

A Novel Type of Cell Death of Lymphocytes Induced by a Monoclonal Antibody without Participation of Complement

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Summary

A monoclonal antibody, RE2, raised by immunizing a rat with cell lysate of a mouse T cell clone, was found to directly kill interleukin 2-dependent T cell clones without participation of serum complement. Fab fragments of RE2 had no cytolytic activity, while the cross-linking of Fab fragments with anti-rat immunoglobulin reconstituted the cytotoxicity. The cytotoxicity was temperature dependent: the antibody could kill target cells at 37°C but not at 0°C. Sodium azide, ethylenediaminetetraacetic acid, and forskolin did not affect the cytolytic activity of RE2, while the treatment of target cells with cytochalasin B and D completely blocked the activity. This suggested that the cell death involves a cytoskeleton-dependent active process. Giant holes on the cell membrane were formed within 5 minutes after the treatment with RE2, as observed by scanning electron microscopy. There was no indication of DNA fragmentation nor swelling of mitochondria during the cytolysis, suggesting that the cell death is neither apoptosis nor typical necrosis. The antibody also killed T cell lymphomas and T and B cell hybridomas only when these cells were preactivated with concanavalin A, lipopolysaccharide, or phorbol myristate acetate. Preactivated peripheral T and B cells were sensitive to the cytotoxicity of RE2, while resting T and B cells were insensitive. These results provide evidence for a novel pathway of cell death of activated lymphocytes by membrane excitation.

Active cell death is an important mechanism in deleting useless or harmful cells from organisms to maintain normal ontogenic and homeostatic pathways (1–3). It is particularly important in the immune system, where lymphoid cells with autoreactive and useless receptors should be excluded in central and peripheral lymphoid organs (4, 5). The best-known mechanisms of cell death are apoptosis and necrosis. The negative selection of developing T cells is known to be the result of apoptotic death of thymocytes having autoreactive TCRs (6). The cytolysis caused by cytotoxic T and NK cells is apoptotic death, where an active process resulting in DNA fragmentation is involved (7). Cytokines such as TNF and lymphotoxin are known to induce apoptosis of target cells (8). On the other hand, immune mechanisms can induce necrotic death of somatic cells with antibody and complement. Autoantibodies can cause necrotic cell death with the aid of complement and macrophages. Necrosis caused by complement attack, for example, is characterized by swelling of cells, rupture of plasma membranes, and swelling of mitochondria (5). Both mechanisms are effectively used in the immune system to maintain and protect the organism.

We found that a particular mAb raised against cell surface components of a T cell clone constantly killed activated lymphoid cells of T and B cell lineage without complement-dependent membrane damage. The resting T and B cells, as well as nonlymphoid somatic cells, were not killed by the antibody. The cell death was distinct from apoptosis and the usual pattern of necrosis. It involved an active process initiated by the cross-linking of cell surface components. Since only the activated T and B cells were killed by the antibody, it was speculated that this process may play a role in avoiding autoreactivity of lymphoid cells under certain circumstances. This report describes properties of this mAb and characteristic features of cell death caused by the antibody.

Materials and Methods

Animals

C3H/HeN (C3H) mice were purchased from Japan SCL Inc. (Hamamatsu, Japan). Sprague-Dawley rats and a hamster were purchased from Charles River Japan Inc. (Atsugi, Japan).

Cells

Splenic Cells. A T cell-enriched population was obtained as spleen cells nonadherent to anti-mouse Ig-coated dishes as described previously (9). To obtain splenic B cells, spleen cells were depleted of T cells by treatment with T cell-specific rabbit anti-mouse brain serum and complement as described previously (10).

T Cell Clones. IL-2-dependent T cell clones with helper and suppressor functions have been established from C3H, C57BL/6, and (B6 × C3H)F₁ mice as previously described (11–13).

B Cell Lines. Immature B cell clones 46.6 and Ig 6.3 and B cell line WEHI 231 of (BALB/c × NZB)F₁ mice were gifts of Dr. T. Takemori (National Institute of Health, Tokyo, Japan) (14).

Lymphoid Tumor Cell Lines. EL-4, RMA (15), and RMA-S (a class I-defective mutant line derived from RMA) (16) were used as T cell leukemic cell lines.

T and B Cell Hybridomas. T cell hybridoma 7C3-13 has been described previously (17). B cell hybridoma LK35.2 was a gift from Dr. A. Singer (National Institutes of Health, Bethesda, MD) (18). B cell hybridoma TA3 was a gift from Dr. N. Shinohara (Mitsubishi-Kasei Institute of Life Science, Tokyo, Japan) (19).

Nonlymphoid Tumor Cells. The B16 melanoma cell line was a gift from Dr. M. Taniguchi (Center for Neurobiology and Molecular Immunology, Chiba University, Chiba, Japan) (20). The MH134 hepatoma cell line was a gift from Dr. H. Fujiwara (Osaka University, Osaka, Japan) (21).

Transfectant. A murine Fas antigen transfected (22, 23) Jurkat cell line, J6 (24), was kindly provided by Dr. S. Nagata (Osaka City Institute of Public Health and Environmental Sciences, Osaka, Japan) (22).

Culture Medium

The medium used for maintaining the cell lines was RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 50 μM 2-ME, and 10% heat-inactivated FCS. The culture medium for IL-2-dependent T cell clones was additionally supplemented with 5% Con A-stimulated culture supernatant of rat spleen cells as an IL-2 source.

Antibodies

Anti-K^k mAb (11-4.1) (25), anti-D^k mAb (15-5-5s) (26), anti-K^b mAb (28-8-6s) (27), anti-β₂-microglobulin (allotype b specific) (S19.8) (28), and anti-CD3 mAb (145-2C11) (29) were purified by protein A-Sepharose (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) or by precipitation with 40% saturation of ammonium sulfate at pH 7.2.

Production of RE2 mAb

A rat was immunized intraperitoneally four times with NP-40 lysates of a MS-S2 (12) T cell clone absorbed with anti-I-J^k-coupled (30–32) Sepharose 4B. 3 d after the last immunization, rat spleen cells were fused with P3U1 nonproducing myeloma cells by the polyethyleneglycol method, and hybridomas were selected by culturing in the presence of a mixture of 100 μM of hypoxanthine, 4 μM of aminopterin, and 16 μM of thymidine. To increase the efficiency of hybridization, 400 U/ml of recombinant IL-6 was added in the selection medium as previously described (33). Hybridomas producing antibodies that reacted with MS-S2 were selected by microfluorimetry. Five hybridomas, RE1 to RE5, were selected and cloned by limiting dilution. All of the mAbs produced by these hybridomas were IgG class and widely cross-reactive with

the molecules of MHC class I family (Kishimoto, H., K. Sano, S. Matsuoka, Y. Asano, and T. Tada, manuscript in preparation).

Production of S27 mAb

A hamster was immunized with immunoprecipitates from lysate of the MS-S2 T cell clone by RE2 mAb. The spleen cells of the hamster were fused with P3U1 cells as described above. Hybridomas were selected by cytolytic activity on the MS-S2 T cell clone.

Papain Digestion of RE2 mAb

RE2 mAb was digested by papain to yield Fab fragments by the method of Rousseaux et al. (34). Briefly, purified RE2 mAb at a concentration of 1.4 mg/ml was digested with mercuripapain (Sigma Chemical Co., St. Louis, MO) in the presence of 1 mM cysteine at the enzyme/protein ratio of 1% (wt/wt) for 18 h at 37°C.

Reagents

The following reagents were used. Forskolin (20 μg/ml; Sigma Chemical Co.) was added to the target cell for 1 h before cytolytic assay where stated. Sodium azide (50 mM), EDTA (30 mM), cytochalasin B (10 μg/ml; Sigma Chemical Co.), or cytochalasin D (15 μg/ml; Sigma Chemical Co.) were added to the assay medium during the cytolytic assay to test the effect of these reagents.

Assay of Cytolytic Activity

Target cells were resuspended at 10⁷/ml in RPMI 1640 supplemented with 2% FCS. mAb was added at 3 μg/ml to the cell suspension after decomplexation. Cells were incubated at 37°C for 1 h unless otherwise stated. The percentage of cell lysis was determined by dye exclusion using trypan blue in duplicate or triplicate and was calculated by the following formula:

$$\text{Percent of cytotoxicity} = \frac{A - B}{A} \times 100,$$

where *A* is the number of live cells after incubation without mAb and *B* is the number of live cells after incubation with mAb. The percentage of cell lysis of T cells adherent to culture dishes was determined by calculating the live cell number after incubating the dishes with mAbs.

Electron Microscopic Study

Scanning Electron Microscopy. To prepare the cells for observation with a scanning electron microscope, MS-S2 cells were incubated with RE2 at 37°C for 0, 5, and 20 min and then washed with and resuspended in PBS containing 2% FCS. The suspension was fixed with 10 vol of 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 2 h. The fixed cells were washed with 0.1 M cacodylate buffer, postfixed for 1 h in 1% OsO₄ at 4°C, and dehydrated in 50–100% ethyl alcohol followed by drying in tertiary butyl alcohol with a freeze-drying instrument (ID-2; Giko, Tokyo, Japan). The cells were mounted on electric conductive double-sided tape (Nisshin-EM, Tokyo, Japan) coated with a gold-palladium coating system (Polaron, England), and they were examined by a scanning electron microscope (model S-430; Hitachi Ltd., Tokyo, Japan).

Transmission Electron Microscopy. MS-S2 cells were incubated with RE2 or RE1 at 37°C for 20 min and fixed by 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3). The cells were postfixed by 1% OsO₄ in 0.1 M cacodylate buffer

(pH 7.3) and stained in 1% uranyl acetate. These cells were dehydrated in a graded series of ethyl alcohol and embedded in Spurr's resin (low viscosity embedding media; Polysciences Inc., War- rington, PA). Silver to gold sections were cut and examined with a transmission electron microscope (model JEM-1200Ex; JEOL Ltd., Tokyo, Japan).

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis

Aliquots of 2×10^6 MS-S2 cells were incubated with RE mAbs for 30 min at 37°C, and 6×10^6 thymocytes were incubated with 1.2 μ M of dexamethasone for 8 h at 37°C. After incubation, the cells were resuspended in TNE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5) containing 0.5% SDS and further incubated for 7 h at 37°C in the presence of 100 μ g/ml of proteinase K (Sigma Chemical Co.). Samples of DNA were prepared by phenolchloroform extraction and ethanol precipitation fol- lowed by RNase treatment. 6 μ g of DNA was electrophoresed in a 2% agarose gel prepared in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.5) containing 0.3 μ g/ml ethidium bromide and visualized by UV light.

Cell Staining and Flow Cytometry

Suspension of normal thymocytes, spleen cells, and a T cell lym- phoma RMA and its MHC class I-defective mutant line RMA-S were stained with RE2 mAb followed by incubation with FITC- conjugated mouse anti-rat Ig. To study the inhibition of binding of anti-class I mAbs, MS-S2 cells were first incubated with RE2 mAb and further incubated with FITC-conjugated anti-K^k (11-4.1) or anti-D^k (15-5-5s) mAbs. Fluorescence analysis of stained cells was performed with a flow cytometer (FACStar plus[®], Becton Dick- inson & Co., Mountain View, CA) (33).

Surface Labeling of Cells, Immunoprecipitation, and Gel Electrophoresis

Cell surface iodination of spleen cells, MS-S2 cells, and 24-2 cells was performed by chemical reaction catalyzed by Iodogen (Pierce Chemical Co., Rockford, IL) as described previously (35). After radiolabeling, the cells were lysed with 1% NP-40. Aliquots of lysates were mixed and precipitated with mAbs coupled to protein A- or protein G-Sepharose as described (32). The immunoprecipi- tates were dissolved in SDS sample buffer (2.3% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8) and analyzed by SDS-PAGE as described by Laemmli (36).

Results

Cytotoxic Activity of RE2 for T Cell Clones. In the course of screening the mAb produced against the immunoprecipi- tate of anti-I-J and the lysate of T cell clone MS-S2, we in- cidentally found that one mAb (RE2) constantly killed mouse IL-2-dependent T cell clones when cells were incubated with the mAb at 4°C for staining and flow cytometric analysis. Fig. 1 shows the result of an experiment where a CD4⁺ Th2 T cell clone MS-S2 was incubated with RE2 mAb at 37°C for various incubation periods. The treatment induced rapid cell lysis at 37°C. 60% of cells were killed even within 15 min, and >90% were killed at 60 min. About 60% of cells were also killed at 60 min, if they were incubated at 4°C. The cytotoxicity was independent of serum comple- ment since the heating of ascitic fluid at 56°C for 30 min did not affect the cytotoxicity. The killing of cells occurred

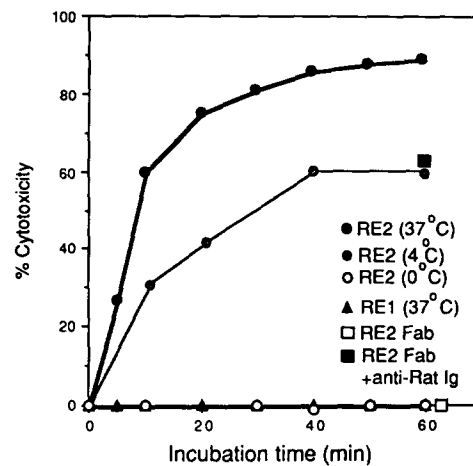


Figure 1. Kinetics of cytotoxic effect of RE2 mAb on T cell clone MS-S2. MS-S2 cells were incubated with RE2 and RE1 mAbs as described in Materials and Methods. Cytotoxic activity of RE2 at 37°C (●), 4°C (○), 0°C (□), and of RE1 at 37°C (▲) were measured at the indicated times. MS-S2 cells were also treated with Fab fragments of RE2 (□). The one group of Fab fragment-treated cells was further treated with anti-rat Ig (■). They were incubated at 37°C for 1 h.

even under serum-free culture conditions. However, no cyto- toxicity was observed when the reaction mixture was kept at 0°C, suggesting the presence of an active process in the target cells. An Ig class-matched control rat mAb RE1 (IgG₁) produced by the same hybridization procedure did not show cytotoxicity under the same conditions. RE1 mAb was shown to react with similar molecular species on the surface of target cells in the immunoprecipitation (see below).

Cytotoxicity of RE2 on Various Cell Populations. The cyto- lytic activity of RE2 was tested on various IL-2-dependent T cell lines, lymphomas, and hybridomas of T and B cell origins (Table 1 A). All IL-2-dependent T cell clones in the suspension were killed at variable degrees. When the mAb was added to the same cells attached to plastic culture dishes, however, RE2 was unable to kill the cells (see asterisk in Table 1 A). This peculiar property will be discussed below. Three lymphomas and hybridomas from stationary culture were not killed by RE2. However, they became sensitive to the cyto- toxicity of RE2 after activation by mitogens such as Con A or LPS. Three transformed B lineage cell lines and several nonlymphoid tumor cell lines tested were insensitive to the treatment with RE2 even after incubation with appropriate mitogenic stimuli. Therefore, the cytolytic effect of RE2 mAb appears to have a preferential effect on activated lymphoid cells.

We also tested the cytolytic activity of RE2 on spleen cells, bone marrow cells, lymph node cells, and thymocytes of C3H mice. Splenic T and B cells and lymph node cells were killed by the antibody only after activation with the appropriate mitogens (Table 1 B). The same results were obtained with the spleen cells of other mouse strains of H-2^b and H-2^d (data not shown). Thymocytes were not killed by RE2 mAb before and after activation with various reagents. Bone marrow cells were not killed. Although RE2 mAb was able to stain all lymphoid cells including thymocytes, spleen cells, bone

Table 1 A. Cytotoxicity of RE2 on Various Cell Lines

Target cells	Origin	Cell type	Percent of cytotoxicity with	
			RE2	RE1
28-4	B6C3F1	CD4 ⁺ Th1	65.3	<1.0
MS-S2	C3H	CD4 ⁺ Th2	90.0	<1.0
MS-S2 (adherent)*			<1.0	<1.0
24-2	B6C3F1	CD4 ⁺ Th2	45.3	<1.0
9-5	B6C3F1	CD4 ⁺ Ts	90.0	<1.0
HD8	C3H	CD8 ⁺ Ts	41.4	4.4
EL4 (not treated)	B6	T cell lymphoma	<1.0	<1.0
(Con A treated)			60.0	<1.0
7C3-13 (not treated)	B10.BR	T cell hybridoma	<1.0	<1.0
(rat Con A sup treated)			92.1	<1.0
TA3 (not treated)	CAF1	B cell lymphoma	<1.0	<1.0
(LPS treated)			45.4	<1.0
LK35.2 (not treated)	B10.BR	B cell hybridoma	<1.0	<1.0
(LPS treated)			<1.0	<1.0
46.6	BALB/c	Pro-B	<1.0	<1.0
Ig6.3	BALB/c	Pre-B	<1.0	<1.0
WEHI231	BALB/c	B	<1.0	<1.0
B16 (not treated)	B6	Melanoma	<1.0	<1.0
(IFN- γ treated)			<1.0	<1.0
MH134 (not treated)	C3H	Hepatoma	<1.0	<1.0
(IL-6 treated)			<1.0	<1.0

Table 1 B. Cytotoxicity of RE2 on Normal Cells

Target cells	Percent of cytotoxicity with	
	RE2	RE1
Thymocytes	<1.0	<1.0
Con A-activated thymocytes	<1.0	<1.0
Anti-CD3-activated thymocytes	<1.0	<1.0
Spleen cells	<1.0	<1.0
Con A-activated spleen cells	40.0	<1.0
Con A-activated splenic T cells	78.9	<1.0
LPS-activated splenic B cells	54.5	<1.0
Normal lymph node cells	<1.0	<1.0
Con A-activated lymph node cells	41.0	<1.0

In both parts of this table, cells were incubated with RE mAbs for 1 h at 37°C. The percentage of cytotoxicity was evaluated as described in Fig. 1. Some tumor cell lines and hybridomas were treated with the indicated reagents or 5% Con A-stimulated culture supernatant of rat spleen cells for 12–24 h before cytolytic assay. Thymocytes and spleen cells of C3H mice were incubated with or without Con A (2 μ g/ml), LPS (5 μ g/ml), and anti-CD3 mAb (3 μ g/ml) for 24–48 h at 37°C.

marrow cells, and lymph node cells, the mAb was able to kill the peripheral lymphoid cells only when they were activated.

Electron Microscopic Findings. The morphology of cells treated with RE2 mAb was studied by scanning electron microscopy. Giant pore formation was observed on the surface of target T cells in an early phase (5–20 min) of killing by RE2 (Fig. 2). As early as 5 min after the incubation with the antibody, giant holes were formed on the surface of target cells. The diameter of the holes reached $\sim 2 \mu$ m. The number of holes observed was variable, but one to six holes were generally observed on the cells. In the transmission electron microscopic analysis, destruction of plasma membrane, dilation of the endoplasmic reticulum, and deformation and condensation of mitochondria were observed, while chromatins of their nuclei were almost intact (Fig. 3). These findings are distinct from those observed in apoptotic or typical necrotic cell death. To further discriminate the RE2-induced cell death from apoptosis, we analyzed the DNA of the antibody-treated cells (Fig. 4). The DNA extracted from the RE2-treated MS-S2 cells showed no degraded DNA, suggesting this type of cell death is different from that of apoptosis.

Requirement of Cross-Linking of Cell Surface Molecules for Cytotoxic Activity of RE2. To determine whether the binding of RE2 mAb to cell surface molecules is sufficient for in-

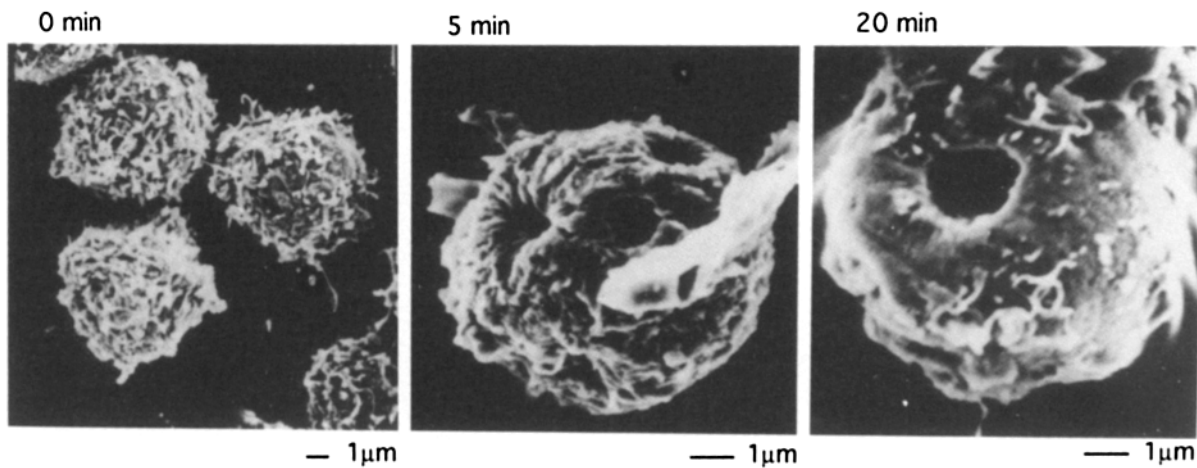


Figure 2. Scanning electron microscopic findings. MS-S2 cells were incubated with RE2 for 0, 5, and 20 min at 37°C. Electron micrographs were taken as described in Materials and Methods. Bars, 1 μ m.

ducing cell lysis, the T cell clone MS-S2 cells were treated with intact RE2 mAb or its Fab fragments at 37°C for 1 h. The treatment of cells with intact RE2 mAb always induced cell lysis, while Fab fragments of RE2 failed to do so. However, cross-linking of the cell-bound Fab fragments with anti-rat Ig reconstituted the cytotoxicity (Fig. 1). These results

indicate that a cross-linking of target molecules is required for triggering of the antibody-mediated, complement-independent cytotoxicity.

Blocking of Cytotoxicity by Cytochalasin. To learn the mechanism of cell death induced by RE2, various reagents were added to the reaction mixture as described in Materials and

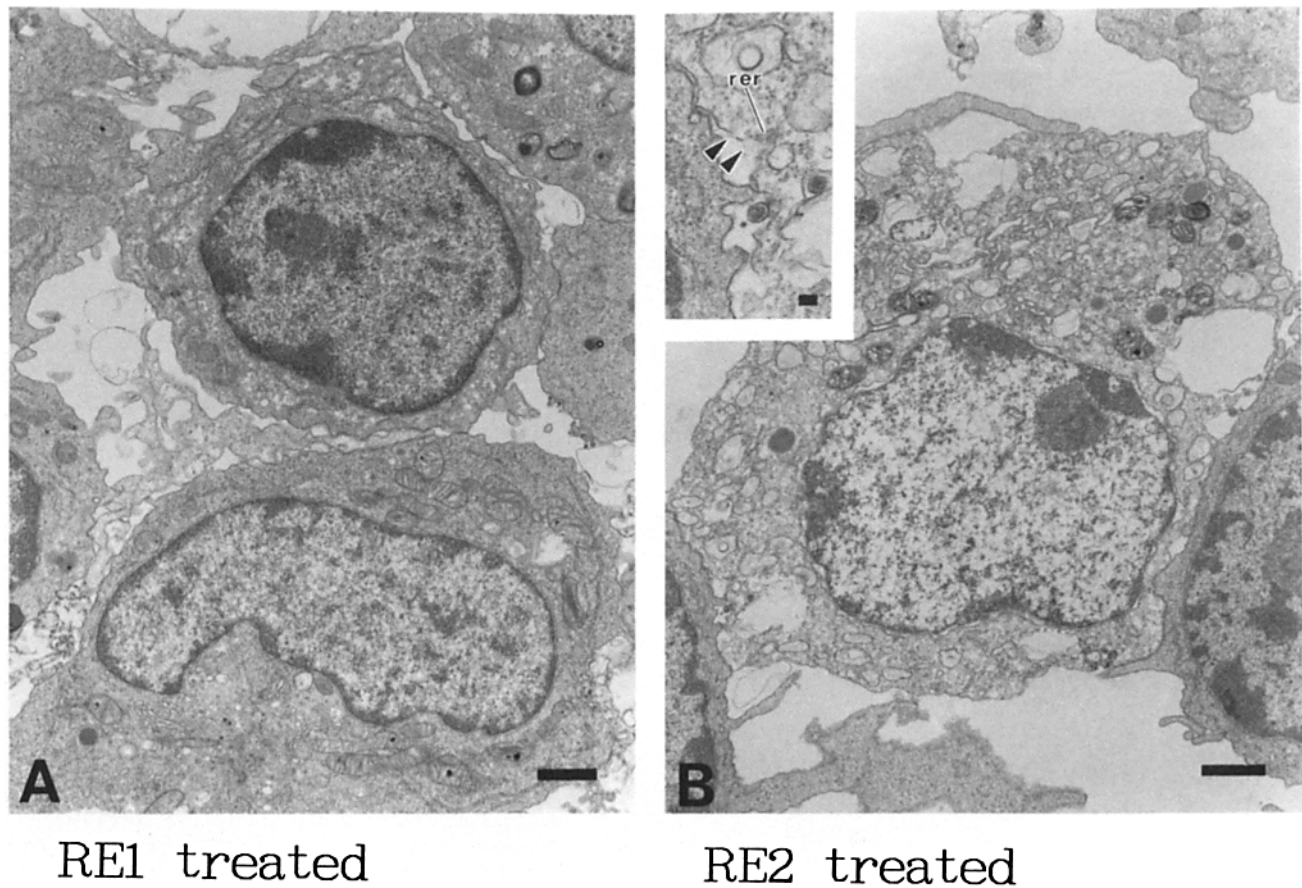


Figure 3. Transmission electron microscopic findings. MS-S2 cells were incubated with RE1 (A) or RE2 (B) for 20 min at 37°C. Electron micrographs were taken as described in Materials and Methods. Arrowheads indicate disruption of plasma membrane. *rer*, rough-surface endoplasmic reticulum. Bars, 1 μ m.

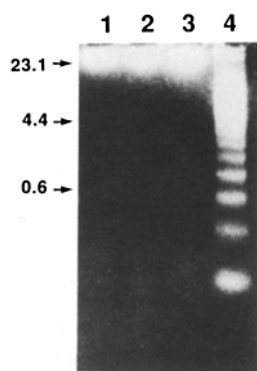


Figure 4. No findings of DNA fragmentation. MS-S2 cells were incubated without mAb (lane 1) or with RE1 mAb (lane 2) and RE2 mAb (lane 3) for 30 min at 37°C. Thymocytes of a C3H mouse were incubated with dexamethasone for 8 h (lane 4). Total DNA was prepared from the treated cells and electrophoresed as described in Materials and Methods.

Methods. As shown in Fig. 5, forskolin, EDTA, and sodium azide failed to interfere with the RE2-induced cell death, suggesting that neither the elevation of cytoplasmic cAMP, Ca^{2+} influx, nor capping was involved in the cell death. In contrast, the addition of cytochalasin B or cytochalasin D completely blocked the cytolysis. As shown in Fig. 6, when cytochalasin D was added before the incubation with RE2, the cytolytic activity of RE2 was completely abrogated. When cytochalasin D was added at various times after incubation with the antibody, the cell lysis was stopped at that time point, and no further cell death was observed.

Expression of the RE2 Determinant on Thymocytes and Spleen Cells. Fig. 7a shows the expression of the RE2 determinant on thymocytes and spleen cells derived from a C3H mouse. The RE2 determinant was expressed on thymocytes and spleen cells of mouse strains with different H-2 haplotypes. It was also detectable on the cell membrane of virtually all the tissues including the liver and thymic supportive tissues, suggesting that the RE2 epitope is ubiquitously expressed on all somatic cells (data not shown). However, tissue cells and tumor cells were insensitive to the cytolytic effect of RE2. Since ubiquitous expression of RE2 on lymphoid cells suggested that the determinant could be a class I molecule, a relationship between RE2 and class I molecules was deduced by a competitive inhibition study of staining. As shown in Fig. 7b, RE2 was able to partially inhibit the staining of H-2^k T cell clone

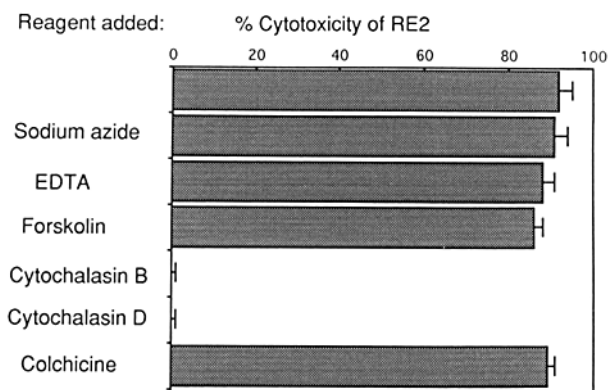


Figure 5. Blocking of cytotoxicity of RE2 by cytochalasins. Cytotoxic activity of RE2 on MS-S2 cells was measured in the presence of various reagents under conditions described in Materials and Methods.

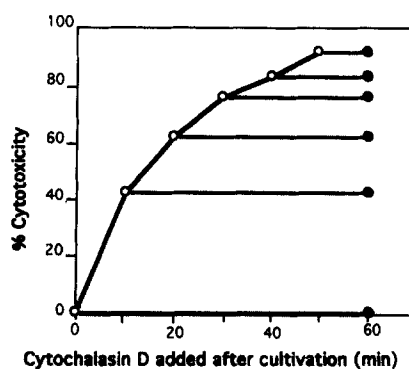


Figure 6. Effect of cytochalasin D on cytotoxicity of RE2 at various times during the assay. 20 μ g/ml of cytochalasin D was added to the cytolytic assay of RE2 on MS-S2 cells at the indicated time, and cytotoxic activity was evaluated at the time when cytochalasin was added (○) and at the end of the assay (60 min) (●).

MS-S2 cells with anti-K^k and anti-D^k mAbs. It was also found that the RE2 determinant is expressed on a T cell leukemic line RMA selected from the C57BL/6-derived RBL-5 lymphoma, but not on an MHC class I defective mutant, that is, RMA-S cells derived from RMA (Fig. 7c). These results indicate that the RE2 determinant is strongly related to class I molecules.

Immunoprecipitation from T Cell Clone MS-S2, Spleen Cells, and T Cell Clone 24-2. The lysate of surface-labeled MS-S2 cells and spleen cells with or without in vitro activation were analyzed by SDS-PAGE after immunoprecipitation with normal rat serum and RE2, RE1, and S27 mAbs. RE2 and S27 precipitated 44-, 60-, and 90-kD molecules from the T cell clone MS-S2 (Fig. 8a). RE2 precipitated 44-kD and 60-kD molecules from freshly isolated spleen cells (Fig. 8b),

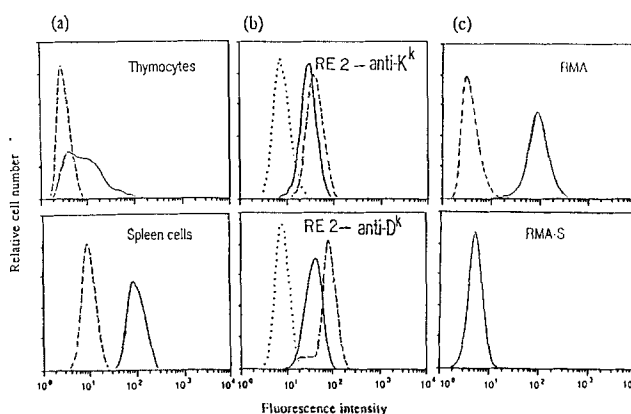


Figure 7. Expression of the RE2 determinant on thymocytes, spleen cells, and cell lines. (a) Thymocytes and spleen cells derived from a C3H mouse, as well as (c) T cell leukemic line RMA cells and MHC class I-defective mutant RMA-S cells were stained with RE2 mAb as described in Materials and Methods. Staining profiles with RE2 are shown by solid lines. Negative controls are shown by dotted lines. (b) MS-S2 cells were incubated with RE2 mAb followed by the staining with anti-K^k or anti-D^k. Staining profiles of anti-class I mAb without or with RE2 incubation are shown by dotted lines and those with RE2 incubation are shown by solid lines.

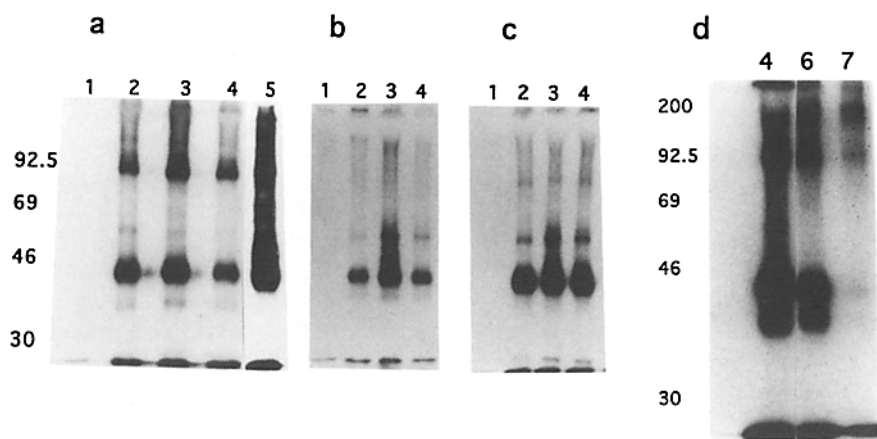


Figure 8. Immunoprecipitation from T cell clone MS-S2, spleen cells, and T cell clone 24-2. (a) T cell clone MS-S2 cells, (b) freshly isolated spleen cells, (c) Con A-activated spleen cells (incubated with 2 μ g/ml of Con A for 24 h), and (d) T cell clone 24-2 cells surface labeled with 125 I by the iodogen method. NP-40 lysate of the cells was subjected to immunoprecipitation with normal rat serum (lane 1), anti-K^k (11-4.1) (lane 2), RE1 (lane 3), RE2 (lane 4), S27 (lane 5), anti- β_2 -microglobulin (lane 6), and anti-K^b (28-8-6s) (lane 7) mAbs.

whereas the antibody precipitated the additional 90-kD molecule from Con A-activated spleen cells (Fig. 8 c). RE1 mAb also precipitated 44- and 60-kD molecules from freshly isolated spleen cells and the additional 90-kD molecule from the activated spleen cells. To further characterize RE2 and class I determinants, immunoprecipitates with RE2, anti- β_2 -microglobulin, and anti-K^b mAbs from the lysates of T cell clone 24-2 (B6C3F1 origin) cells were analyzed by SDS-PAGE. RE2, anti- β_2 -microglobulin (allotype b specific), and anti-K^b mAbs precipitated both 44- and 90-kD molecules (Fig. 8 d). When immunoprecipitates with RE2 were analyzed by 15% SDS-PAGE, 14-kD molecules, which have similar molecular masses as β_2 -microglobulin, were detected (data not shown).

Discussion

During the course of our study searching for a xenogenic antibody specific for mouse T cell membrane molecules, we incidentally found that one mAb, RE2, induced a complement-independent cell lysis of a T cell clone used for screening of mAbs. The nature of the cytotoxicity was different from necrosis and apoptosis in various aspects: there were giant holes on the surface of target cells induced within 5 min, and mitochondrial condensation and deformation instead of swelling were observed. There were no apoptotic bodies in dead cells, and the structure of the nucleus was preserved after the destruction of the cell membrane.

There are two reports on mAbs that induce apoptotic death of lymphocytes. Anti-Apo-1 and anti-Fas can induce complement-independent cell death in activated or malignant human lymphocytes and fibroblasts (22, 37). The cell death induced by these mAbs has been defined as apoptosis since a typical DNA fragmentation was observed in attacked cells. The cell death induced by RE2 is different since no DNA fragmentation has been detected in treated cells. This report described the characteristic features of the cell death induced by RE2 compared with those of necrotic and apoptotic cell death.

Our results indicated that the cross-linking of cell surface molecules reactive with RE2 mAb induced complement-independent cell lysis. We are unable to determine the molecule responsible for this cell death caused by RE2. The mAb

RE2 immunoprecipitated 90-, 60-, and 44-kD molecules from activated spleen cells and T cell clones. However, the same 60- and 44-kD molecules could be precipitated even from resting spleen cells and some T cell lines that were not sensitive to the cytolytic activity of RE2. It was noted that the 90-kD molecule was detectable only on the cells that were sensitive to the cytolytic activity of RE2. However, all of the 44-, 60-, and 90-kD proteins were immunoprecipitated by a noncytotoxic rat mAb RE1. Anti-class I or β_2 -microglobulin mAb (11-4.1 and S19.8) having no cytolytic activity also precipitated the same 90-kD molecule from the activated T cells. RE2 determinant was not expressed on a class I-defective leukemic cell line. RE2 blocked the staining of MHC class I on T cell clones. The results indicated that RE2 recognizes an epitope that is on an MHC class I polymorphic region detectable by conventional anti-class I antibodies or a molecule closely associated with MHC class I after activation. The possibility remains that lymphocytes are induced to death only when a unique structure in class I heavy chain dimers is recognized (38). Since anti-class I antibodies, as well as rat mAb RE1 having similar specificities to RE2, exhibited no cytolytic activity, the target molecule involved in cytolysis may not simply be the MHC class I itself. Also, since the antibody could not kill somatic cells other than lymphocytes, the target molecules may be uniquely expressed on T and B cells. A hamster mAb (S27) obtained by immunizing the RE2 immunoprecipitates of T cell lysate expressed the same complement-independent cytolytic activity (data not shown). Again, this hamster antibody was found to precipitate the same class I molecules from T cell clones and activated lymphoid cells. These results suggested that RE2 mAb can recognize a structure on class I molecules that is involved in the association with unknown membrane molecules responsible for the cytotoxicity. Such molecules may be present only in T and B cells, and they can associate with class I only after activation. Indeed, the cytolytic activity of RE2 was completely blocked by cytochalasin B or cytochalasin D, which have no influence on class I molecules themselves. The involvement of a cytoskeleton-dependent active process was suggested not only by experiments with cytochalasins. If the target cells were attached to the culture dish, they became resistant

to the cytotoxic activity of RE2. Actin filaments of cells are known to be depolymerized by cytochalasin B and cytochalasin D. In the condition where the cells adhere to the plastic wall, they are known to assemble each other to form stress fibers (39). The fact that cytochalasins immediately stopped the RE2-induced killing suggests that a cytoskeleton-dependent pathway is essential for the cytotoxicity.

The cell death observed with RE2 did not involve the apoptotic process with DNA fragmentation. This indicates that the site of action of RE2 mAb is different from that of anti-Fas and anti-Apo-1. The kinetics of cell death and target cell distribution are also different. RE2 could not kill or stain the murine Fas antigen-transfected Jurkat cell J6 (data not shown).

We were unable to define the mechanism of this RE2-induced cell death. However, it represents a novel type of cell death caused by an antibody apart from complement-dependent necrosis and -independent apoptosis. Since RE2 mAb kills only activated but not resting T and B cells, such a mechanism may be involved in an exclusion of autoreactive lymphoid cells at the developmental and effector limbs of the immune response. The formation of the target complex of RE2 on the cell surface of activated lymphoid cells may have a physiologic role for the lymphocyte turnover, although the triggering may involve other mechanisms. The analysis of the sensitivity of dividing lymphoma cells may help us to develop a therapeutic device for leukemic cells.

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