THE ROLE OF DIVALENT CATIONS IN ANTIBODY-DEPENDENT MACROPHAGE-MEDIATED TUMOR LYSIS

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1. Introduction

Macrophages, in addition to lymphocytes, play an important role in tumor rejection [1,2], and activated or stimulated macrophages can lyse tumor cells in vitro. So far two types of tumor lysis by macrophages have been reported:
(i) Direct lysis [3,4];
(ii) Lysis in the presence of antibody [5,6];
but the mechanisms of these actions are unknown.

We investigated the mechanism of the latter type of cytolysis in a C3H/He mouse-MM46 tumor system [6–9]. In this system, immune or stimulated macrophages lysed tumor cells in the presence of syngeneic antitumor antibody [6,8]. Macrophages recognized tumor cells which were coated with antibody through the surface Fc-receptors (submitted). This effector–target interaction was necessary for cytolysis by the macrophages (submitted). To determine what reaction leading to tumor lysis occurs after the effector–target interaction, we tested various compounds as inhibitors of cytolysis. We have found that EDTA inhibited antibody-dependent tumor lysis by macrophages and studied the role of divalent cations in the reaction.

2. Materials and methods

2.1. *Mice, tumor and immunization*

MM46, a transplantable ascites tumor from a spontaneous mammary carcinoma in a C3H/He mouse, was passaged weekly in the peritoneal cavity of C3H/He mice. Mice resistant to MM46 tumor were obtained by inoculation of tumor cells attenuated with mitomycin C (Kyowa Hakko Kogyo, Tokyo) and then 4 challenges of fresh tumor cells [7]. Immune sera of resistant mice were purified by gel filtration and then by two types of ion-exchange column chromatography to obtain the IgG 2a fraction active in cytolysis [6].

2.2. *Macrophage-mediated cytolysis*

Macrophage-mediated cytolysis was assayed by determining ^{51}Cr-release from labeled MM46 tumor cells on incubation with glycogen-stimulated macrophages and purified antitumor antibody at 37°C for 8 h, as in [9].

2.3. *Chemicals*

Ethylenediaminetetraacetic acid (EDTA, Wako Pure Chem. Ind., Tokyo), ethyleneglycolbis-(2-aminoethylether) tetraacetic acid (EGTA, Nakarai Chem., Kyoto), $CaCl_2 \cdot 2\,H_2O$ (Wako), $MgCl_2 \cdot 6\,H_2O$ (Koso Chem., Tokyo), $MnCl_2 \cdot 4\,H_2O$ (Koso), $ZnSO_4 \cdot 7\,H_2O$ (Koso) and $Ba(NO_3)_2$ (Koso) were of the highest grade available. RPMI-1640 medium (Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, 100 U/ml of penicillin and 60 μg streptomycin/ml (RPMI–FCS) was used for the cytolysis assay. This assay medium contained ~0.8 mM Ca^{2+} and 0.5 mM Mg^{2+} [10]. Calcium- and magnesium-free medium (CMF) was prepared using Eagles MEM-amino acids and vitamins medium (Nissui Seiyaku) and divalent cation-free Hank's balanced salt solution. FCS dialysed thoroughly against divalent cation-free salt solution was added to the medium (CMF–FCS) used for cytolysis assay.

3. Results

3.1. *Effects of EDTA and EGTA on cytolysis*

Antibody-dependent tumor lysis by macrophages was examined in the presence of EDTA or EGTA

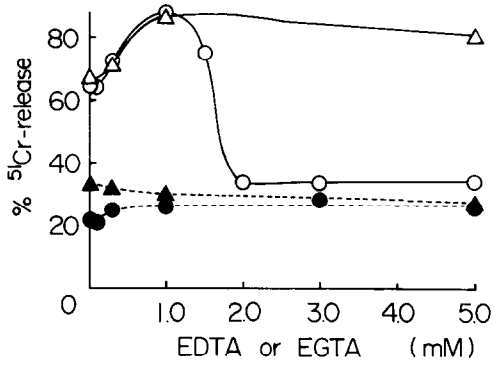

Fig.1. Effects of EDTA and EGTA on antibody-dependent cytolysis by macrophages. In the presence of the indicated concentration of EDTA (○) or EGTA (△), ^{51}Cr-labeled tumor cells (7.5×10^3) were incubated with glycogen-stimulated macrophages (2×10^5) and purified antitumor antibody (1 μg) in 0.5 ml of RPMI–FCS at 37°C for 8 h. Then the radioactivity of the supernatant was determined. As a control, ^{51}Cr-labeled tumor cells and macrophages were incubated in the absence of antibody (●,▲).

$$\%\ ^{51}\text{Cr-release} = \frac{\text{Experimental count}}{\text{Max. releasable count (freeze–thaw 3 times)}}$$

Max. releasable count, 6452 ± 148 cpm.

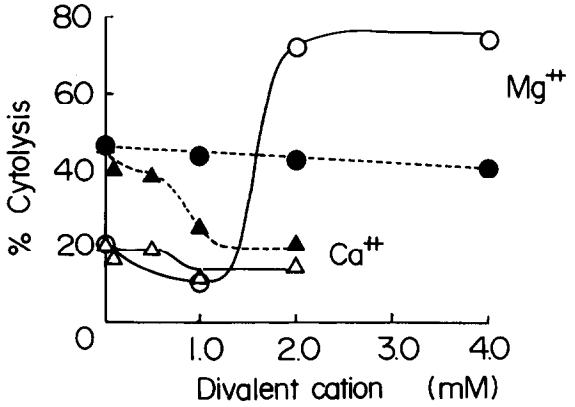

Fig.2. Effects of Mg^{2+} and Ca^{2+} on EDTA-inhibited cytolytic activity. In the presence of 3 mM EDTA, the indicated concentrations of $MgCl_2$ (○) and $CaCl_2$ (△) were added to the cytolytic assay mixtures in fig.1. Divalent cations were also added to the assay mixtures in the absence of EDTA (●,▲).

$$\%\ \text{Cytolysis} = \frac{\text{Exp. count} - \text{control count (without antibody)}}{\text{Max. count} - \text{control count (without antibody)}}$$

Maximum releasable count, 4774 ± 218 cpm; control count, 1346 ± 24 cpm.

using RPMI–FCS. As shown in fig.1, EDTA at >2 mM inhibited the cytolysis activity, but EGTA did not. Neither EDTA nor EGTA affected ^{51}Cr-release in control cultures without antitumor antibody. The enhancing effects of 1 mM EDTA and 1 mM EGTA, shown in fig.1, suggest that RPMI–FCS does not provide optimal concentrations of divalent cations for the cytolysis.

3.2. *Reversion of cytolysis by Mg^{2+} but not Ca^{2+}*

Next we examined whether EDTA actually inhibits the cytolysis by chelating divalent cations. For this, we studied the effect of adding $CaCl_2$ or $MgCl_2$ to the cytolysis assay mixtures in the presence of 3 mM EDTA using RPMI–FCS. Fig.2 shows that the cytolysis activity that was inhibited by 3 mM EDTA was restored by 2 mM Mg^{2+}, but not Ca^{2+}, and that Mg^{2+} increased the activity to more than the control level in RPMI–FCS. In RPMI–FCS in the absence of EDTA, addition of Mg^{2+} had no effect on the cytolysis activity, whereas addition of Ca^{2+} was inhibitory.

For more precise determination of the Mg^{2+}-requirement in antibody-dependent tumor lysis mediated by macrophages, we used CMF medium and dialysed FCS (CMF–FCS). Fig.3 shows that no cytolysis activity was detected in CMF–FCS, and that addition of graded concentrations of Mg^{2+}, but not of Ca^{2+}, induced the cytolysis activity. Thus Mg^{2+} is necessary for antibody-dependent tumor lysis by macrophages, but Ca^{2+} is not necessary and is rather inhibitory.

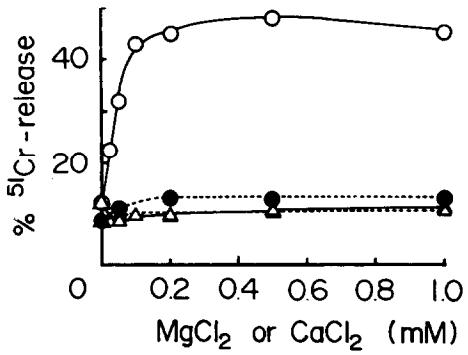

Fig.3. Effects of Mg^{2+} and Ca^{2+} on antibody-dependent cytolysis activity of macrophages in CMF–FCS. The indicated concentrations of $MgCl_2$ (○) and $CaCl_2$ (△) were added to the cytolysis assay mixtures in CMF–FCS. As a control, these cations were added to the assay mixtures without antibody (●,▲). Maximum releasable count, 5530 ± 266 cpm.

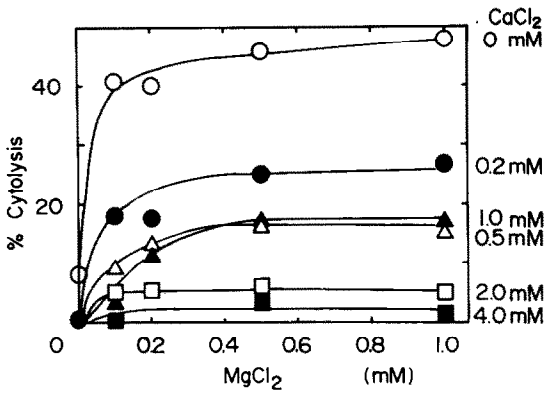

Fig.4. Inhibitory effect of Ca^{2+} on Mg^{2+}-induced cytolysis. Experimental procedures were as for fig.3.

The inhibitory effect of Ca^{2+}, shown in fig.2, was examined in more detail by adding various concentrations of $CaCl_2$ to CMF–FCS containing $MgCl_2$. Fig.4 shows that Ca^{2+} decreased the maximum level of the cytolysis activity induced by Mg^{2+}, but that Ca^{2+} did not change the concentration of Mg^{2+} required for cytolysis. Thus Ca^{2+} inhibits the cytolysis non-competitively with Mg^{2+}.

3.3. *Effects of other divalent cations on cytolysis*

Finally, we examined whether Mg^{2+} could be replaced by other divalent cations. Various divalent cations at 0–2 mM were added to the cytolysis reaction mixtures in CMF–FCS. The results in fig.5 show that Mn^{2+} and Ba^{2+} were not effective for the cytolysis activity, and that Zn^{2+} was, by itself, cytotoxic to target tumor cells. Only Mg^{2+} was effective for induction of antibody-dependent tumor lysis mediated by macrophages.

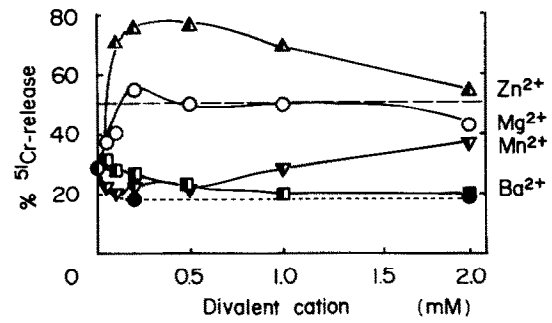

Fig.5. Effects of other divalent cations on antibody-dependent cytolysis activity of macrophages in CMF–FCS. Experimental procedures were as for fig.3. In the presence of Mn^{2+} (▼), Ba^{2+} (■) and Zn^{2+} (▲), experimental ^{51}Cr-release was the same as control ^{51}Cr-release (without antibody). Mg^{2+}: (○) with antibody; (●) without antibody.

4. Discussion

In this work we showed that antibody-dependent tumor lysis by macrophages is dependent on Mg^{2+}, but not Ca^{2+}, and that Ca^{2+} is rather inhibitory to cytolysis. Mg^{2+} at 0.2 mM was sufficient for expression of cytolysis activity (fig.3). This finding is consistent with results of a reversion experiment in the presence of EDTA (fig.2) showing that 2 mM Mg^{2+} was sufficient for expression of cytolysis activity in the presence of 3 mM EDTA in RPMI–FCS (0.5 mM Mg^{2+} and 0.8 mM Ca^{2+}). Mg^{2+} had a specific effect, since other divalent cations, and noteably Mn^{2+} which can replace Mg^{2+} in another system [15], were not effective.

Several investigators have studied the role of divalent cations in cytolysis, especially T cell-mediated cytolysis [10–14] and antibody-dependent cell-mediated cytolysis [15,16], though not yet in macrophage-mediated cytolysis. The process of T cell-mediated cytolysis has been resolved into 3 successive steps: recognition, post-recognition hit and target disintegration [10,12,13]. These 3 steps require Mg^{2+} or Ca^{2+}, Ca^{2+}, and not cations, respectively [10,12]. Thus Ca^{2+} is essential in this system. On the other hand, in antibody-dependent cytolysis mediated by non-T cells, different systems of cytolysis had different cation requirements [15,16]; lysis of antibody-coated sheep red blood cells mediated by mouse spleen cells required Mg^{2+}, the same lysis mediated by human blood cells required no cations, and lysis of Chang cells mediated by human blood cells required Ca^{2+}. The reason for these different cation-requirements is unknown, since the nature of the effector cells was not well defined in these systems. In this respect, we clearly demonstrated a Mg^{2+} requirement for antibody-dependent tumor lysis mediated by macrophages, which suggests that a Mg^{2+} requirement may be peculiar to macrophage-mediated cytolysis or antibody-dependent macrophage-mediated cytolysis.

A new and interesting finding in this work was that Ca^{2+} was inhibitory, but not competitive with Mg^{2+}. The mechanisms of the effects of these two divalent cations are unknown. We have found that antibody-dependent cytoadherence between macrophages and tumor cells was induced even in the

absence of Mg^{2+} and Ca^{2+}, and that it was not inhibited by Ca^{2+} (unpublished). Mg^{2+} seems to be essential for the post-cytoadherence step, lytic events, and Ca^{2+} seems to inhibit the post-cytoadherence step. Further studies are in progress on the site of binding and the effect of Mg^{2+}.

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