

REJECTION OF ASCITES TUMOR ALLOGRAFTS

II. A PATHWAY FOR CELL-MEDIATED TUMOR DESTRUCTION IN VITRO BY PERITONEAL EXUDATE LYMPHOID CELLS*

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In previous communications (1-4) we reported that peritoneal lymphoid cells from BALB/c mice which had rejected ascites leukemia EL4 were uniquely efficient in destroying the tumor in vitro. The effector cell population was identified as nonadherent small- to medium-sized lymphocytes. The inclusion of normal serum in the medium was obligatory for cytolytic reactivity. We now report on the pathway of the in vitro destruction of EL4 leukemic cells by peritoneal effector lymphocytes.

Materials and Methods

Tumor, Mice, and Immunization.—Leukemia EL4 was maintained in an ascites form in male C57BL/Spr mice. 8-12-wk old male BALB/c mice were injected intraperitoneally with 2.5×10^7 EL4 cells. Nonadherent peritoneal exudate lymphoid cells (PEC)¹ were obtained 11 days after immunization as described before (2).

Cell-Mediated Cytotoxicity against Tumor Cells.—In vitro cytotoxicity against the tumor was determined, as already described (2), by incubating ⁵¹Cr-Na₂CrO₄-labeled EL4 cells with nonadherent PEC in phosphate-buffered saline supplemented with 10% heat-inactivated fetal calf serum and comparing the amount of radioactivity released with that obtained by freeze-thawing, three times, a known number of labeled EL4 cells. Values given in text figures indicate the mean and range of triplicate cultures.

Physical Manipulations.—Under standard conditions, 1-ml cultures were rocked for 6 cycle/min on a rocking platform (Bellco Glass Inc., Vineland, N. J.) at 37°C. Rocking was stopped by transferring cultures to a stationary position. Conversely, rocking was initiated for stationary cultures by placing them on the rocking platform. Three incubation temperatures were used: 36.5 ± 1 (37), 24 ± 2 (25), and 7 ± 2 (7)°C. Unavoidable delay time for manipulations was 3 ± 2 min.

RESULTS

Fate of PEC during Target Cell Destruction.—An understanding of the pathway followed during the cytolytic reaction requires knowledge of the fate of

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¹ Abbreviations used in this paper: PEC, immune nonadherent peritoneal exudate lymphoid cells; T cell, thymus-derived cell.

the effector cell as well as that of the target. Previous experiments (4-9) indicated that effector cells could kill repeatedly, which suggested that they were neither inactivated nor killed during the cytolytic reaction. Direct evidence obtained by reacting ^{51}Cr -labeled PEC with EL4 cells strengthens the conclusion that effector cells were not killed during the reaction. Label (Table I) was released from the target, but not from the effector cells during cytolysis. If all data were taken into account, the ^{51}Cr released when both effector cell and target were labeled could be accounted for by destruction of the target cells alone.

TABLE I
 ^{51}Cr Release from PEC during Target Cell Destruction

Cells*	Radioactivity released			
	Total†	Experimental		
		45 min	90 min	
		<i>cpm ± variation</i>		
k + T	24,012 +428 -647	4968 +182 -269	11,624 +384 -465	
K + t	1289 +54 -50	202 +10 -6	243 +26 -34	
K + T	24,993 +536 -763	5037 +200 -273	11,396 +450 -468	
T	23,676 +666 -565	880 +36 -50	1478 +160 -122	
K	1358 +49 -49	241 +31 -19	293 +7 -6	

* 2×10^5 PEC, ^{51}Cr -labeled (K) or nonlabeled (k), were reacted with 2×10^5 EL4, ^{51}Cr -labeled (T), or nonlabeled (t) under standard conditions.

† Values obtained by freezing and thawing the appropriate labeled cells three times.

Contribution of Collision and Temperature to Cell-Mediated Cytotoxicity.—The impact of temperature and rocking on the rate of cell-mediated tumor destruction in vitro is illustrated in Fig. 1. Efficient killing of ^{51}Cr -labeled EL4 cells was obtained only in those cultures maintained at 37°C and rocked. The near-background levels observed at 25 and 7°C with rocking indicated that cytolysis was a temperature-dependent process. We have previously suggested (5, 2, 4) that the rate of cell-mediated cytotoxicity depends upon the number of lymphocyte-target cell interactions. Rocking most likely facilitated PEC-EL4 collisions (10) and, therefore, the number of successful interactions.² Cultures incubated at 37°C without rocking showed only low levels of release within the time of the experiments.

² The finding that the rate of EL4 destruction by PEC is enhanced by increasing the concentration of either type of cell, without rocking (G. Berke, unpublished results), is in line with this interpretation; see reference 5 for discussion.

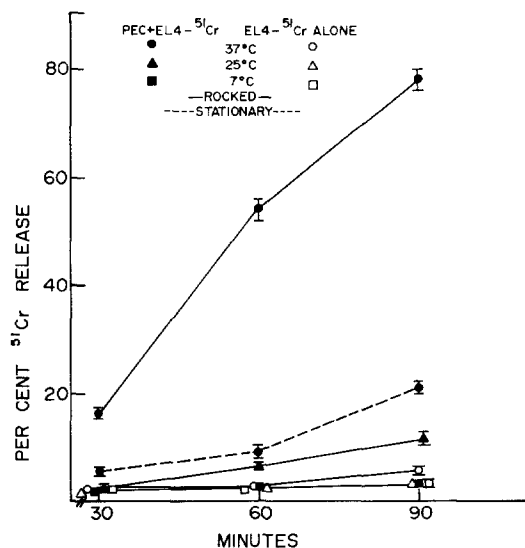


Fig. 1. Effect of rocking and temperature on the cytolytic reaction. 1-ml cultures containing 1.5×10^5 PEC and 5×10^4 ^{51}Cr -labeled EL4 cells were incubated with rocking (—) at 37 (●), 25 (▲), or 7°C (■), and without rocking (-----) at 37°C. Control cultures contained ^{51}Cr -labeled EL4 cells alone (open symbols). The per cent ^{51}Cr released was determined by comparing experimental with freeze-thaw values (2). Vertical bars indicate ranges in all figures.

The dependence of the lytic reaction upon lymphocyte-tumor cell interaction was further analyzed. Four groups of cultures containing mixtures of immune PEC and ^{51}Cr -labeled EL4 cells were incubated for 30 min at 37°C; two of the groups were rocked. After 30 min, rocking was stopped for one group. At the same time, rocking was initiated for another group that had not been rocked before. The results of the "stop-rock" experiment (Fig. 2) show that release of label continued at essentially full rate for 30 min after rocking had ceased. More information about the time requirement for initiation of killing was given by the "start-rock" experiment. Here, where effector and target cells were allowed to equilibrate at 37°C for 30 min (Fig. 2), killing was initiated within 10 min after onset of rocking. These findings indicated a lag period of about 10 min for the initiation of cytolysis and a continuation of the process for at least 30 min after new interactions had essentially ceased.

Since the previous experiments (Fig. 1) showed that the reaction as a whole was dependent upon temperature, and led us to imply the formation of an intermediate phase dependent upon interactions (Fig. 2), experiments were performed to determine the specific contribution of temperature to cell-mediated cytolysis. Cultures that had been rocked at 37°C and then transferred to a stationary platform continued to release label; however, when the temperature of the cultures was lowered to 7°C the rate of release fell to base-line values (Fig. 3). Since cooling abrogated the release of label, one might expect label

to be released upon subsequent warming. The results of such a warm-up experiment, Fig. 4, showed that label was indeed released upon warming from 7 to 37°C without further rocking. These results supported the existence of an intermediate stage and indicated that the sequence of events leading to the

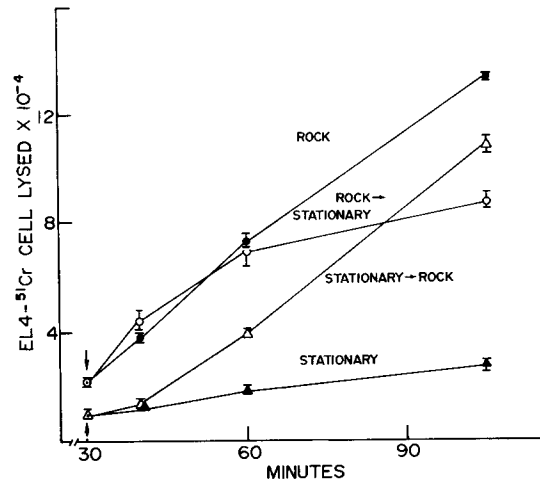


FIG. 2. Rock, stop-rock analysis. 1-ml cultures containing an equal number of PEC and ⁵¹Cr-labeled EL4 cells (2.5×10^5) were incubated at 37°C with or without rocking. After 30 min, rocked cultures were divided (↓) into two groups; one continued to be rocked (●) while the other was now held stationary (○). Stationary cultures were similarly divided (↑); one-half remained stationary (▲) and the second half was then rocked (△).

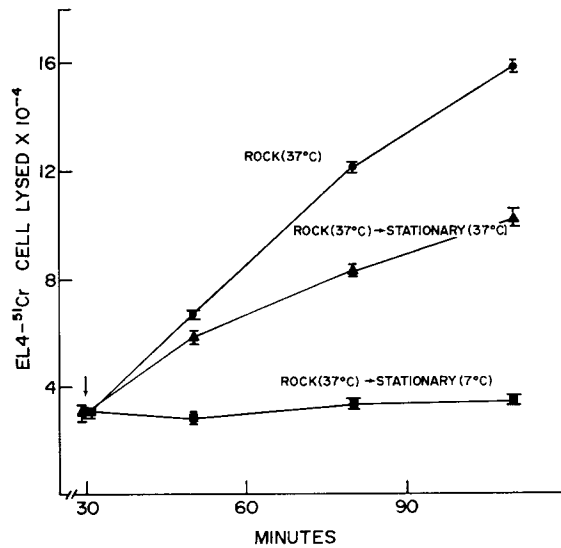


FIG. 3. Temperature dependence of cytolysis. 1-ml cultures containing an equal number of PEC and ⁵¹Cr-labeled EL4 cells (2.5×10^5) were rocked at 37°C for 30 min. The cultures were then divided (↓) into three groups; one continued to be rocked at 37°C (●), while the second and third were now held stationary either at 37°C (▲) or 7°C (■).

decomposition of the target (as reflected by ^{51}Cr release) was controlled by temperature.

The contribution of PEC-tumor cell interactions, as well as temperature, to the initiation of the reaction, was investigated in a series of 11 separate experiments; one of the most comprehensive is summarized in Table II and expressed graphically in Fig. 5. Interestingly, the reaction could be initiated at low temperature, although it could not be completed. The initiation was primarily dependent upon rocking, whereas completion of the reaction was absolutely

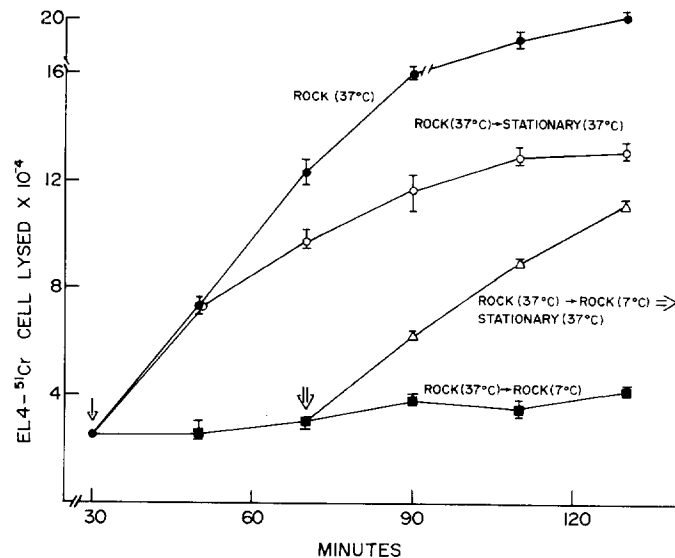


FIG. 4. Temperature regulation of cytolysis. All cultures (1 ml) containing an equal number of PEC and ^{51}Cr -labeled EL4 cells (2.5×10^5) were rocked at 37°C . After 30 min, cultures were divided (\downarrow): one group continued to be rocked (\bullet) while the second was held stationary (\circ); both groups were at 37°C . The third group was rocked at 7°C (\blacksquare) for 40 min and then further divided (double arrow) into two groups: one continued to be rocked at 7°C while the second was held stationary at 37°C (\triangle).

dependent on temperature. All the other experiments were in excellent agreement with the data presented.

DISCUSSION

We have shown that the effects of temperature and lymphocyte-target collision in cell-mediated cytolysis are complementary but not interchangeable. Lymphocyte-target interaction can occur at low or high temperature. The irreversible series of events which is thereby initiated is completed only at high temperature. These events result in target cell but not lymphocyte destruction. The terminal phase of ^{51}Cr release from target cells can be totally interrupted by cooling, and partially suppressed by reagents such as ethyl-

TABLE II
Effects of Rocking and Temperature on Cytolysis*

Conditions of culture		Cytolysis	
First 40 min	Last 50 min	60 min	90 min
		($\times 10^{-4}$ EL4 cells)	
Rock at 37°C (●—●)	Rock at 37°C	9.5	12.5
	Stationary at 37°C (○---○)	8.7	11.1
Stationary at 37°C (△—△)	Stationary at 37°C	2.4	3.2
	Rock at 37°C (▲—▲)	4.6	9.5
Rock at 25°C (□---□)	Rock at 25°C	1.9	3.4
	Rock at 37°C (■—■)	5.4	10.1
	Stationary at 37°C (□---□)	4.8	7.1
Stationary at 25°C (ovals with dots)	Stationary at 25°C	0.9	1.3
	Rock at 37°C (filled ovals)	4.5	9.1
	Stationary at 37°C (open ovals)	1.8	2.8

* See Fig. 5 for a graphical presentation of the data.

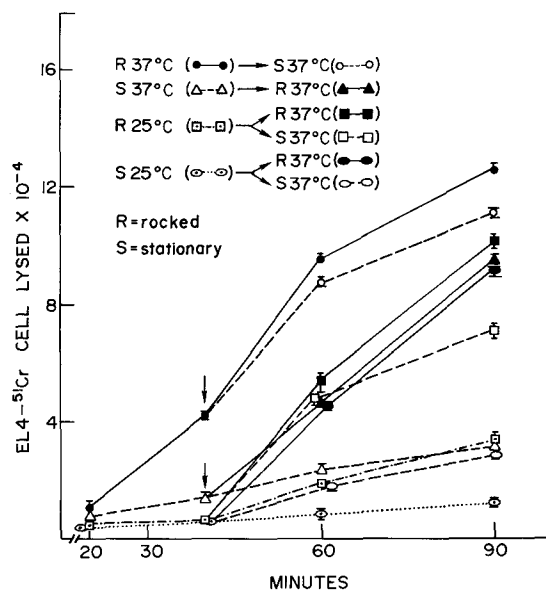


FIG. 5. Rock, stop-rock, temperature analysis. 1-ml cultures containing an equal number of PEC and ^{51}Cr -labeled EL4 cells (1.67×10^5) were incubated at 25 or 37°C with or without rocking. After 40 min, cultures were divided (\downarrow) into the following groups: continuously rocked at 37°C (●—●), rocked at 37°C and then stationary at 37°C (○---○), stationary at 37°C (△---△), stationary at 37°C and then rocked at 37°C (▲—▲), continuously rocked at 25°C (□—□), rocked at 25°C and then rocked (■—■) or stationary (□---□) at 37°C, stationary at 25°C (ovals with dots), and, finally, stationary at 25°C and then rocked (filled ovals) or stationary at 37°C (open ovals).

enediaminetetraacetate (EDTA) and anti-target cell alloantibody which interfere with lymphocyte-target cell interaction (11). All available evidence strongly indicates that the lymphocyte does not blast the target cell immediately upon contact and suggests a working hypothesis for lymphocyte-mediated target cell destruction; for convenience, the components of the pathway for cell-mediated cytolysis will be discussed as individual subjects.

Cycling.—It has been previously suggested that effector lymphocytes are not destroyed after they are confronted with target cells (7, 5-9; for a contrary opinion see reference 12), but can lyse target cells repeatedly (5, 4). The data presented here provide direct evidence that under the conditions of these experiments effector lymphocytes are not killed during cytolysis. In addition, our "stationary-start rock" approach (Fig. 2) allows an estimate of 10 min for initiation of cytolysis. The same lag period was observed in another system (5, 8).³

Conjugation.—From several lines of evidence obtained in different systems, it is known that effector lymphocytes can attach specifically to target cells (13-18) and remain conjugated for a finite time possibly through their uropod (8). The inclusion of normal serum in the medium, although mandatory for cytolysis (2), is not required for the initial attachment.⁴ Attachment has been found to be reversible since conjugated lymphocytes can be recovered by trypsinization (15, 16) or by EDTA or low pH.⁴ In addition, spontaneous detachment of lymphocytes has been observed through cinematography (8).

The molecular species that play a role in attachment are partially known. The specificity of attachment (13-16) strongly implicates the cell surface transplantation antigens of the target cells. Ca^{++} and Mg^{++} ions are necessary for the cytolytic reaction (19) and EDTA can prevent interaction between lymphocytes and target cells or dissociate conjugates already formed.⁴

Wilson inferred that lymphocyte-target cell interaction could occur at low temperature (20). Although functional lymphocyte-to-target cell adsorption is hardly demonstrable around 4°C (15).⁴ Our more sensitive "rock, warm-up" approach shows that lymphocyte-target cell interaction can definitely occur at 25°C, and even to some extent at 7°C (11), temperatures where no lysis is observed. These observations clearly separate conjugation from the subsequent process of target cell destruction.

Cytolysis.—The rate and extent of cell-mediated cytolysis is dependent upon the concentration of effector lymphocytes (20, 21, 2, 5, 6), the concentration of target cells (5, 4), and upon the total number of successful lymphocyte-target cell collisions (5, 4). We have shown, in the first report of this series, that

³ It must be noted that the lag period is influenced by several factors, e.g. susceptibility of the target, method used for estimation, temperature, or medium, which may form the basis for discrepancies among various reports (20, 5, 9, 10).

⁴ Stulting, R. D., and G. Berke. Nature of lymphocyte-tumor interaction: a general method for cellular immunoabsorbents. Manuscript submitted for publication.

the rate of target cell destruction is also strongly dependent upon the presence of normal heated serum from various mammalian species.

The possibility that antibody is involved in this system, playing a role in conjugation, in cytolysis, or in both, must be seriously considered (see reference 22 for review). Unfortunately, we have not as yet been able to obtain evidence for the participation of antibody and a considerable body of evidence suggests that free antibody is not involved. When BALB/c anti-EL4 serum collected at various times after single or multiple injections was included in the reaction mixture, suppression rather than potentiation occurred. The degree of suppression was proportional to the amount of antibody added⁵ (21, 19; see reference 23 for further discussion). Furthermore, the addition of soluble transplantation antigens, capable of neutralizing fluid phase antibody, failed to interfere with cell-mediated cytotoxicity.⁶ These experiments speak against the participation of free antibody in the reaction. Cell-mediated cytotoxicity comparable to that of BALB/c anti-EL4 PEC has also been observed with PEC from the *H-2^b* mutant H(Z1).⁷ The H(Z1) mice are serologically indistinguishable from C57BL/6 and do not make any detectable antibodies against C57BL/6 tissues (24), or against EL4 leukemic cells.⁷ Finally, the demonstration that the small- to medium-sized effector PEC in our system are θ -positive suggests that this population is comprised of thymus-derived (T) lymphocytes. The addition of anti- θ -C3H serum and complement is accompanied by a reduction in effector cell function proportional to the number of lysed PEC.⁸ Although postulated (25; see in addition reference 26 for discussion), there has been no precise demonstration that lymphocyte-bound antibodies are responsible for allograft rejection or for T cell-mediated target cell destruction *in vitro*. Our previous findings (2) speak against the involvement of "cytophilic antibody"; however, work is currently in progress to further evaluate the possible existence of a functional antibody on immune PEC.

We have presented quantitative evidence that the tumor cells pass through a presumptive intermediate stage before their death (complete release of ⁵¹Cr). "Stop-rock" and "stop-rock, temperature" experiments at 37 and 25°C in which ⁵¹Cr release continued despite cessation of rocking, or was apparent upon warm-up, supported the involvement of an intermediate stage of the target cells during the reaction. The release of ⁵¹Cr from target cells at the intermediate stage was found to be a highly temperature-dependent process. Moreover, the

⁵ Todd, R. F., and G. Berke. Antibody interference in cell-mediated cytotoxicity. Manuscript in preparation.

⁶ Berke, G. Unpublished observations.

⁷ Berke, G., and D. B. Amos. 1972. Cytotoxic lymphocyte in the absence of detectable antibodies. Manuscript submitted for publication.

⁸ Sullivan, K. A., G. Berke, and D. B. Amos. Serological characterization of lymphocytes involved in ascites allograft rejection. Manuscript submitted for publication. Anti- θ -C3H serum was produced in (A/ θ AKR \times AKR/*H-2^b*)F₁ mice against A leukemia and was kindly provided by Dr. E. A. Boyse, Sloan-Kettering Institute, New York.

appearance of radioactivity in the medium appears to be a rate-limiting factor. EDTA and anti-target cell antibody experiments (11) suggest that a pre-destructive reaction occurs while the target cell is associated with the effector lymphocyte and that the temperature-dependent process may occur in the absence of continuous lymphocyte-target cell association. The microscopic events that follow initial lymphocyte-target cell contact have been explored by microcinematography (8). In these experiments, too, the target cell at the moment of lysis often had no associated lymphocyte, although interactions always preceded lysis.

SUMMARY

A pathway for cell-mediated tumor destruction in vitro by immune peritoneal exudate lymphoid cells has been proposed. The union of lymphocytes and tumor cells precedes the formation of an intermediate phase leading to lysis. The initial interaction is only partially temperature dependent. The cytolytic process per se is highly temperature dependent, being negligible at 25°C but proceeding rapidly at 37°C. ⁵¹Cr release from tumor cells is demonstrable within 10 min at 37°C and can be reversibly arrested by cooling. Once initiated, lysis is largely independent of additional interactions and continues at almost full rate for 30 min. The effector cells are not lysed and appear to be free to enter into further effector cycles.

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