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Limited specific T-cell mediated cytolysis in the absence of extracellular Ca^{2+}

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Summary. Mouse lymphocytes sensitized either *in vitro* or *in vivo* with allogeneic cells were analysed for their cytolytic activity in complete or Ca^{2+}-deprived medium. The deprived medium was obtained by adding EGTA in excess of the molar Ca^{2+} concentration but not exceeding that of Ca^{2+} plus Mg^{2+}. The cytolysis in complete medium was between five and thirty-fold greater in terms of lytic units, than that in Ca^{2+}-deprived medium. This applied equally for effectors sensitized *in vivo* or *in vitro* although the overall lytic capacity of the former was greater. The cytolysis in Ca^{2+}-deprived medium was judged to be Mg^{2+} dependent, as little or no cytolysis was seen if EDTA was added to cultures in a molar concentration exceeding that of Ca^{2+} plus Mg^{2+}. Direct comparison of cytolysis in complete and deprived medium showed no difference between effectors in the two situations with respect to (a) adherence to nylon wool; (b) sensitivity to lysis by anti-Thy-1.2 antiserum and complement; (c) specificity of lysis; (d) rate of cytolysis with time when effector cell numbers were adjusted to give equal levels of cytolysis at any one time; and (e) shape and angle of the slope of ^{51}Cr release against log increase in effector cell numbers where target cell numbers were constant.

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INTRODUCTION

In recent years a number of approaches have been devised to study the process of T-cell mediated cytolysis. These have been extensively reviewed (Golstein & Smith, 1977; Henney, 1977; Martz, 1977). This paper uses changes in cation concentration to analyse this form of cytotoxicity. Conventionally, three stages have been recognized in T-cell mediated cytolysis, which have been shown to have relative if not absolute differences in divalent cation requirement. For the first of these stages, the initial effector–target cell interaction, divalent cations are required to form strong specific binding between effectors and targets. Mg^{2+} is more efficient in this respect than Ca^{2+} (Stulting & Berke, 1973; Golstein & Smith, 1976; Plaut, Bubbers & Henney, 1976). Recent results, however, indicate that specific recognition *per se* may occur in the absence of divalent cations but be strengthened by non-specific interactions requiring Mg^{2+} (MacLennan & Golstein. 1978a; Shortman & Golstein, 1979). For the last stage of T-cell mediated cytolysis, target cell disintegration, divalent cations are apparently not required (Golstein & Smith, 1976) and the presence of the effector cell is no longer necessary (Martz & Benacerraf, 1973).

The second 'lethal hit' stage of T-cell mediated cytolysis is characterized by its relative dependence on extracellular Ca^{2+} (Golstein & Smith, 1976, 1977).

This property has been exploited in the 'calcium pulse' technique to study the action of drugs on this key stage of cytolysis (Golstein & Smith, 1977; Golstein, Foa & MacLennan, 1978; MacLennan & Golstein, 1978b). Martz (1975, 1977), however has warned that the requirement for the extracellular Ca^{2+} is not absolute if interaction is induced by cocentrifugation of high-specific-activity effector populations and target cells. Recently we have carried out experiments where a limited but significant level of cytolysis occurred in the absence of free calcium ions in the medium. This cytolysis, however, was minimal or absent unless Mg^{2+} was present in cultures. In this report, we compare cytolyis seen in the presence of extracellular calcium and magnesium ions with that seen in media with Mg^{2+} alone. The specificity of lysis and the nature of the effectors in both systems are compared.

MATERIALS AND METHODS

Media

All experiments were carried out in RPMI-1640 (GIBCO Bio-cult, Glasgow, Scotland) supplemented with 1% dialysed foetal bovine serum (DFBS, batch 802.42, Eurobio, Paris, France). Cells were prepared by washing twice in low phosphate-buffered saline (LPBS) which is an aqueous solution containing 137 mM NaCl, 2·7 mM KCl, 1·5 mM KH_2PO_4 and 3·2 mM Na_2HPO_4 but no divalent cations.

In vivo *sensitized effector cells*

C57Bl/6 mice (H-2b) were sensitized by intraperitoneal injection of 3×10^7 P815 (H-2d) mastocytoma cells. The resulting H-2b anti-H-2d (b→d for short) effector cells were harvested 10–11 days later by washing out the peritoneal cavity of these mice with three changes of 7 ml of LPBS. In many experiments the sensitized peritoneal cells were purified on nylon wool columns as described (Julius, Simpson & Herzenberg, 1973) with minor modifications. The cells were washed twice in LPBS and counted before resuspension in medium for the cytolysis assay.

DBA2 mice were immunized in the same way with the C57Bl/6 lymphoma EL4 to yield d→b effector cells.

Effector cells sensitized in vitro

Mouse splenic responder cells (2×10^7) and irradiated (2000 rad) allogeneic spleen cells (2×10^6) in 20 ml of RPMI 1640 with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine and 0·02 mM mercaptoethanol, were incubated for 5 days in an upright plastic flask (Falcon 3013) in a moist atmosphere of 5% CO_2 in air at 37°. Combinations of C57Bl/6 and DBA2 splenocytes were used to induce b→d and d→b sensitized spleen cells. At the end of incubation the cells were washed twice in LPBS before counting and resuspension in medium for the cytolysis assay.

Labelled target cells

P815 and EL4 maintained as continuous *in vitro* lines were used as targets. These cells ($2–5 \times 10^6$) were labelled overnight with 200 μCi of $Na_2CrO_4^{2-}$ in 10 ml RPMI 1640 plus 10% foetal bovine serum and washed twice in LPBS before enumeration and resuspension in medium.

Cytotoxicity tests

The precise conditions (especially in terms of divalent cations) for each group of experiments varied and these conditions are indicated in the results and legends to figures and tables. All experiments were carried out in Cooke V-shaped-well microtitre plates with 10^4 labelled target cells per well. After the addition of both effector and target cells (and competitor cells in some experiments) the plates were centrifuged at 200 *g* for 2 min to facilitate cell to cell contact. The final volume per well was 200 μl. The cytolytic reaction was carried out at 37° in a moist atmosphere of 5% CO_2 in air. At the end of incubation the culture plates were again centrifuged. The radioactivity of an 100 μl aliquot from each well was expressed as percentage of the initial radioactivity of 5×10^3 target cells. Cytolysis was calculated as the average percentage of ^{51}Cr released from target cells in triplicate wells.

Treatment with anti-Thy-1.2 antibody and complement

Anti-Thy-1.2 antibody was derived from serum of BALB/c mice carrying a hybridoma producing anti-Thy-1.2. This was a kind gift from Dr P. Lake of the Department of Zoology, University College, London. Complement was fresh rat serum absorbed with a 25% volume of packed C57Bl/6 spleen cells.

Cells to be treated with anti-Thy-1.2 antibody were suspended at a concentration of 3×10^7/ml in RPMI 1640 plus 1% DFBS. Fifty-microlitre aliquots of this supension were added to dilutions of anti-Thy-1.2 in 200 μl at 4°. They were incubated for 30 min at this temperature before the addition of 100 μl of absorbed rat serum as complement. The suspension was incu-

bated at 37° for 20 min, resuspended in 5 ml medium, centrifuged, and the pellet was taken up in 500 μl. The number of viable cells in each tube was assessed by trypan blue exclusion and the cytolytic capacity of 50 μl of these suspensions (not readjusted for cell numbers) was tested.

RESULTS

Cytolysis by sensitized peritoneal lymphocytes in the absence of extracellular Ca^{2+}

Nylon-wool column-purified sensitized lymphocytes from C57Bl/6 mice were tested for their capacity to lyse ^{51}Cr-labelled P815 cells in medium containing dilutions of EGTA. EGTA has approximately 1000 times higher affinity for Ca^{2+} than Mg^{2+}. Consequently in RPMI 1640, which contains between 0·4 and 0·5 mM of each of these ions, after the addition of 0·5 mM EGTA the medium would be expected to contain no free Ca^{2+} but almost as much free Mg^{2+} as medium without EGTA. In the experiment described in Fig. 1, cytolysis was greatest in the absence of chelating agent. Around the point where all free calcium was taken up by EGTA the cytolysis diminished abruptly. The level of cytolysis, however, which remained when the molar concentration of EGTA was well in excess of that of Ca^{2+} was still well above the d-non-immune spleen cell control. In terms of lytic units, in this experiment the cytolysis in medium without EGTA, was 14 times that with EGTA in excess of calcium.

A second point to note in this experiment is that even when the EGTA concentration was well in excess of the sum of the concentrations of Mg^{2+} and Ca^{2+} the level of cytolysis was not greatly different from that with 0·5 mM EGTA. As EGTA has some affinity for Mg^{2+} this might have indicated that the cytolysis seen in the absence of free Ca^{2+} in the medium was in fact totally independent of extracellular divalent cations.

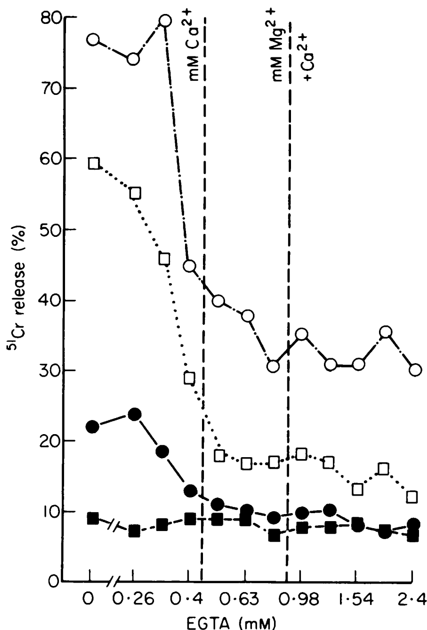

Figure 1. Cytolysis by nylon-wool-purified b→d peritoneal lymphocytes towards P815 target cells in the presence of various dilutions of EGTA. The basic medium was RPMI 1640 + 1% DFBS. This contains 0·4–0·5 mM of both Ca^{2+} and Mg^{2+}. Cultures with > 0·5 mM EGTA should contain no free Ca^{2+}. ○, 40 × 10^4 b→d/well; □, 8 × 10^4 b→d/well; ●, 1·6 × 10^4 b→d/well; ■, 40 × 10^4 d non-immune spleen mononuclear cells/well. Chelating agent was added in 100 μl to each well followed sequentially by effector cells in 50 μl then 10^4 P815 targets in 50 μl. The interrupted vertical line represents the mM concentrations of Ca^{2+} and Ca^{2+} plus Mg^{2+} in the medium. The cytolysis test was for 4 h at 37°.

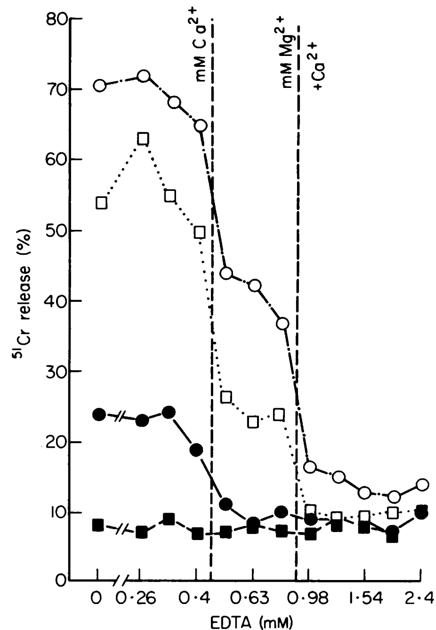

Figure 2. Cytolysis by b→d nylon-wool-purified peritoneal lymphocytes towards P815 target cells in the presence of various dilutions of EDTA. The symbols and cytolysis test conditions are the same as in Fig. 1.

In similar experiments, however, using EDTA instead of EGTA, cytolysis was almost totally abolished when the molar concentration of EDTA exceeded the sum of the concentrations of Mg^{2+} and Ca^{2+} (Fig. 2). The difference between the effects of EGTA and EDTA was even more obvious in the type of experiment described in Fig. 3, in which the concentration of Mg^{2+} was six times higher than the concentration of Ca^{2+}. Greater concentrations of EDTA than EGTA were required before cytolysis fell from the maximum level to the first plateau. Above the molar concentration of Ca^{2+} plus Mg^{2+} a further decline in cytolysis occurred with EDTA (as in Fig. 2) but not with EGTA. This steep fall associated with EDTA concentrations just in excess of that of Mg^{2+} plus Ca^{2+} supports the hypothesis that the cytolysis seen in the absence of free Ca^{2+} is Mg^{2+} dependent. In further experiments (not shown), concentrations of EGTA as high as 11 mM did not reduce cytolysis more than did EGTA just in excess of the Ca^{2+} concentration. In these experiments, the concentrations of both Mg^{2+} and Ca^{2+} were between 0·4 and 0·5 mM. This finding is used in the discussion to argue against a participation in cytolysis of Ca^{2+} leaking from intracellular sources.

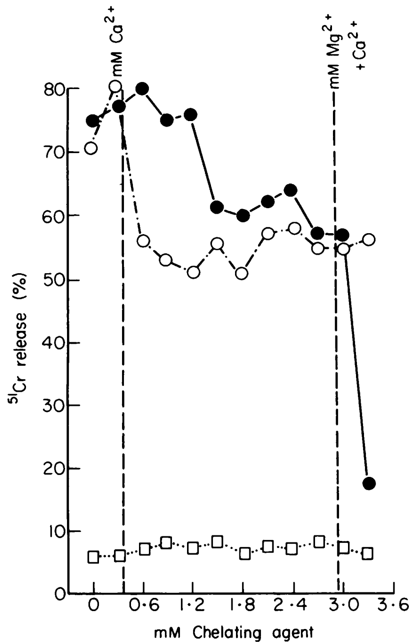

Figure 3. Cytolysis by b→d nylon-wool-purified peritoneal lymphocytes towards P815 target cells in the presence of various dilutions of chelating agent. The basic medium was RPMI 1640 + 1% DFBS + 2 mM Mg^{2+}. The vertical lines represent the concentrations of Ca^{2+} and Ca^{2+} + Mg^{2+} in the medium. •, 15 × 10^4 b→d/well with dilutions of EDTA; ○, 15 × 10^4 b→d/well with dilutions of EGTA; □, 15 × 10^4 d non-immune spleen mononuclear cells with dilutions of EDTA. Duration of the cytolysis test was 4·5 h but the conditions otherwise were as in Figs 1 and 2.

The kinetics of the cytolytic reaction in medium with Ca^{2+} and Mg^{2+} compared with that in medium with Mg^{2+} alone

The rate of cytolysis by dilutions of effector cells against 10^4 targets was assessed in RPMI + 1% DFBS or RPMI + 1% DFBS + 1·5 mM Mg^{2+} and 1·0 mM EGTA (Fig. 4). When equal numbers of effector cells were used the rate of cytolysis was far more rapid when free Ca^{2+} was present in the medium. When equal numbers of lytic units were used, however, the kinetics of the reaction was the same for cytolysis in medium with Mg^{2+} and Ca^{2+} compared with that with Mg^{2+} only.

Specificity of cytolysis seen in Mg^{2+}-containing Ca^{2+}-deprived medium

Figure 5 shows good levels of specific lysis by b→d and

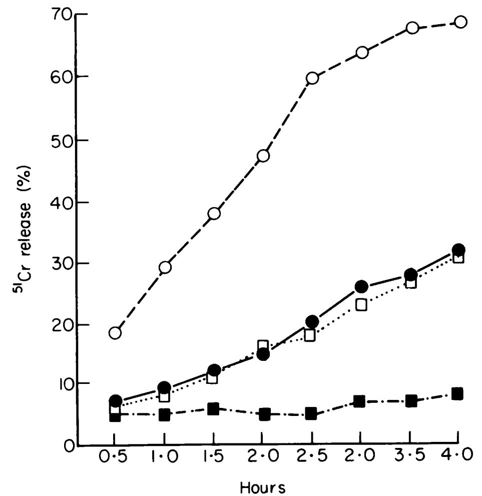

Figure 4. The rate of cytolysis mediated by nylon-wool-purified b→d peritoneal lymphocytes in complete and Ca^{2+}-deprived medium. Cultured in RPMI 1640 plus 1% DFBS: ○, b→d 20 × 10^4/well. □, b→d 2 × 10^4/well. ■, d-non-immune spleen mononuclear cells 20 × 10^4/well. Cultures in RPMI 1640 plus 1% DFBS plus 1·5 mM Mg^{2+} and 1 mM EGTA, i.e. $[Mg^{2+}] > [EGTA] > [Ca^{2+}]$; •, b→d 20 × 10^4/well. All wells had 10^4 labelled P815 as targets.

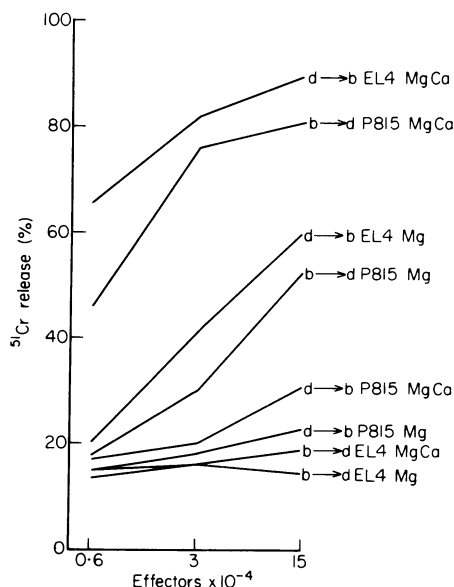

Figure 5. Specificity of cytolysis mediated in complete or Ca^{2+} deprived medium by nylon-wool-purified peritoneal lymphocytes sensitized against allogeneic tumour cells. Side lettering from left to right indicates: 1. b→d or d→b effectors; 2. tested against P815 (H-2d) or EL4 (H-2b) tumour targets; 3. in Mg Ca (RPMI 1640 plus 1% DFBS) or Mg (RPMI 1640 plus 1% DFBS, 1·5 mM Mg^{2+} and 1 mM EGTA). All cultures had 10^4 labelled target cells. Cytolysis was allowed to proceed for 5 h at 37° before harvesting.

d→b peritoneal effector cells of their allogeneic tumour target cells. With EL4 target cells, the number of d→b effector cells required to produce 50% ^{51}Cr release with Mg^{2+} and Ca^{2+} was $0{\cdot}23 \times 10^4$ and with Mg^{2+} only was $6{\cdot}5 \times 10^4$, i.e. a twenty-seven-fold difference. Cytolysis against syngeneic tumour cells was only marked with d→b effector cells tested on P815 in the presence of both Ca^{2+} and Mg^{2+}. This experiment, therefore, demonstrates the specificity of Mg^{2+}-dependent, Ca^{2+}-independent cytolysis.

Unlabelled tumour cells or normal spleen cells were shown to inhibit this cytolysis competitively in a specific way (Table 1) which further establishes its specificity and demonstrates that it was not directed against antigens only found on tumour cells.

Table 1. Specificity of Ca^{2+}-independent-Mg^{2+}-dependent kill assessed by addition of competitor cells

Targets / Effectors	EL4 b→d	EL4 d→b	P815 b→d	P815 d→b
Competitors × 10^4				
d-NIS				
220	19	53	33	24
73	19	60	41	20
24	23	54	44	17
8	25	66	49	21
0	16	60	50	21
b-NIS				
220	14	31	57	21
73	17	45	54	20
24	16	61	57	25
8	16	62	52	22
0	13	60	52	24
P815				
30	16	60	25	17
10	16	65	33	21
3·3	21	57	42	20
0	17	60	50	21
EL4				
30	16	29	57	21
10	17	43	59	21
3·3	15	53	54	21
0	13	60	52	24

Labelled and unlabelled target cells were mixed in wells before the addition of effectors. Each well had 10^4 labelled targets and 15×10^4 nylon-wool-purified peritoneal lymphocytes. The results are shown as percentage ^{51}Cr release from targets in cultures carried out for 5 h at 37° in RPMI 1640 plus 1% DFBS, 1·5 mM Mg^{2+} and 1 mM EGTA, i.e. $[Mg^{2+}] > [EGTA] > [Ca^{2+}]$. b-NIS and d-NIS are spleen mononuclear cells from normal non-immunized C57Bl/6 and DBA/2 mice respectively.

The nature of the effector cells

Sensitized peritoneal cells were passed through nylon-wool columns and the passed cells were tested for cytolytic activity in different cation conditions. Table 2 shows that in terms of percentage lytic activity recovered after column fractionation, no consistent difference was detected between Ca^{2+}-dependent and Ca^{2+}-independent cytolysis.

Table 3 shows that pre-treatment of effector cells with anti-Thy-1.2 antibody and complement specifically ablated both the cytolytic activity observed in the presence of Mg^{2+} and Ca^{2+} and that with Mg^{2+} only.

Table 2. Nylon-wool separation and specific cytolysis by b→d peritoneal lymphocytes

		Lytic units per 10^6 lymphoid cells in medium		Lytic activity recovered (%) in medium	
	Yield (%)	Ca^{2+}-deprived	Complete	Ca^{2+}-deprived	Complete
Expt 1					
Original		10·2	88		
Post-nylon	46	4·2	106	19	55
Expt 2					
Original		2·5	86		
Post-nylon	52	1·7	26	35	16
Expt 3					
Original		6·8	159		
Post-nylon	56	8·3	147	68	52

Cytolysis was carried out with dilutions of effector cells and 10^4 P815. The number of cells required to produce 50% ^{51}Cr release from targets (i.e. 1 lytic unit) was determined for cells before and after nylon-wool purification, in either complete medium (RPMI 1640 plus 1% DFBS) or Ca^{2+}-deprived medium (RPMI 1640 plus 1% DBFS plus 1·5 mM Mg^{2+} plus 1 mM EGTA). Yields are calculated as percentage of the number of initial lymphoid cells. Activated macrophages as identified under phase contrast were excluded from counts. Cytolysis was allowed to proceed for 4·5 h at 37°.

Table 3. Specificity of lysis of nylon-wool-purified b→d peritoneal lymphocytes by anti-Thy-1.2 and rat complement

	Medium	C′	anti-Thy-1.2	C′ anti-Thy-1.2	C′+anti-Thy-1.2 abs. AKR brain	C′+anti-Thy-1.2 abs. C57Bl/6 brain
Percentage yield	80	77	81	1·0	1·5	86
Lytic units/10^6 lymphoid cells in complete medium	166	166	110	No cytolysis detected	No cytolysis detected	143
Lytic units/10^6 lymphoid cells in Ca^{2+} deprived medium	7·1	6·3	4·0	No cytolysis detected	No cytolysis detected	6·7

Details of anti Thy-1.2 and complement treatment are given in Methods. Brain absorption was made with 1:20 anti-Thy-1.2 incubated with an equal volume of packed brain tissue washed three times in LPBS. Absorption was for 30 min at 4° with occasional mixing. Lytic units were calculated as in Table 2. Complete medium: RPMI 1640 plus 1% DFBS. Ca^{2+}-deprived medium: RPMI 1640 plus 1% DFBS plus 1 mM EGTA% plus 1·5 mM Mg^{2+}. Cultures were incubated for 4·5 h at 37°.

Moreover (Fig. 6), the lytic titre of anti-Thy-1.2 in the presence of complement was found to be the same for (a) all nylon-wool-purified b→d peritoneal exudate cells, (b) cytolysis in the presence of Mg^{2+} and Ca^{2+}, (c) cytolysis in the presence of medium with Mg^{2+} but no free Ca^{2+}.

Cytolysis in the absence of extracellular Ca^{2+} by spleen cells sensitized *in vitro*

Several experiments showed that spleen cells, sensitized *in vitro* to allogeneic irradiated splenocytes, were able to lyse target cells specifically in medium contain-

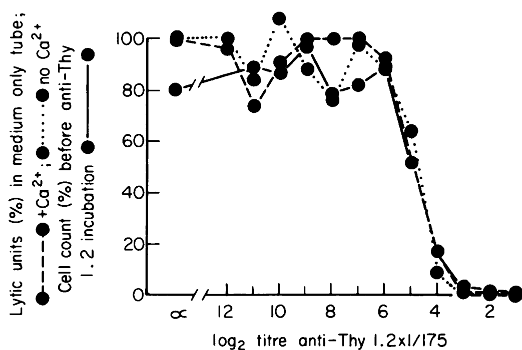

Figure 6. The susceptibility of nylon-wool-purified peritoneal lymphocytes to lysis by anti-Thy-1.2 and complement. Results are expressed as percentage of cell numbers or percentage of lytic units before treatment with anti-Thy-1.2 and complement. Details of anti-Thy-1.2 treatment and effector cell numbers used in cytolysis are given in the Methods. •——•, % initial cell count; • - - - •, % lytic units in RPMI 1640 plus 1% DFBS; • . . . •, % lytic units in RPMI 1640 plus 1% DFBS, 1·5 mM Mg^{2+} and 1 mM EGTA. Cell-mediated cytolysis was allowed to proceed for 4 h at 37°.

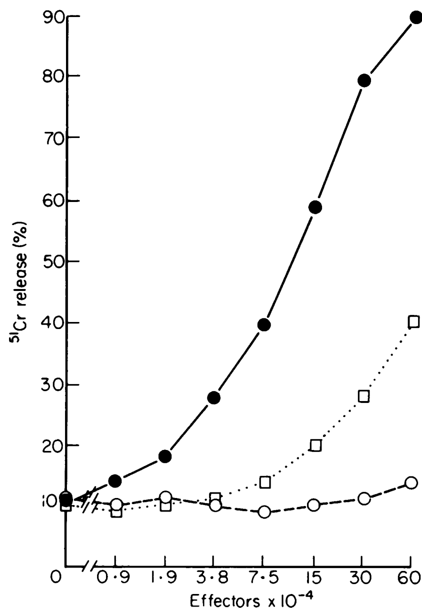

Figure 7. Cytolysis of P815 in complete or Ca^{2+}-deprived medium by spleen cells sensitized against allogeneic irradiated splenocytes for 5 days *in vitro*. Cultured in RPMI 1640 plus 1% DFBS: ○, b→d •, d→b. Cultures in RPMI 1640 plus 1% DFBS plus 1·5 mM Mg^{2+} and 1 mM EGTA: □, b→d. Cultures were for 6 h at 37° with 10^4 labelled-P815.

ing Mg^{2+} but no free Ca^{2+}. Figure 7 shows such an experiment where cytolysis was allowed to proceed in RPMI 1640 + 1% DFBS as well as in the same medium supplemented with 1·5 mM Mg^{2+} and 1 mM EGTA. The lytic capacity of a given number of peritoneal lymphocytes sensitized *in vivo* was consistently greater than that of spleen cells sensitized *in vitro*. The relative cytolysis, however, in complete and Ca^{2+}-depleted media was similar for cytolytic cells prepared in these two ways, i.e. cytolysis with Ca^{2+} and Mg^{2+} was five to thirty times greater in terms of lytic units per given number of cells than that with Mg^{2+} alone, for effectors sensitized either *in vivo* or *in vitro*.

DISCUSSION

This study indicates that populations of lymphoid cells, sensitized either *in vitro* or *in vivo* against allogeneic cells, can show specific cytolytic activity in the absence of extracellular Ca^{2+}. Such cytolysis, however, is between five and thirty times less effective in terms of lytic units than that seen when both Ca^{2+} and Mg^{2+} are present in the medium. Three main explanations for these findings will be discussed: (1) cytolysis seen in the absence of free extracellular Ca^{2+} might depend on leakage of intracellular Ca^{2+} from either targets or effectors; (2) cytolysis in Ca^{2+}-deprived medium might be due to a different population of cells from that cytolytic in the presence of both Ca^{2+} and Mg^{2+}, (3) T-cell mediated cytolysis could depend on one or several biochemical reactions which function optimally in the presence of extracellular Ca^{2+} but can proceed at a reduced rate in the presence of Mg^{2+}.

Taking the first of these explanations, it can be argued that Ca^{2+} is released from intracellular sources in sufficient amounts to saturate extracellular EGTA. This possibility, however, seems most unlikely as cytolysis was seen in medium with 0·5 mM Mg^{2+}, 0·5 mM Ca^{2+} and 11 mM EGTA. One can, on the other hand, postulate the existence of pockets of medium trapped between target and effector cells. If the volume of such pockets was small it is conceivable that Ca^{2+} from inside the cell could rise to levels in excess of 11 mM EGTA. The uniform level of cytolysis, however, observed after EGTA concentration has exceeded that of Ca^{2+} in the medium (0·5 mM) by up to 11 mM militates against this explanation.

If one rejects the first hypothesis, is there evidence in favour of the second explanation that the apparent Ca^{2+}-independent component of cytolysis and the

Ca^{2+}-dependent one are due to different subsets of effector cells? The data which are presented provide no evidence in favour of such a dichotomy, i.e. cytolysis both in the presence of and without extracellular Ca^{2+} was mediated by cells with (a) a similar range of adherence to nylon wool, (b) equal sensitivity to anti-Thy-1.2 antiserum and complement, (c) similar specificity and (d) similarity of dose-response curves as a function of either time or effector cell numbers. Therefore, although two distinct populations cannot be excluded, the third explanation has to be considered, that T-cell mediated cytolysis includes a heavily Ca^{2+}-dependent rate-limiting step at the lethal hit stage (Golstein & Smith, 1976) which can however proceed to some degree with Mg^{2+} alone, via the same on perhaps alternative divalent-cation-dependent pathways. This seems to us the most satisfactory explanation in the light of available evidence.

The observation of cytolysis occurring at concentrations of EGTA well in excess of those of Mg^{2+} is in itself of interest. Although the binding constant of this chelating agent is greater for Ca^{2+} than for Mg^{2+} by approximately three orders of magnitude, in free solution EGTA chelates Mg^{2+}. This finding, therefore, might suggest that Mg^{2+} is not required. This, however, does not seem to be the case, as near complete inhibition of cytolysis occurred when the concentration of EDTA in the medium reached the combined molar concentrations of Ca^{2+} and Mg^{2+} (Figs 2 and 3). A likely interpretation of the contrasted effect of EDTA and EGTA on the Ca^{2+}-independent component of cytolysis is that (a) Mg^{2+} is required and (b) the binding affinity for Mg^{2+} of the relevant cell system is higher than that of EGTA but lower than that of EDTA. It is interesting that in a strictly Mg^{2+}-dependent non-T cytolytic system, a similarly EGTA-resistant component may also exist (Golstein & Gomperts, 1978).

We have only seen marginal levels of cytolysis when the free divalent cations are chelated with EDTA. Martz (1975), however, in a careful study noted that marked specific T-cell mediated cytolysis could be induced in the presence of excess EDTA if the effector and target cells were subjected to prolonged cocentrifugation. Arguably such conditions of forced cell interaction are not often used experimentally. On the other hand the conditions used in the present study are frequently employed.

From a practical point of view, although cytolysis can occur to a significant extent in the presence of Mg^{2+} alone, Ca^{2+} remains a powerful marker for the lethal hit stage of T-cell mediated cytolysis. Some caution should be exercised by controlling for Ca^{2+}-independent cytolysis and keeping this to minimum levels if one wishes to study the lethal hit stage of cytolysis using divalent-cation manipulation. The effector to target cell ratios should not be too high. The length of the initial recognition stage in the presence of Mg^{2+} alone and the length of the Ca^{2+} pulse should be kept to a minimum. Also, to make sure that no further lethal hit events occur during the target cell disintegration stage, EDTA (Mauel, Rudolf, Chapuis & Brunner, 1970; Henney & Bubbers, 1973; Martz, 1975; MacDonald, 1975) rather than EGTA should be added, or the effector cells should be destroyed with antiserum and complement (Martz & Benacerraf, 1973) or thermal inactivation (Miller & Dunkley, 1974; Wagner & Röllinghoff, 1974; Golstein & Smith, 1977). Finally it is advisable to include controls in which effector cell function is abrogated before the addition of Ca^{2+}. Within these limitations the calcium pulse technique remains a valuable tool for analysing the lethal hit stage of T-cell mediated cytolysis in spite of the existence of the limited amount of Ca^{2+}-independent cytolysis described in this report.

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