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The mechanism of T-cell mediated cytotoxicity

VIII. ZEIOSIS CORRESPONDS TO IRREVERSIBLE PHASE (PROGRAMMING FOR LYSIS) IN STEPS LEADING TO LYSIS

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Summary. The combined use of an improved technique for inactivating cytotoxic T cells during the lytic reaction, with time lapse cinematography and isotope release assay, have shown that the initiation of the morphological phase of zeiosis corresponds to the time when the target cell is irreversibly programmed to lyse. It is suggested that rubidium release occurs during the phase of zeiosis. The rate of release of chromium is the result of two phases of variable length, The reversible phase (before programming for lysis) and the irreversible phase from the initiation of zeiosis to the final lytic event. The time required for programming for lysis to occur depends on the number of T cells reacting with the target cell. Thus at high ratios in tubes, where multiple interactions are possible, most target cells are programmed to lyse within 10 min. However, under conditions when T-cell:target-cell conjugates are kept in suspension to prevent multiple interactions, programming for lysis can take several hours. This provides an explanation for the apparent difference in timing of zeiosis and programming for lysis in previous publications.

It is also shown that further T-cell interactions with the target cell after programming for lysis (i.e. during the irreversible phase), markedly influence the rate of chromium release. This provides an explanation for the fact that chromium release takes at least 3 h tc reach plateau levels after inactivation of the T cells, whereas at high effector cell ratios, maximum levels of chromium release can occur within 1 h.

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INTRODUCTION

In the study of the mechanism of T-cell cytotoxicity two apparently different points of view have emerged. On the one hand, time lapse observations have shown a variable period of time after T-cell contact, varying from a few minutes up to several hours in which the target cell remains morphologically and functionally normal, before undergoing spectacular zeiosis (blebbing) leading to lysis (Sanderson, 1976 a, b). On the other hand T-cell inactivation experiments with chromium release assays indicate a period of a few minutes after contact in which the T cell delivers a 'lethal hit' (Wagner & Rollinghoff, 1974) and the target cell is then 'programmed to lyse' (Martz, 1975, 1977).

This paper describes experiments which reconcile these points of view and show that the morphological event of zeiosis corresponds to the irreversible phase, when the target is programmed to lyse.

These experiments have been made possible by an improvement of the T-cell-inactivation technique using antibody and complement (Martz & Benacerraf,

1973; Sanderson & Taylor, 1975). In this technique a monoclonal IgM anti-Thy 1.2 antibody is allowed to react with the T cells before mixing with the target cells. This does not result in any detectable change in their ability to lyse target cells. When complement is added the T cells are rapidly inactivated allowing accurate timing of the T-cell-independent events in the lytic cycle.

MATERIALS AND METHODS

Full details of the system have been published previously (Sanderson, 1976a, b). In brief, cytotoxic T cells were purified from peritoneal exudates of C57Bl. 10 mice immunized with P815 mastocytoma. The P815 cells were labelled with ^{51}chromium or double labelled with ^{51}chromium and ^{86}rubidium. Assays were carried out in 1·5 ml polypropylene tubes [W. Sarstedt (UK) Ltd, Leicester; No. 690] in a total volume of 100–200 μl, with 10^4 targets/tube. The tubes were centrifuged at 400 *g* for 2 min to induce contacts. Temperature and timings are given with each experiment. Suspension experiments were carried out, by resuspending the cells with 500 μl of 10% Ficoll 400 (Pharmacia, Fine Chemicals AB, Uppsala, Sweden) using a vortex mixer. Controls with each experiment showed that effectors and targets suspended in this medium (without centrifugation) did not interact as no chromium was detectable over a 4 h incubation at 37°.

Cytotoxic T cells were inactivated using a monoclonal IgM anti-Thy 1.2 (F7D5) described by Lake, Clarke, Khorshidi & Sunshine (1979) (obtainable from Olac Ltd, Bicester). Purified peritoneal exudate cells (2×10^6/ml) were mixed with the antibody at a dilution of 1:1000 and incubated at 22° for 30 min. The cells were then diluted to the required concentration, but were not washed. Preliminary experiments showed that this antibody did not alter the cytotoxic activity of the T cells. The T cells were then inactivated at different times by adding guinea-pig serum as a source of complement (volume 100 μl at a dilution of 1:5). The time required for inactivation of the T-cell activity could not be measured accurately, but was less than 2 min. Complement added at time zero (immediately after centrifugation at 22°) completely inhibited chromium release in a 4 h assay, while cells not treated with complement reached maximum levels of release.

Time lapse cinematography was carried out using a chamber formed between glass coverslips separated by a sheet of silicone rubber with a 5 mm diameter hole. Filming was at 12 frames/min, using a $\times 25$ phase contrast objective.

Percentage specific release was calculated from percentage isotope release: $[(t-m)/(\max-m)] \times 100$, where t = experimental value, m = medium control, max = maximum value when isotope release reached plateau levels with high ratios of effectors. Inhibition of chromium release was calculated as the percentage of control (c) values (T cells not inactivated); $[(t-m)/(c-m)] \times 100$. Experiments were analysed by analysis of variance and Duncan's multiple range test, on log transformed percentage chromium release values.

RESULTS AND DISCUSSION

To determine the relationship between programming for lysis and zeiosis, effectors and targets (ratio 5:1) were centrifuged and incubated for 5 min at 37°. This should allow programming for lysis to take place in approximately half the target cells (Martz, 1976). The cells were then transferred to the viewing chamber and complement added. Filming began within 2 min, by which time most of the lymphocytes were visibly dead. A small number of lymphocytes remained viable throughout the filming, these were assumed to be non-T cells. As can be seen from Table 1, every target cell which lysed was found to be in the phase of zeiosis at the beginning of the film. This indicates that the T cell is required up to the phase of zeiosis, and any cells not in zeiosis at the time the T cell are inactivated do not enter this phase at a later time, and do not lyse. Programming for lysis therefore corresponds to the initiation of zeiosis, and once initiated the T cell is no longer required for the lysis of the target. Table 1 shows that this occurs at a variable time after zeiosis. The time at which chromium release occurs is difficult to establish, but at high magnification, bubbles of cytoplasmic material can be seen bursting out of the cell. This sometimes occurs during zeiosis, but in some cases cells become quiescent but retain phase contrast until lysis and swelling. This represents a modification of my previous assertion that lysis and zeosis always coincide (Sanderson, 1976b). This new analysis makes it clear that only in some cells is this correct. In some cells lysis occurs soon after contact, but in others it occurs at some later time. It seems likely that chromium release can occur at any time after the initiation of zeiosis, thus the rate of release of chromium is the result of two variable periods; the time before zeiosis

Table 1. Time lapse cinematography records of morphological changes in individual cells after inactivating T cells immediately before filming

Cell*	Time (min) after beginning of film	
	Duration of zeiosis†	Time of final swelling
A	0–10	10
B	7–11‡	11
C	0–12	12
D	0–8	13
E	0–15	15
F	0–3	15
G	0–3	18
H	0–11	19
I	16–20‡	20
J	0–3	21
K	0–17	27
L	0–15	30
M	10–17‡	34
N	0–7	37
O	0–12	38
P	0–1	50
Q	0–39	—§

*Pooled data from individual cells in two films on the same cell preparation. Three other films gave similar data.

†Zero is the beginning of filming and represents 5 min at 37° after centrifugation and 2 min at 22° (for addition of complement transferring to viewing chamber and selecting field).

‡Cells entered field in zeiosis, at time shown.

§Not swollen at end of film (90 min).

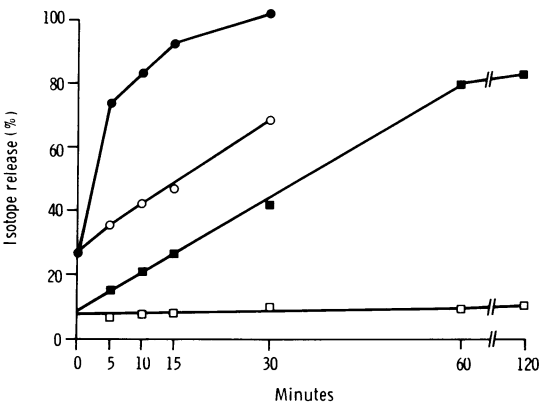

Figure 1. Release of ^{86}Rb and ^{51}Cr from double-labelled P815 target cells with T cells at an effector to target cell ratio of 20:1. ^{86}Rb medium control (○) and in presence of T cells (●). ^{51}Cr medium control (□), and in presence of T cells (■). The difference between medium control and T cells is significant at all time points. Chromium release at 60 and 120 min is not significantly different.

and the time between zeiosis and lysis. The fact that chromium release has no lag phase and is measurable within 5 min (Fig. 1) indicates that some target cells pass through the two phases very rapidly. The variable time between contact and swelling shown in Table 1 is similar to data obtained by direct observation of cell conjugates (Zaguary, Bernard, Thierness, Feldman & Berke, 1975; Grimm & Bonavida, 1979).

By recording the number of lysed target cells in conjugates at different times, it is possible to establish the rate at which lysis takes place and the time after contact to reach maximum values (Grimm & Bonavida, 1977). This showed that different populations of T cells showed different lytic efficiency against one type of target, and that different types of target cell were lysed with different efficiency by the same T-cell preparation. Zeiosis was not recorded in those experiments (as this would required uninterrupted observation), however, it would be interesting to know if this heterogeneity in timing lies in the interval between contact and zeiosis or between zeiosis and lysis. At least in one case, the difference in susceptibility during the cell cycle, it is the interval between contact and zeiosis which is important (Sanderson, 1976b; Sanderson & Thomas, 1976).

Martz (1976) found that programming for lysis corresponded closely to the release of rubidium from target cells. His inactivation technique involved a 10 min incubation in EDTA to separate T cells from target cells, before resuspending in dextran containing medium to prevent further interactions. This meant that precise timing was not possible, and so the question has been re-examined using the more rapid inactivation technique with antibody and complement.

Figure 1 shows release of ^{51}Cr and ^{86}Rb in the presence of a high ratio of purified T cells. Spontaneous release of chromium is low and, in the presence of T cells, release is approximately linear with no lag phase, reaching maximum values at 60 min. On the other hand the spontaneous release of Rb is rapid, and in the presence of T cells is non-linear, with most release in the first 5 min. After 15 min the difference between cytotoxic and spontaneous release is maximal, thus Rb release must be assayed at 15 min. By

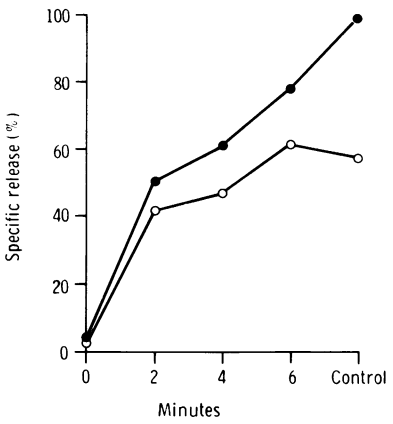

Figure 2. Effect of inactivating T cells at different times on ^{86}Rb release (o, determined at 15 min, medium 33·3%, maximum 107%) and ^{51}Cr release (•, determined at 4 h, medium 5·2%, maximum 91%). At each time point the number of cells programmed to lyse (Cr release) is higher than the percentage Rb release, suggesting that Rb release occurs after programming for lysis. The value of Rb release after inactivation at 6 min is not significantly different from control value (5% level).

comparison with other experiments (see below) programming for lysis must be complete well before 10 min in this experiment. However, only 68% (specific release) of rubidium has occurred at 10 min, suggesting that rubidium release occurs later than programming for lysis.

The time required for programming for lysis was estimated by inactivating the T cells at different times and assaying chromium release when plateau levels were reached at 4 h. It was consistently found that the proportion of rubidium released at 15 min, was lower than the proportion of cells programmed to lyse. For example, when the T cells were inactivated at 6 min, approximately 60% of the Rb was released at 15 min, whereas nearly 80% of the cells went on to lyse (Fig. 2). This suggests that Rb release does not coincide precisely with programming for lysis but that some of the events detected by rubidium release are occurring after the 15 min assay period. These two types of experiment suggest that rubidium release, like zeiosis, occurs after programming for lysis.

The problem remaining is why the interval between contact and zeiosis was observed to be as long as several hours, when Martz had described programming for lysis as complete with 10 min. That Martz is correct can be seen from Fig. 2 where 50% of the cells are programmed to lyse by 2 min. It seemed possible

Table 2. Effect on 4 h chromium release values from suspended conjugates, by inactivating T cells at different times

Time (a) (min)	Ratio (effector to target cell) 1:1	2:1	5:1	10:1
0	6 (b)	13	10	16
5	28	46	49	61
10	39	46	54	59
20	39	49	58	75
30	53	50	73	85
60	51	75	81	80
120	89(n.s.)	82	93(n.s.)	82
Control value (c)	16·8	25·6	50·8	64·3
Apparent half time (d)	45	25	5	<5

*Time when complement added to cells suspended in medium containing Ficoll.

†Percentage of control value, calculated; $[(t-m)/(c-m)] \times 100$, where t = test value, c = control value, m = medium control (10·8%).

‡Control value (T cells not inactivated) after subtracting medium control value.

§Time required to allow 50% programming for lysis. Estimated from graphical plots.

n.s., Not significantly different from control value. (All other values are significantly different from control values at 5% level or greater.)

that the time required for programming for lysis was very dependent on the number of effectors interacting with a target cell. This possibility was discussed when the time relationship between contact and zeiosis was first reported (Sanderson, 1976b) and at that time it was recognized that with high ratios of effectors, lysis of the entire population could occur in much less than 3 h.

To investigate this point a modification of the dispersion technique (Martz, 1975) was used. Effectors and targets at different ratios were centrifuged and held at 22° for 5 min, to allow contacts to form with minimum programming for lysis. They were then resuspended in medium containing Ficoll, incubated at 37° and complement added at different times to inactivate the T cells. Table 2 shows that T-cell inactivation, even after 2 h, caused inhibition of chromium release, indicating that some programming for lysis was occurring after this time. Estimation of the half time for each ratio showed that the lower the ratio of effectors as targets, the longer the time required to achieve 50% programming for lysis. These results

show that the large apparent difference in timing of zeiosis and programming for lysis lies in the different cell interactions possible in the different techniques. Thus when programming for lysis is defined in a system where cells interact in a pellet at the bottom of a tube, multiple cell interactions cause rapid programming for lysis in all the target cells. However, when T-cell inactivation experiments are carried out under conditions like those used for time lapse studies (low ratios and low cell density) it is found that programming for lysis is not complete 2 h after contact, in agreement with the time lapse data for zeiosis (Sanderson 1976b).

One further problem arising from these types of experiments is the fact that when cells are allowed to interact for a few minutes before inactivating the T cells, chromium release does not reach plateau levels until 3–4 h (Wagner & Rollinghoff, 1974; Martz, 1975). This would suggest that even with high ratios of effectors, maximum release of chromium would require at least 3 hours. However, it is well known (Cerrotini & Brunner, 1974) that maximum levels of chromium release can be reached within 1 h (Fig. 1). One explanation for this could be that T-cell interactions continuing after programming for lysis would increase the rate of chromium release. This point was investigated using high ratios of effectors to target cells and inactivating the T cells at different times (Fig. 3). When the T cells were inactivated at 10 min, chromium release reached maximum values by 4 h, indicating that all targets were programmed to lyse by 10 min. The control rate chromium release was much faster, reaching maximum values within 2 h. This indicates that intact T cells increased the rate of chromium release from target cells after programming for lysis was complete. It can be seen from the 1 h values in Fig. 3 that the rate of chromium release in the presence of T cells was at least double the rate compared to tubes in which the T cells were inactivated at 10 min.

The events taking place during this irreversible phase are unknown. However, progress to lysis is temperature dependent (Berke, Sullivan & Amos, 1972; Sanderson & Taylor, 1975) suggesting that some metabolic or catabolic events are required. It is possible that continued T-cell activity after programming for lysis increases the rate of these events, thus shortening the time before lysis occurs.

These results show that the interaction between T cells and target cells is complex. Although killing is a one-hit phenomenon, in the sense that one T cell can kill one target cell, the rate of cell lysis is the result of

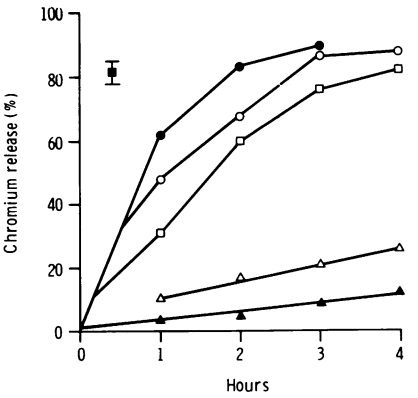

Figure 3. Release of ^{51}Cr obtained with high ratio of effectors to target cell (20:1) when T cells inactivated at different times. Control rate of release (●), spontaneous release (▲). Inactivation with complement at time zero (△), 10 min (□), and 30 min (○). Values for 20 min were intermediate between 10 and 20 min values, and have been omitted. Isotope release obtained for time zero inactivation, represents programming for lysis during the 5 min preincubation at 22°. Maximum values obtained by lysing the P 815 cells with anti-H2d and complement are shown (■) with least significant differences at 5% level.

two variable periods of time. Firstly a reversible phase after initial contact, before the initiation of zeiosis. This varies from a few minutes to several hours when only one T cell interacts with each target cell, but all target cells pass through this phase in a few minutes under conditions when multiple T-cell interactions with each target cell are possible. Secondly, from about the time of the initiation of zeiosis, the target cells will progress irreversibly to complete lysis. The timing of this phase varies between different individual target cells and is decreased by the continued presence of intact T cells.

Although the rate of chromium release is proportional to the number of effectors, and approximately linear with time as would be expected of a simple first-order reaction, it would appear to be an oversimplification to equate the process with first-order enzyme reactions, as the factors determining the rate of cell lysis do not fit any simple kinetic model. This raises considerable doubt that analysis of chromium release data using enzyme kinetic equations, will provide any useful understanding of the mechanism of killing or the properties of the T-cell receptor.

REFERENCES

CEROTTINI J.C. & BRUNNER K.T. (1974) Cell mediated cyto-

toxicity, allograft rejection and tumour immunity. *Adv. Immunol.* **18,** 67.

BERKE G., SULLIVAN K.A. & AMOS B. (1972) Rejection of ascites allografts. II. A pathway for cell mediated tumour destruction in vitro by peritoneal exudate lymphoid cells. *J. exp. Med.* **136,** 1594.

GRIMM E.A. & BONAVIDA B. (1977) Studies on the induction and expression of T cell mediated immunity. VI. Heterogeneity of lytic efficiency exhibited by isolated cytotoxic T lymphocytes prepared from highly enriched populations of effector–target conjugates. *J. Immunol.* **119,** 1041.

GRIMM E.A. & BONAVIDA B. (1979) Mechanism of cell mediated cytotoxicity at the single cell level. I. Estimation of cytotoxic T lymphocyte frequency and relative lytic efficiency. *J. Immunol.* **123,** 2861.

LAKE P., CLARK E.A., KHORSHIDI M. & SUNSHINE G.H. (1979) Production and characterisation of cytotoxic Thy 1 antibody secreting hybrid cell lines. Detection of T cell subsets. *Eur. J. Immunol.* **9,** 875.

MARTZ E. (1975) Early steps in specific tumor cell lysis by sensitised mouse T lymphocytes. *J. Immunol.* **115,** 261.

MARTZ E. (1976) Early steps in specific tumor cell lysis, by sensitised mouse T lymphocytes. II. Electrolyte permeability increase in the target cell membrane concomitant with programming for lysis. *J. Immunol.* **117,** 1023.

MARTZ E. (1977) Mechanism of specific tumour cell cell lysis by alloimmune T lymphocytes: Resolution and characterization of discrete steps in the cellular interaction. *Cont. Top. Immunobiol.* **7,** 301.

MARTZ E. & BENACERRAF B. (1973) An effector cell independent step in target cell lysis by sensitised mouse lymphocytes. *J. Immunol.* **111,** 1538.

SANDERSON C.J. (1976a) The mechanism of T cell mediated cytotoxicity. I. The release of different cell components. *Proc. Roy. Soc. Lond.* (B) **192,** 221.

SANDERSON C.J. (1976b) The mechanism of T cell mediated cytotoxicity. II. Morphological studies of cell death by time-lapse microcinematography. *Proc. Roy. Soc. Lond.* (B), **192,** 241.

SANDERSON C.J. & TAYLOR G. (1975) The kinetics of ^{51}Cr release from target cells in cell mediated cytotoxicity and the relationship to the kinetics of killing. *Cell Tissue Kinet.* **8,** 23.

SANDERSON C.J. & THOMAS J.A. (1976) The mechanism of T cell mediated cytotoxicity III. Changes in target cell susceptibility during the cell cycle. *Proc. Roy. Soc. Lond. (B),* **194,** 417.

WAGNER H. & ROLLINGHOFF M. (1974) T-cell mediated cytotoxicity: discrimination between antigen recognition, lethal hit and cytolysis phase. *Eur. J. Immunol.* **4,** 745.

ZAGURY D., BERNARD J., THIERNESS N., FELDMAN M. & BERKE G. (1975) Isolation and characterisation of individual functionally reactive cytotoxic T lymphocytes: conjugation, killing and recycling at the single cell level. *Eur. J. Immunol.* **5,** 818.