THE ROLE OF Mg^{2+} and Ca^{2+} IN THE DIRECT MACROPHAGE-MEDIATED TUMOR CELL LYSIS

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1. Introduction

Macrophages and lymphocytes possibly play an important role in the host defense against neoplasms [1,2]. In vitro the process of the tumor cell lysis by specific T lymphocytes has been resolved into 3 steps. Several investigators have established that one of these steps is Mg^{2+}-dependent, and that another one is Ca^{2+}-dependent [3,4]. In vitro macrophages destroy tumor cells by 2 types of cytolysis: a direct cytolysis and a cytolysis in presence of antibodies. The mechanisms of these 2 types of macrophage-mediated cytolysis are unknown.

In order to investigate the role of Ca^{2+} and Mg^{2+} in the direct macrophage-mediated tumor cell lysis, we used 2 approaches:
(i) With various concentrations of chelating agents;
(ii) With media of defined cationic content.
It was found that Mg^{2+} inhibited the tumor cell lysis by BCG-activated macrophages, whereas Ca^{2+} had no effect on the cytolysis. These results indicate that the mechanism of the direct macrophage-mediated tumor cell lysis could be different from the mechanism of the T lymphocyte-mediated cytolysis.

2. Materials and methods

2.1. *Chemicals*

Ethylene diamine tetraacetic acid disodium salt (EDTA), $CaCl_2 \cdot 2\,H_2O$ and $MgCl_2 \cdot 6\,H_2O$ were purchased from Merck (Darmstadt). Ethylene diamine tetraacetic acid calcium disodium salt (CaEDTA) and ethyleneglycol–bis-(2-aminoethyl) tetraacetic acid (EGTA) were obtained from Fluka (Buchs).

2.2. *Macrophages and tumor cells*

The macrophages were adherent peritoneal cells from BDIX rats, purified by washing. Activated macrophages were harvested 5–10 days after the last of 2 intraperitoneal injections of BCG, 1 mg/injection, 1 month apart. A permanent cell line, DHD K-12, established from a dimethylhydrazine-induced BDIX rat colon carcinoma [5] was used as target cells. Tumor cells ($5–10 \times 10^6$) were incubated with 50 μCi [^{3}H]thymidine for 18 h. They were then washed with 30 mM EDTA, trypsinised and washed with Ham F-10 supplemented with 10% FCS or with HBSS supplemented with 2% FCS.

2.3. *Macrophages-mediated cytolysis assay*

Macrophages (10^6/well) were incubated 1 h at 37°C in a microtest 3040 tissue culture plate (Falcon, Oxnard CA). They were washed with 30 mM EDTA to remove Ca^{2+} and Mg^{2+} and with HBSS to remove EDTA. The labelled tumor cells (10^5/well) were seeded with or without preincubated macrophages. After 72 h at 37°C the cultures were washed 4 times to remove non-adherent cells. The remaining viable adherent cells were lysed with 1 N sodium hydroxide and the radioactivity was counted. The cytolytic activity of the macrophages was calculated in percentage according to the formula $100 \times [(a - b)/a]$ where a is the mean radioactivity of the cancer cells and b is the mean radioactivity of the cancer cells cultivated with macrophages.

2.4. *Incubation media*

Ham F-10 culture media, Ca^{2+}- and Mg^{2+}-free · Hank's balanced salt sodium (HBSS), and fetal calf serum (FCS) were purchased from Microbiological Assoc. (Walkersville MD). Total [Ca^{2+}] and total

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$[Mg^{2+}]$ in the different incubation media were determined by atomic absorption spectroscopy. Equilibrium concentrations of each cation, with or without complexing agents in the different incubation media were calculated with a computer program according to [6]. The K_d-values of EDTA for Mg^{2+} and Ca^{2+} used were $10^{-8.69}$ M and $10^{-10.7}$ M, respectively [7]. The pH of the incubation media varied from 7.4–7.9. The absence of endotoxin in the incubation media has been checked by the *Limulus* amebocyte lysate test (Microbiological Assoc.).

3. Results and discussion

Macrophages activated in vivo by BCG were cytolytic in vitro on tumor cells in Ham F-10 medium supplemented with 10% FCS. In this mixture total $[Ca^{2+}]$ and total $[Mg^{2+}]$ were 1 mM and 0.8 mM, respectively. As shown in fig.1, addition of EDTA or EGTA up to 0.6 mM, significantly enhanced the macrophage-mediated cytolysis. Below 0.6 mM, EDTA and EGTA were weakly toxic for tumor cells. It was not possible to investigate the effect of higher concentrations as their toxic effect exceeded 25%. It has been verified that, with the concentrations used, neither EDTA nor EGTA detached the tumor cells. It was calculated that the addition of EDTA in the culture medium up to 0.4 mM, induced a diminution of free Ca^{2+} and free Mg^{2+} of 0.38 mM and 0.01 mM, respectively. The complexes formed were CaEDTA 0.395 mM and MgEDTA 0.005 mM. These results suggest that the macrophage-mediated cytolysis is either inhibited by free Ca^{2+} and/or free Mg^{2+}, or enhanced by their complexes with EDTA.

To determine the role of free Ca^{2+} or Mg^{2+} in the enhancing effect of EDTA, we added $CaCl_2$ or $MgSO_4$ to the 0.4 mM EDTA culture medium. The necessary quantities of the 2 cations to add to 1 liter of the incubation medium in order to restore their initial levels, were calculated as 0.42 mmol of Ca^{2+} and 0.03 mmol Mg^{2+}. Actually, the addition of these quantities of $CaCl_2$ or $MgSO_4$ to EDTA mixtures did not modify the EDTA enhancing effect (table 1). These results showed that the enhancement of the macrophage-mediated cytolysis was not due to the diminution of free Ca^{2+} and free Mg^{2+} but could be due to the EDTA complexes.

To ensure that the effect obtained with EDTA was an exclusive property of EDTA complexes, macrophage-mediated cytolysis were performed with increasing concentration of CaEDTA. The results

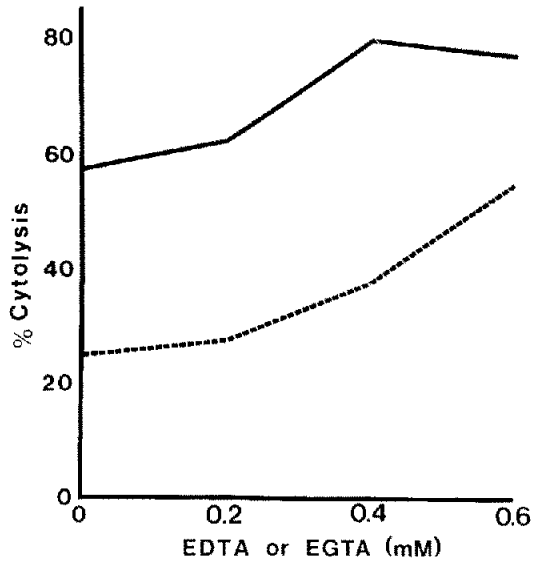

Fig.1. Effects of EDTA (——) and EGTA (– – –) on the direct macrophage-mediated tumor cell lysis. Each point represents the mean of 8 results.

Table 1
Effects of Ca^{2+} or Mg^{2+} on the EDTA-stimulated cytolytic activity of macrophages

Culture medium (final conc. mM)			Residual cpm ($\times 10^2$)[a]		Cytolysis[b] (%)
EDTA	Ca^{2+}	Mg^{2+}	no mϕ[c]	mϕ	
0	0	–	124 ± 8	89 ± 3	28
0.4	0	–	98 ± 6	46 ± 7	53
0.4	0.4	–	100 ± 3	43 ± 4	57
0.4	0.45	–	105 ± 4	44 ± 4	58
0.4	0.5	–	94 ± 2	44 ± 2	53
0	–	0	115 ± 6	88 ± 3	23
0.4	–	0	87 ± 5	46 ± 5	47
0.4	–	0.02	90 ± 5	51 ± 9	43
0.4	–	0.03	92 ± 9	50 ± 8	46
0.4	–	0.04	104 ± 2	56 ± 6	46

[a] Mean of 8 wells ± SD

[b] The cytolytic effect of activated macrophages was highly significant in all experiments ($p < 0.001$, Student's t-test). The effect of EDTA on the macrophage-mediated cytolysis was highly significant ($p < 0.01$, Mann and Whitney's U-test). Neither Ca^{2+} nor Mg^{2+} had a significant effect on the EDTA enhancement

[c] mϕ, macrophages

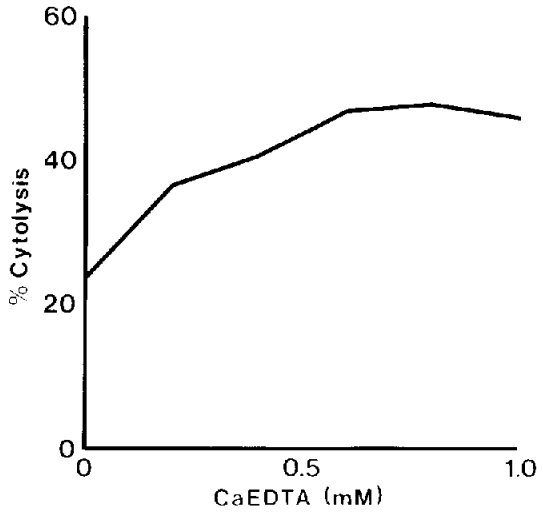

Fig.2. Effects of CaEDTA on the macrophage-mediated tumor cell lysis. Each point represents the mean of 8 results.

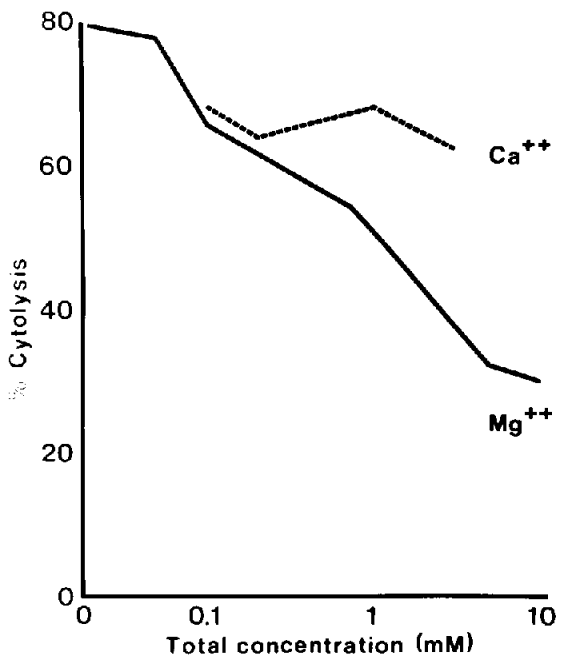

Fig.3. Effects of Ca^{2+} and Mg^{2+} on the macrophage-mediated tumor cell lysis. The cytolysis was measured in Ca^{2+}- and Mg^{2+}-free HBSS with 1% FCS. [Mg^{2+}] varied from 0.02–1 mM at 1 mM Ca^{2+} (——). [Ca^{2+}] varied from 0.1–3 mM at 0.8 mM Mg^{2+} (– – –). Each point represents the mean of 8 results.

shown in fig.2 confirm that CaEDTA up to 1 mM significantly enhanced the macrophage-mediated cytolysis. CaEDTA was not toxic on tumor cells, and it did not detach them. It was calculated that when 0.8 mmol CaEDTA was added to 1 liter medium, actual CaEDTA was 0.794 mM, MgEDTA was 0.00596 mM, free Ca^{2+} remained unchanged and free Mg^{2+} decreased to 0.01 mM. As was shown before, this decrease did not affect the macrophage-mediated cytolysis. Therefore the enhancement of the macrophage-mediated cytolysis observed with an addition of CaEDTA was actually due to the complexes and probably to CaEDTA. MgEDTA was not assayed because Ca^{2+} displaces Mg^{2+} from MgEDTA complexes to give a mixture of CaEDTA and MgEDTA complexes.

Since the evaluation of the roles of Ca^{2+} and Mg^{2+} in the macrophage-mediated cytolysis by the use of EDTA is greatly complicated by the effect of CaEDTA on the macrophage activity, it has been examined in the absence of EDTA. To assay low levels of Ca^{2+} and Mg^{2+}, experiments have been performed in Ca^{2+}- and Mg^{2+}-free HBSS supplemented with only 1% FCS, and graded concentrations of the two cations. In these experiments, increasing Mg^{2+} from 0.02–10 mM, at 1 mM Ca^{2+}, significantly inhibited the macrophage-mediated cytolysis (fig.3). On the contrary, increasing Ca^{2+} from 0.1–3 mM, at 0.8 mM Mg^{2+}, had no significant effect on the macrophage-mediated cytolysis. Whatever the Mg^{2+} level tested it was not toxic for the tumor cells. Ca^{2+} at <0.1 mM were cytotoxic, and at >3 mM, $CaCl_2$ was not soluble. The decrease of FCS from 10–1% led to a weak enhancement of the cytolysis (mean 6%, not significant), without cytotoxic effect.

We have shown that direct macrophage-mediated tumor cell lysis was inhibited by Mg^{2+} and was Ca^{2+}-independent. Furthermore, we observed that CaEDTA enhanced the macrophage cytolytic activity. To our knowledge, no study has been performed on the role of Ca^{2+} or Mg^{2+} in the direct macrophage-mediated cytolysis. In [8], the effects of EDTA and EGTA in antibody-dependent macrophage-mediated cytolysis was investigated. It was concluded that Mg^{2+}, but not Ca^{2+}, was necessary. Nevertheless, one cannot assert that this Mg^{2+}-dependence is the property of the cytolysis itself and not a character of the antibody recognition by the Fc receptor. Besides, the non-macrophage- and non-T cell-mediated cytolysis of antibody-coated sheep red blood cells requires Mg^{2+} but not Ca^{2+} [9]. Investigators who studied the spe-

cific tumor cell lysis by sensitized T lymphocytes found that Mg^{2+} is sufficient to support the adhesion between T lymphocytes and tumor cells, whereas programming the tumor cell for lysis is strongly Ca^{2+}-dependent [3,4]. Our results indicate that the mechanism of the direct macrophage-mediated cytolysis could be different from the mechanism of the lymphocyte-mediated cytolysis. According to [10], the macrophage-mediated tumor cell lysis is due to a fusion of the membranes of the 2 cells, allowing a transfer of the macrophage lysosomes into the tumor cell. Ca^{2+} appears to play an important role as a regulator of membrane fusion: in certain cases, Ca^{2+} restricts the lateral mobility of anionic phospholipids, but in other cases Ca^{2+} enhances the lateral mobility of membrane lipids [11]. Thus the Ca^{2+} role is variable. In most systems, Mg^{2+} is ineffective in natural membrane fusion phenomena, but it has been reported to inhibit the fusion of cultured muscle cells [12]. The inhibiting effect of Mg^{2+} on the macrophage-mediated cytolysis is consistent with the fusion hypothesis in [10].

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