hepatitis in mice. (A) Serum levels of GOT and GPT in C57BL/6 mice ($n = 6$) 24 h after an intravenous injection of Con A, with or without administration of mAb RE2. Closed and open bars represent serum levels of GOT and GPT, respectively. (B) Histopathology of massive liver cell necrosis seen in a mouse 24 h after an intravenous injection of Con A. (C) Absence of Con A–induced liver cell necrosis in a mouse treated with mAb RE2. (D) Reduction of CD69-positive activated CD4^{+} and CD8^{+} T cells and NK1.1^{+} CD3^{+} NKT cells in the liver of mice given Con A and mAb RE2 (bottom), compared with findings in mice given Con A alone (top). NKT cells were examined by triple staining of cells with mAbs to CD69, NK1.1, and CD3, and gated CD3^{+} cells were analyzed for CD69 and NK1.1, using FACStar[PLUS™].

tivated lymphocytes in the manner seen with mAb RE2. This can be attributed to the unique specificity of mAb RE2 that recognizes a monomorphic cross-reactive epitope of the MHC class I α2 domain on both class I K and D molecules (our unpublished data).

Because of its rapid and specific cytotoxic activity to activated lymphocytes, we then attempted to apply this mAb for therapy of Con A–induced hepatitis, an experimental fulminant hepatitis model in mice. Con A causes severe liver cell necrosis in mice within 6 h after an intravenous injection. Development of this disease is mediated by activated T cells (22, 23) and NKT cells (18) and both TNFα and IFN-γ are thought to be critical mediators (18, 24). As shown in Fig. 4, a significant elevation of serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels and histologically evident massive liver cell necrosis were observed in C57BL/6 mice 24 h after an intravenous injection of Con A. In contrast, mice given one intraperitoneal administration of mAb RE2 soon after the Con A treatment showed an almost complete inhibition of such liver cell injuries. Polyclonal rat IgG used as a control was without influence. As mononuclear cells obtained from liver tissues of Con A–treated mice expressed up-regulated MHC class I molecules (unpublished data), we then examined influences of mAb RE2 on activated T cells and NKT cells in the liver of Con A–treated mice. As shown in Fig. 4 D, CD69^{+}-activated CD4^{+} and CD8^{+} T cells and CD69^{+}NK1.1^{+}CD3^{+} NKT cells were all to a great extent reduced in mAb RE2-treated mice, compared with findings in mice treated with Con A alone. As a control, mice were treated with mAb RE2 alone. There were no changes in the number and the frequency of CD4^{+} and CD8^{+} T cells and NKT cells obtained from liver tissues between mAb RE2-treated and nontreated mice (unpublished data).

Our present findings may be important in two respects. First, mAbs that have potential to induce the present form of cell death could be used as an effective means for treating subjects with a variety of disorders mediated by aberrant activated or transformed lymphocytes such as transplant rejection, autoimmune diseases, allergic diseases, and lymphoma/leukemias. Second, this form of cell death may play a part in immune regulation. In addition to the well-established role for antigen presentation, MHC class I may be involved at least in part in activation-induced cell death, through mechanisms by which activated lymphocytes are eliminated by CD8^{+} T cells potentially recognizing the monomorphic portion of class I epitope such as RE2. Involvement in the immune surveillance mechanism for transformed lymphocytes is also possible, as most lymphocyte cell lines are sensitive to the cytotoxicity of mAb RE2 (4).

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