細胞表面抗原に対するモノクローナル抗体によって補体非存性に導かれるリンパ球の新しいタイプの細胞死

A novel type of cell death of lymphocytes induced by a monoclonal antibody against cell surface antigens without participation of complement

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A novel type of cell death of lymphocytes induced by a monoclonal antibody against cell surface antigens without participation of complement[1]

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6Abbreviations used in this paper:

Con A;	Concanavalin A
EDTA;	ethylenediaminetetraacetic acid
FCS;	fetal calf serum
FITC;	fluorescein isothiocyanate
Ig;	immunoglobulin
LPS;	lipopolysaccharide
mAb;	monoclonal antibody
MHC;	major histocompatibility complex
NP-40;	Nonidet P-40
SDS;	sodium dodecyl sulfate
PAGE;	polyacrylamide gel electrophoresis
TcR;	T cell receptor
Th;	T helper cells
Ts;	T suppressor cells

Abstract

A monoclonal antibody, RE2, raised against immunoprecipitates formed by mouse anti-I-J monoclonal antibody (mAb) and the lysate from a T cell clone was found to kill IL2 dependent T cell clones without participation of serum complement. Fab fragments of RE2 had no cytotoxic activity, while the cross-linking of Fab fragments with anti-rat Ig reconstituted the cytotoxicity. The cytotoxicity was temperature-dependent where the antibody could kill target cells at 37°C but not at 0°C. Sodium azide, EDTA, forskolin and Zn^{2+} did not affect the cytotoxic activity of RE2, while the treatment of cells with cytochalasin B and D completely blocked the activity. This suggested that the cell death involves a cytoskeleton-dependent active pathway. Giant holes on the cell membrane were formed within 5 min after the treatment with RE2 as observed by a 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 pre-activated with Con A, LPS or PMA. Activated peripheral T and B cells were sensitive to the cytotoxicity of RE2, while resting T and B cells were insensitive. Non-lymphoid cells were not killed by RE2 even after stimulation. These results suggest that there is a novel pathway of cell death of activated lymphocytes involving cytoskeleton-dependent event which may act to exclude the autoreactivity under certain circumstances.

Introduction

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 auto-reactive and nonsense T cell receptor (TcR) (6). The cytolysis caused by cytotoxic T and NK cells is the apoptotic death where an active process resulting in the 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. Auto-antibodies 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.

In the course of studies of rat monoclonal antibodies (mAb) specific for a mouse cell surface antigen I-J (9,10) (Kishimoto, H., et al. manuscript in preparation), we found that a particular mAb constantly killed activated lymphoid cells of T and B cell lineage without the participation of complement. The resting T and B cells, as well as non lymphoid 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 to avoid autoreactivity of lymphoid cells under certain circumstances. The present report describes properties of this mAb and characteristic features of cell death caused by it.

Materials and Methods

Animals C3H/HeN (C3H) mice were purchased from Japan SLC Inc., Hamamatsu, Japan. Sprague-Dawly 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 non-adherent to anti-mouse immunoglobulin (Ig)-coated dishes as described previously (11). To obtain splenic B cells, spleen cells were depleted of T cells by the treatment with T cell-specific rabbit anti-mouse brain serum and complement as described previously (12). *T cell clones:* IL2-dependent T cell clones with helper and suppressor functions have been established from C3H, C57BL/6 and (B6 x C3H)F_1 as previously described (13-15). *B cell lines*: Immature B cell clones 46.6 and Ig 6.3 and a B cell line WEHI 231 of (BALB/c x NZB)F1, were gifts by Dr. T. Takemori, National Institute of Health, Tokyo, Japan (16). *Lymphoid tumor cell lines:* BW5147, EL-4, RMA (17) and RMA-S (a class I-defective mutant line derived from RMA) (18) were used as T cell leukemic cell lines. *T and B cell hybridomas*: A T cell hybridoma 7C3-13 has been described previously (19). A B cell hybridomas LK35.2 was a gift from Dr. A. Singer, National Institutes of Health, Bethesda, MD (20). A B cell hybridoma TA3 was a gift from Dr. N. Shinohara, Mitsubishi-Kasei Institute of Life Science, Tokyo, Japan (21). *Nonlymphoid tumor cells:* A B16 melanoma cell line was a gift from Dr. M. Taniguchi, Center for Neurobiology and Molecular Immunology, Chiba University, Chiba, Japan (22). MH134 hepatoma cell line was a gift from Dr. H. Fujiwara, Osaka University, Osaka, Japan (23). *Transfectant:* A murine Fas antigen (24,25)-transfected Jurkat cell line

(J26) was kindly presented by Dr. S. Nagata, Osaka City Institute of Pablic Health and Environmental Sciences, Osaka, Japan.

Culture medium The medium used for maintaining the cell lines was RPMI1640 medium (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 50μM 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (FCS). The culture medium for IL2-dependent T cell clones was additionally supplemented with 5% Con A-stimulated culture supernatant of rat spleen cells as an IL2 source.

Antibodies Anti-K^k mAb (11-4.1) (26), anti-D^k mAb (15-5-5s) (27) and anti-CD3 mAb (145-2C11) (28) 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 4 times with Nonidet P-40 (NP-40) lysates of a MS-S2 T cell clone with anti-I-J^k-coupled Sepharose 4B. Three days after the last immunization spleen cells of the rat 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 hypoxantine, 4 μM of aminopterin and 16 μM of thymidine. In order to increase the efficiency of hybridization, 400 U/ml of recombinant IL-6 was added in the selection medium as previously described (29). 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 the MHC class I family. (Kishimoto, H., et al. 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 in the same way as above. Hybridomas were selected by the cytotoxic 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 (30). 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 to protein ratio of E/S = 1 %(w/w) for 18 h at 37°C.

Reagents The following reagents were used. Forskolin (20μg/ml, Sigma) was added to the target cell for 1 h before cytolytic assay where stated in the text. $ZnSO_4$ (80μM), sodium azide (50mM), ethylenediaminetetraacetic acid (EDTA, 30mM), of cytochalasin B (10 μg/ml, Sigma) or cytochalasin D (15 μg/ml, Sigma) were added into the assay medium during the cytolytic assay in order to test the effect of these reagents.

Assay of cytolytic activity Target cells were resuspended at $1x10^7$/ml in RPMI1640 supplemented with 2% FCS. MAb was added at 3μg/ml to the cell suspension after decomplementation. The cells were incubated at 37°C for 1h unless otherwise stated in the text. The percentage of cell lysis was determined by dye exclusion using trypanblue in duplicate or triplicate, and was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{A - B}{A} \times 100$$

where, A = number of live cells after incubation without mAb

B = number of live cells after incubation with mAb

The percentage of cell lysis of T cell adherent to culture dishes were determined by calculating the live cell number after incubating the dishes with mAbs .

Electron microscopic study *Scanning electron microscope*: 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 minutes, and then washed with, and resuspended in, phosphate buffered saline containing 2% FCS. The suspension was fixed with 10 volumes of 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 2h. The fixed cells were washed with 0.1 M cacodylate buffer, post-fixed for 1 h in 1% OsO_4 at 4°C and dehydrated in 50% ethyl alcohol to 100% ethyl alcohol followed by drying in tertiary buthyl alcohol by a freeze-drying instrument (ID-2, Giko, Tokyo, Japan). The cells were mounted on an electric conductive double-sided tape (carbon tape, Nisshin-EM, Tokyo, Japan), coated with a gold-palladium coating system (Polaron, England) and examined by a scanning electron microscope (S-430, Hitachi, Tokyo, Japan). *Transmission electron microscope:* MS-S2 cells were incubated with RE2 or RE1 at 37°C for 20 min, and were fixed by 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (pH7.3). The cells were postfixed by 1% OsO_4 in 0.1M cacodylate buffer (pH7.3) and stained in 1% uranyl acetate. These cells were dehydrated in a graded series of ethyl alcohol, and embedded in Spurr's resin (Spurr's low-viscosity embedding media, Polysciences, Inc., Warrington, PA). Silver to gold sections were cut and examined by a transmission electron microscope (JEM-1200Ex, JEOL, Tokyo, Japan).

Analysis of DNA fragmentation by agarose gel electrophoresis Aliquots of $2x10^6$ MS-S2 cells were incubated with RE mAbs for 30 min at 37°C and $6x10^6$ thymocytes were incubated with 1.2μM of dexamethasone for 8 h at 37°C. After incubation the cells were

resuspended in TNE buffer (10 mM Tris, 100mM NaCl, 1mM EDTA, pH 7.5) containing 0.5% sodium dodecyl sulfate (SDS) and further incubated for 7 h at 37°C in the presence of 100 µg/ml of protenase K (Sigma). Samples of DNA were prepared by phenol-chloroform extraction and ethanol precipitation followed by RNase treatment. Six µg of DNA were electrophoresed in a 2 % agarose gel prepared in TAE buffer (40mM Tris-acetate, 2mM EDTA, pH7.5) containing 0.3 µg/ml ethidium bromide and visualized by UV light.

Cell staining and flow cytometry Suspension of normal thymocytes, spleen cells and a T cell lymphoma RMA and its MHC class I-defective mutant line RMA-S were stained with RE2 mAb followed by the incubation with fluoresceine isothiocyanate (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 Dickinson, Montain View, CA). The data analysis was carried out by using the Consort 30 (Becton Dickinson) as previously described (29).

Surface labeling of cells, immunoprecipitation and gel electrophoresis Cell surface iodination of spleen cells and MS-S2 cells was performed by the chemical reaction catalyzed by Iodogen (Pierce Chemical Co., Rockford, IL) as described previously (31). 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 immunoprecipitates were disolved in SDS sample buffer (2.3% SDS, 10%glycerol, 62.5 mM Tris, pH6.8) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (33).

Results

Cytotoxic activity of RE2 for T cell clones During the screening of the mAb produced against immunoprecipitate of anti-I-J and lysate of a T cell clone MS-S2, we incidentally found one mAb constantly killed T cell clones when cells were incubated with the mAb for staining and FACS analysis. Fig.1 shows the result of an experiment where the T cell clone MS-S2 was incubated with RE2 mAb at 37°C for a different incubation time. The treatment induced a rapid cell lysis at 37°C. Sixty percent of cells were killed even within 15 min, and more than 90% were killed at 60 min. The cytotoxicity was independent of serum complement as the heating of anti-serum at 56°C for 30 min did not affect the cytotoxicity. No cytotoxicity was observed when the reaction mixture was incubated at 0°C. An Ig class-matched control antibody RE1 produced by the same hybridization procedure did not show the cytotoxicity under the same condition.

Cytotoxicity of RE2 on various cell populations The cytotoxic activity of RE2 was tested on various IL2-dependent T cell lines, lymphomas and hybridomas of T and B cell origins (Table 1). All IL2-dependent T cell clones tested were killed at variable degrees if they were suspended. In contrast, they were not killed, if they were adherent to the culture dish. Four lymphomas and hybridomas from stationary culture were not killed by RE2, while after the treatment with appropriate reagents they were changed to be killed by the mAb. Three transformed B lineage cell lines and several nonlymphoid tumor cell lines tested were insensitive to the treatment with RE2 even after the incubation with apropriate reagents. Therefore, the cytotoxic effect of RE2 mAb appears to have a preferential effect on activated lymphoid cells.

To further extend the above observation to normal lymphoid cells, we tested the cytotoxic activity of RE2 on spleen cells, bone marrow cells, lymph node cells and thymocytes of C3H. Splenic T and B cells and lymph node cells were affected by the antibody after activation with the appropriate reagent (Table 2). 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 regents. Bone marrow cells were not killed. Although RE2 mAb was able to stain all lymphoid cells including thymocytes, spleen cells, bone marrow cells and lymph node cells, it was able to kill only activated peripheral lymphoid cells.

Electron microscopic findings The morphology of cells treated with RE2 mAb was studied by a scanning electron microscopy. The pore formation was observed on the surface of target T cells in the early phase (5 to 20 min) of killing by RE2 (Fig.2a). The diameter of the holes reached approximately 2 μm. The number of holes observed was variable. In transmission electron microscopic analysis, the deformation and condensation of mitochondrias, and swelling of endoplasmic reticulum were observed, while chromatins of their nuclei were almost intact (Fig.2b). These findings are distinct from those observed in apoptotic or typical necrotic cell death.

Absence of DNA fragmentation To further discriminate the RE2-induced cell death from apoptosis, we analyzed DNA of the antibody-treated cells (Fig.3). DNA extracted from the RE2-treated MS-S2 cells showed no degraded DNA. The pattern was similar to that observed in control groups which include the untreated and control mAb (RE1)-treated cells.

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 to induce the cell lysis, MS-S2 cells were treated with intact RE2 or its Fab fragments at 37°C for 1h. The treatment of cells with intact RE2 induced the cell lysis, while Fab fragments of RE2 failed to induce the cell lysis. The cross-linking of fragments with anti-rat Ig reconstituted the cytolysis (Fig.4). These results indicate that the binding of the antibody to RE2 molecules is insufficient to induce cell lysis. The cross-linking of the molecules is required for triggering of the antibody-mediated complement-independent cytolysis.

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 Methods. As shown in Fig5, the addition of forskolin, EDTA, and sodium azide during the cytolytic assay did not interfere with the cytotoxic activity of RE2, indicating that the process did not involve the elevation of cytoplasmic cyclic AMP, Ca^{2+}influx, nor capping. Zinc ion, which is known to block apoptosis (34), did not interfere with the cytolysis caused by RE2. In contrast, the addition of cytochalasin B or cytochalasin D completely blocked the cytotoxic activity of RE2. As shown in Fig 6, when cytochalasin D was added prior to the incubation with RE2, the cytolytic activity of RE2 was completely abrogated. If cytochalasin D was added at a various time point after incubation with the antibody, the cell lysis was stopped at the point, and no further cell death was observed.

Expression of the RE2 determinant on thymocytes and spleen cells Figure 7a shows the expression of the RE2 determinant on thymocytes and spleen cells derived from C3H mouse. The RE2 determinant was expressed on thymocytes and spleen cells of virtually all mouse strains to various degrees, although the gene coding for the determinant could not be mapped in a single locus (Kishimoto et al, manuscript in preparation).

Staining profiles of T and B cells after activation were not changed. The results indicate that the activation induces a physical change of cell surface molecules reactive with RE2, the crosslinking of which leads to the active process of cytolysis.

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. MS-S2 cells were first incubated with RE2 mAb followed by the further staining with anti-K^k and anti-D^k mAbs (Fig.7b). RE2 inhibited the staining of the cells with anti-K^k and anti-D^k, indicating that RE2 mAb bound to class I molecules.

To further confirm this point, the reactivity of RE2 mAb to a class I defective cell line RMA-S was tested. The RE2 determinant is expressed on T cell leukemic line RMA cells, but not on MHC class I defective mutant RMA-S cells (Fig.7c). This result demonstrates that the determinant recognized by RE2 mAb is on MHC class I molecules. It was further demonstrated that the activated RMA but not RMA-S cells were killed by RE2 in the absence of complement (data not shown), indicating that the class I molecules are involved in the cell lysis.

Immunoprecipitation from a T cell clone MS-S2 and spleen cells

The lysate of surface labeled MS-S2 cells and spleen cells with or without in vitro activation were analysed by SDS-PAGE after immunoprecipitation with normal rat serum, RE2, RE1 and S27 mAbs. RE2 and S27 precipitated 44K, 60K and 90K molecules from the T cell clone (Fig. 8a). RE2 precipitated 44K and 60K molecules from freshly isolated spleen cells (Fig. 8b), whereas the antibody precipitated the additional 90K molecule from Con A-activated spleen cells (Fig.8c). RE1 mAb also precipitated 44K and 60K molecules from freshly isolated

spleen cells, and the additional 90K molecule from the activated spleen cells.

Discussion

During the course of our study searching for a xenogenic antibody specific for a mouse T cell membrane molecules, I-J (32,35), we incidentally found that one mAb, RE2, induced a complement-independent cell lysis of a T cell clone used for screening of mAb. 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 nucleus was preserved after the destruction of cell membrane. There are 2 reports on mAb that induce apoptotic death of lymphocytes. Anti-Apo-1 and anti-Fas had an ability to induce complement-independent cell death in activated or malignant human lymphocytes and fibroblasts (24,36). 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 as no DNA fragmentation has been detected in treated cells. The present report described the characteristic features of the cell death induced by the RE2 in comparison 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 90K, 60K and 44K molecules from activated spleen cells and T cell clones. However, the same 60K and 44K molecules could be precipitated even from resting spleen cells and some T cell lines which were not sensitive to the cytotoxic activity of RE2. The 90K molecule was precipitated only from

activated spleen cells and long term cultured T cell clones, and the molecule should be formed as a result of T cell activation. This 90K molecule was not the responsible antigen for RE2-induced cell death, as the same 90K molecule could be precipitated by another mAb RE1 which was obtained by the same immunization procedure. An anti-class I mAb (11-4.1) having no cytotoxic activity also precipitated the same 90K molecule from the activated T cells. RE2 blocked the staining of MHC class I on T cell clones. The results indicated that RE2 recognizes an epitope which 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. Since anti-class I antibodies as well as rat mAb RE1 having similar specificities to RE2 exhibited no cytotoxic activity, the target molecule may not be the class I MHC itself alone. Also, since the Ab could not kill somatic cells other than lymphocytes, the target molecules may be uniquely expressed on T and B cells. A hamster mAb obtained by immunizing the RE2 immunoprecipitates of T cell lysate expressed the same complement-independent cytotoxic 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 which 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 can associate with class I only after activation. Indeed, the cytotoxic 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 are adhered to the plastic wall, they are known to assemble each other to form stress fibers (37). 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 the murine Fas antigen-transfected Jurkat cell, J26.

At present 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 a 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 turn-over, although the triggering may be 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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Legends for Figures

Fig. 1. Kinetics of cytotoxic effect of RE2 mAb on a T cell clone MS-S2. MS-S2 cells were incubated with RE2 and RE1 mAb as described in Materials and Methods. Cytotoxic activity of RE2 at 37°C (●), 0°C (○) and of RE1 at 37°C (▲) were measured at the indicated time period.

Fig. 2. Electron microscopic findings. (a) *Scanning electron micrograph :* MS-S2 cells were incubated with RE2 for 0, 5 and 20 min at 37°C. (b) *Transmission electron micrograph:* MS-S2 cells were incubated with RE2 or RE1 for 20 min at 37°C. Electron micrographs were taken as described in Materials and Methods.

Fig. 3. No findings of DNA fragmentation. MS-S2 cells were incubated without mAb (lane1), or with RE1 mAb (lane2) and RE2 mAb (lane3) for 30 min at 37°C. Thymocytes of C3H mouse were incubated with dexamethasone for 8 h (lane4). Total DNA was prepared from the treated cells and electrophorased as described in Materials and Methods.

Fig. 4. Requirement of cross-linking of cell surface molecules for cytotoxic activity of RE2. MS-S2 cells were treated with whole molecules and Fab fragments of RE2. The one group of Fab fragments-treated cells was further treated with anti-rat Ig. The all treated cells were incubated at 37°C for 1 hr.

Fig. 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 the conditions described in Materials and Methods.

Fig. 6. Effect of cytochalasin D on cytotoxicity of RE2 at various time periods during the assay. Twenty μg/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 (O) and end of the assay (60 min) (●).

Fig. 7. Expression of the RE2 determinant on thymocytes, spleen cells and cell lines. (a) Thymocytes and spleen cells derived from C3H mouse, and (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, respectively.

Fig. 8. Immunoprecipitation from a T cell clone MS-S2 and spleen cells. (a) T cell clone MS-S2 cells, (b) freshly isolated spleen cells, (c) Con A-activated spleen cells (incubated with 2 μg/ml of Con A 24h) were surface labelled with ^{125}I by Iodogen method. NP-40 lysate of the cells were subjected to immunoprecipitation with normal rat serum (lane1), anti-K^k (11-4.1) (lane2), RE1 (lane3), RE2 (lane4) and S27 (lane5) mAbs.

Table 1. Cytotoxicity of RE2 on various cell lines

Target cells	Origin	Cell type	%Cytotoxicity with	
			RE2	RE1
28-4	B6C3F1	$CD4^+$Th1	65.3	0.0
MS-S2	C3H	$CD4^+$Th2	90.0	0.0
MS-S2 (adherent)			0.0	0.0
24-2	B6C3F1	$CD4^+$Th2	45.3	0.0
9-5	B6C3F1	$CD4^+$Ts	90.0	0.0
HD8	C3H	$CD8^+$Ts	41.4	4.4
BW5147 (not treated)	AKR	T cell lymphoma	5.7	3.1
(Con A-treated)			49.2	8.0
EL4 (not treated)	B6	T cell lymphoma	0.0	0.0
(Con A-treated)			60.0	0.0
7C3-13 (not treated)	B10.BR	T cell hybridoma	0.0	0.0
(Con A sup-treated)			92.1	0.0
TA3 (not treated)	CAF1	B cell lymphoma	0.0	0.0
(LPS-treated)			45.4	0.0
LK35.2 (not treated)	B10.BR	B cell hybridoma	0.0	0.0
(LPS-treated)			0.0	0.0
46.6	BALB/c	prepre-B	0.0	0.0
Ig6.3	BALB/c	pre-B	0.0	0.0
WEHI231	BALB/c	B	0.0	0.0
B16 (not treated)	B6	melanoma	0.0	0.0
(Interferon γ-treated)			0.0	0.0
MH134 (not treated)	C3H	hepatoma	0.0	0.0
(IL6-treated)			0.0	0.0

Cells were incubated with RE mAbs for 1 h. at 37°C. % cytotoxicity were evaluated as described in Materials and Methods. Some tumor cell lines and hybridomas were treated with the indicated reagents for 12-24 h before cytolytic assay.

Table 2. Cytotoxicity of RE2 on normal cells.

Target cells	% Cytotoxicity with	
	RE2	RE1
Thymocytes	0.0	0.0
Con A-activated thymocytes	0.0	0.0
Anti-CD3-activated thymocytes	0.0	0.0
Spleen cells	0.0	0.0
Con A-activated spleen cells	40.0	0.0
Con A-activated splenic T cells	78.9	0.0
LPS-activated splenic B cells	54.5	0.0
Normal lymph node cells	0.0	0.0
Con A-activated lymph node cells	41.0	0.0

Thymocytes and spleen cells of C3H (38) were incubated with or without Con A (2μg/ml), LPS (5μg/ml), PMA (100ng/ml) and anti-CD3 mAb(3μg/ml) for 24-48h at 37°C. The treated cells were tested for their susceptibility to RE mAbs. % cytotoxicity were evaluated as described in Materials and Methods.

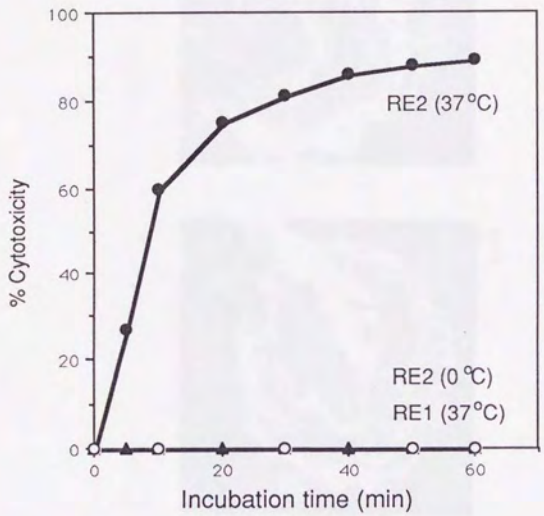
100
80
60
40
20
0
% Cytotoxicity
RE2 (37°C)
RE2 (0°C)
RE1 (37°C)
0
20
40
60
Incubation time (min)

Fig.1

Fig.2a

Fig.2b

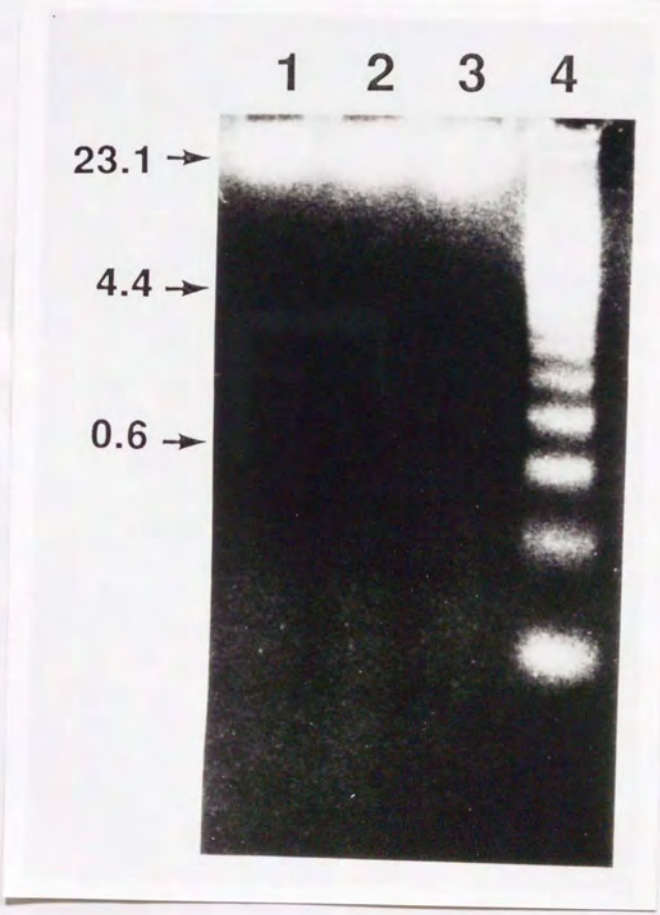

Fig.3

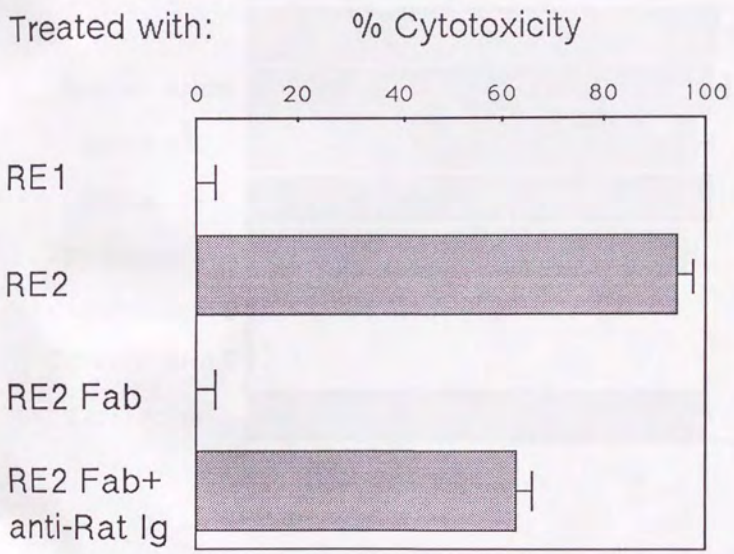
Treated with:
% Cytotoxicity
0
20
40
60
80
100
RE1
RE2
RE2 Fab
RE2 Fab+
anti-Rat Ig

Fig.4

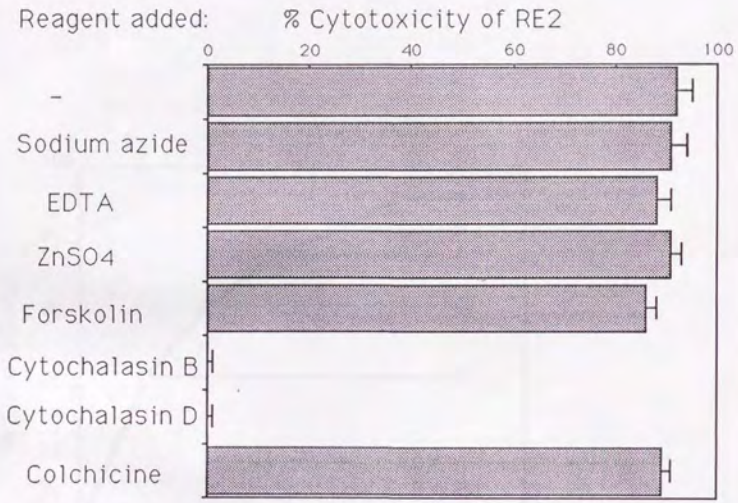
Reagent added:
% Cytotoxicity of RE2
0
20
40
60
80
100
-
Sodium azide
EDTA
ZnSO4
Forskolin
Cytochalasin B
Cytochalasin D
Colchicine

Fig.5

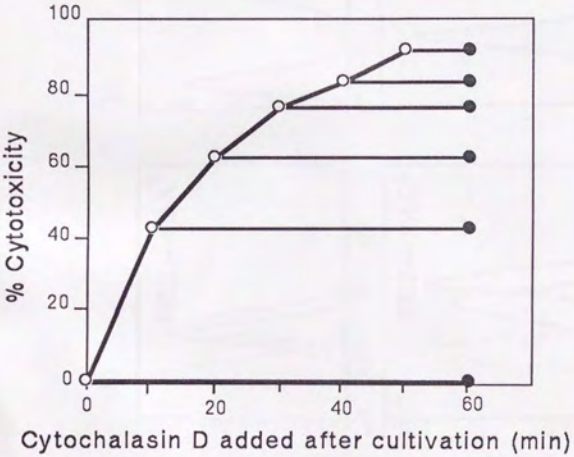
100
80
60
40
20
0
% Cytotoxicity
0
20
40
60
Cytochalasin D added after cultivation (min)

Fig.6

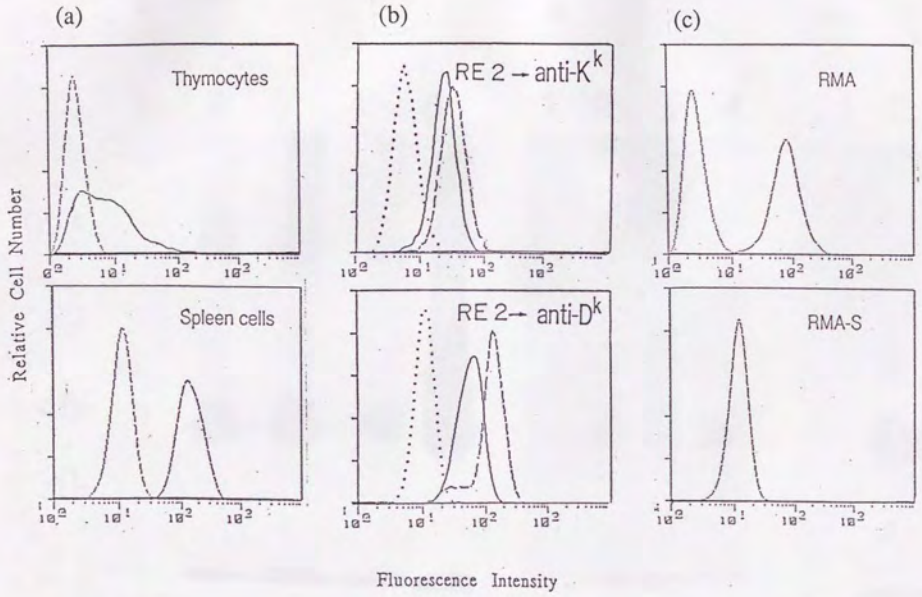
(a)
(b)
(c)
Thymocytes
RE 2 – anti-K^k
RMA
Spleen cells
RE 2 – anti-D^k
RMA-S
Relative Cell Number
Fluorescence Intensity

Fig. 7

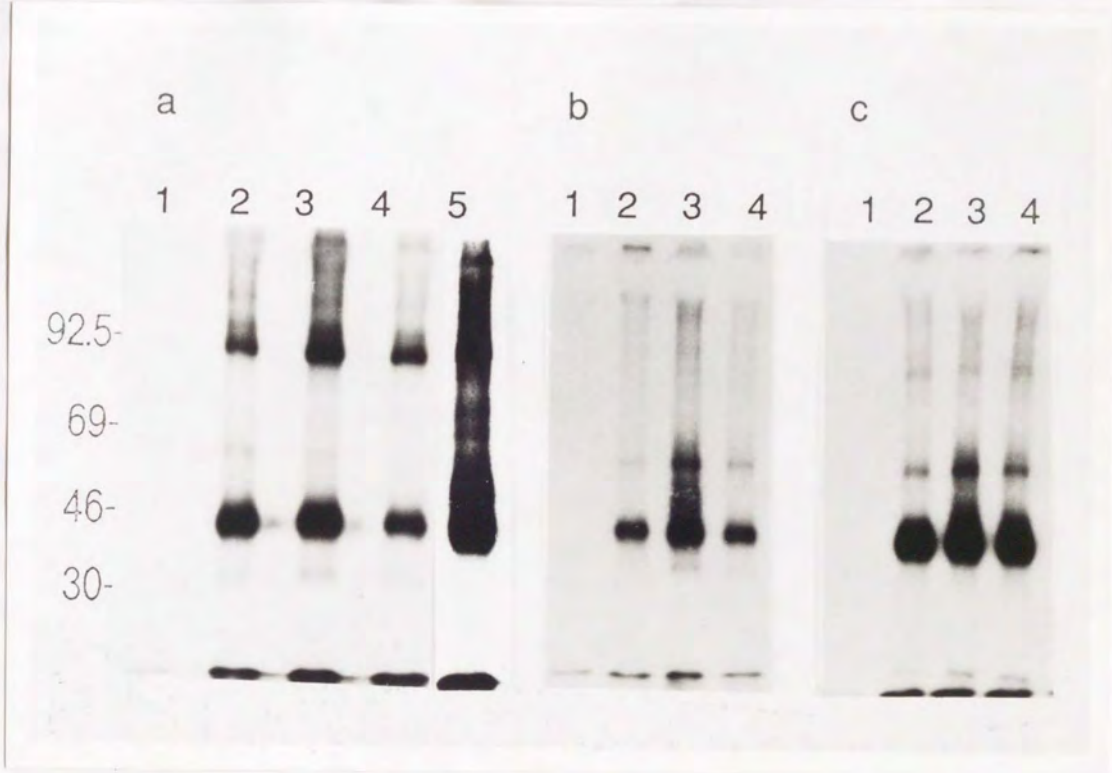

Fig.8

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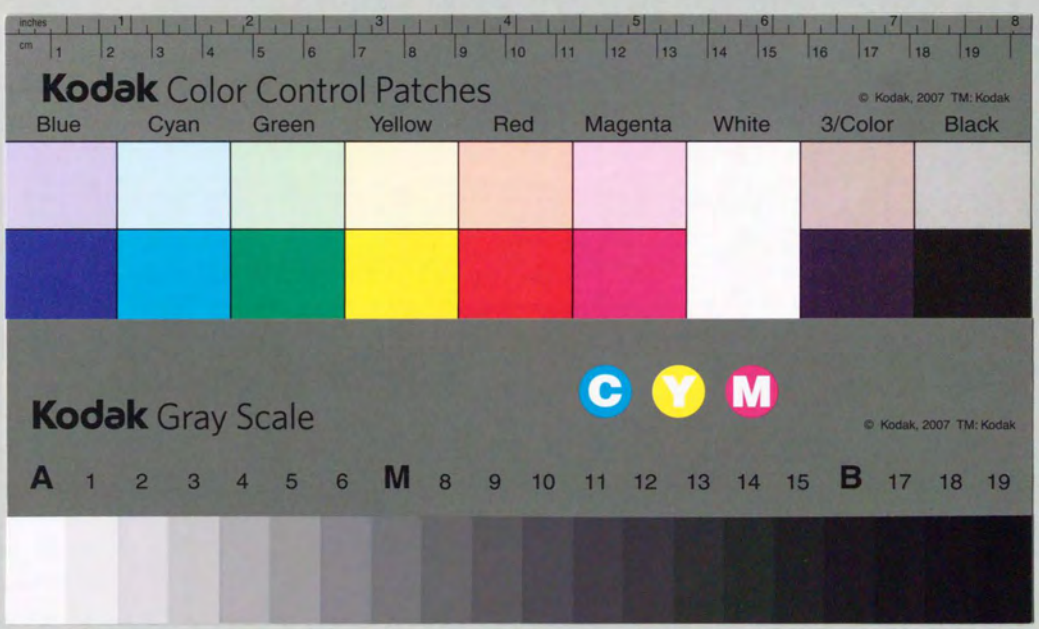
松岡様

against cell surface antigens without participation of complement


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19