Proc. Nat. Acad. Sci. USA
Vol. 71, No. 1, pp. 177–181, January 1974

Interruption of the Sequential Release of Small and Large Molecules from Tumor Cells by Low Temperature During Cytolysis Mediated by Immune T-Cells or Complement

(lymphocyte-mediated lysis/target cells/killer cells)

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ABSTRACT **Specific lysis of tumor cells by thymus-derived lymphocytes from alloimmunized mice (T-effector specific lysis) was studied with target cells labeled with isotopes attached to both small (^{14}C-labeled nicotinamide) and large (^{51}Cr-labeled) molecules. The results confirm and extend previous reports that target cells release small molecules considerably earlier than large molecules during T-effector specific lysis. After interruption of T-effector specific lysis by specific antibody and complement directed against the *killer* cells, or by ethylenediaminetetraacetic acid, release of both isotopes continued, eventually reaching identical levels of specific release, the value of which represents the fraction of the target cell population which had been committed to die at the time these treatments were applied. On the other hand, release of both isotopes during T-effector specific lysis stops immediately when the cultures are cooled to 0°. Thus, while ethylenediaminetetraacetic acid or specific complement-mediated lysis of the killer cells merely prevents the initiation of any new damage to target cells, cooling to 0° also stops the lytic process in already-damaged target cells.**

The colloid osmotic phase of target cell lysis induced by specific antibody and complement was similarly stopped at 0° in tumor cells, but not in erythrocytes. Thus, in tumor target cells, both T-effector specific lysis and complement cause a sequential release of progressively larger molecules which can be immediately stopped at any point by cooling to 0°.

It is now generally recognized that cells of the thymus-derived lymphocyte series (T-effectors) play a crucial role in immune destruction of tissues. In addition to being responsible for the specificity of target cell recognition *in vivo*, T-effectors are capable of direct cytotoxicity (1, 2). This report is the fourth from our laboratory (3–5) concerning the mechanism of T-effector specific lysis (TSL), a subject which has recently been reviewed by Cerottini and Brunner (1) and Henney (6).

Our own studies have been directed towards elucidating the way in which target cell damage is initiated and progresses to completion as evidenced by the release of ^{51}Cr label. In a previous study (4), we resolved the killing process into two steps: (*i*) a rapid, initial interaction which requires the intact killer cell and commits the target cell to eventual lysis, but which does not cause the immediate release of much, if any, ^{51}Cr, and (*ii*) a slower, subsequent process, not requiring the intact killer cell and terminating in the breakdown of membrane integrity to the extent of release of ^{51}Cr label. This resolution was achieved by lysing the killer cells (with specific antiserum and complement) at various times during their action on target cells. We subsequently reported that step (*ii*), the part of the process which occurs independently of the intact killer cell (hence, the "killer-cell-independent phase") is reversibly prevented by low temperature (5).

These findings raised the possibility that step (*i*) involved the production of electrolyte leaks in the cell membrane too small for the loss of ^{51}Cr label [known to be bound to large molecules (7)]. In order to explore this possibility further, an indicator of increased membrane permeability for small molecules was needed. Indeed, Henney has demonstrated that ^{86}Rb and adenosine 5′-triphosphate are released earlier than ^{51}Cr during TSL (7). However, P815 cells loaded with ^{86}Rb or ^{42}K spontaneously released half of either approximately every 40 min, which severely limits kinetic studies.

In order to overcome these problems, we sought an isotopically labeled compound which would be readily taken up by the cell, but not rapidly lost, and which would not become attached to or incorporated in polymeric material. We reasoned [after Rotman and Papermaster (8)] that such a molecule should be uncharged when taken up, but become charged once in the cell to reduce the rate of spontaneous loss. [^{14}C]-nicotinamide, which would seem to meet these theoretical requirements, has been successfully employed in the present study to resolve these events.

MATERIALS AND METHODS

Media. PBES: phosphate (0.005 M)-buffered Earle's salts without bicarbonate, pH 7.2. FCS: fetal-calf serum (Grand Island Biological Company, Grand Island, N.Y.) heat inactivated at 56° for 90 min. MEMS: Eagle's minimal essential medium, Earle's salts containing penicillin (100 units/ml) and streptomycin (100 μg/ml) and 10% FCS. L15S: L15 medium (GIBCO) plus penicillin and streptomycin and 10% FCS.

Effector Cells. Preparation and storage in liquid nitrogen of spleen cells from sensitized and control C57BL/6J male mice have been described (4).

Target Cells and Labeling. P815 mastocytoma cells were maintained by ascites passage in syngeneic DBA/2J or semi-syngeneic (C3H/J × DBA/2J)F1 female mice. Labeling with ^{51}Cr alone was done essentially as described previously (3).

Abbreviations: [^{14}C]Nic, all molecules labeled with ^{14}C which are released from cells previously incubated with [^{14}C]nicotinamide; PVP, polyvinylpyrrolidinone K-30; TSL, T-effector specific lysis; Ab + C, specific antibody and complement.

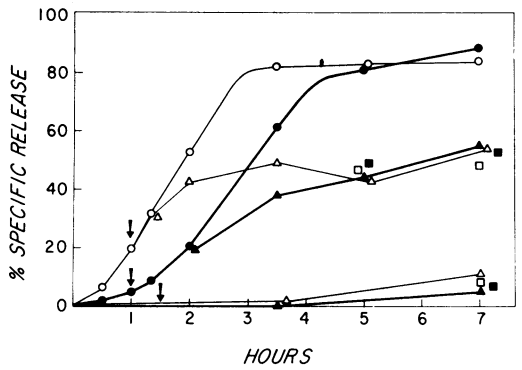

FIG. 1. ^{14}C-Specific release versus ^{51}Cr-specific release during the killer-cell independent phase of specific T-lymphocyte-mediated lysis of tumor cells: P815 cells were labeled with [^{14}C]nicotinamide and $Na_2{}^{51}CrO_4$. *Open symbols* and *thin lines* represent ^{14}C-specific release. *Closed symbols* and *heavy lines* represented ^{51}Cr-specific release. C57BL/6J mouse-spleen cells were incubated with 2.5×10^4 P815 cells at a ratio of 32:1 in 0.1 ml of MEMS (*circles*). Where Ab + C is specified below, spleen cells were lysed by addition of an alloantiserum previously described (DBA/2J mouse antiserum to C57BL/6J cells, final dilution 1:100, ref. 4) together with guinea-pig complement (final dilution 1:6). Ab + C was added to some cultures after 1 hr of incubation (*arrows*) and isotope release was followed subsequently (*triangles*). Some control cultures, initially including unlabeled target cells, also received Ab + C at 1 hr; at 1.5 hr (*arrow*), labeled target cells were added, and isotope release was followed subsequently (*triangles*) to verify the efficacy of the Ab + C treatment. To some cultures, EDTA (final concentration 0.01 M) was added prior to incubation (*squares* near right abscissa) or at 1 hr of incubation (*squares* near 50% specific release). Each point represents the average of duplicate cultures; two comparable experiments yielded similar results. Release of isotope at 7 hr from cultures containing unsensitized spleen was 15% for ^{51}Cr and 51% for ^{14}C.

For double labeling, 5×10^6 twice-rinsed cells were resuspended in 10 ml of MEMS and placed in a 10-cm diameter, bacteriological, polystyrene petri dish. After addition of 50 μCi of [carbonyl-^{14}C]nicotinamide (60 Ci/mol, Amersham/Searle Corp., Arlington Heights, Ill.), the culture was incubated at 37° overnight. The following morning, 0.1 ml of $Na_2{}^{51}CrO_4$ was added, and 2 hr later, the cells were rinsed three times in MEMS and used for experimental cultures. This procedure gave about 1000 beta cpm for each isotope in 2×10^4 cells.

Assay for Cell-Mediated Killing (Double-Labeled Target Cells). Cultures were set up and processed essentially as described previously (4). Both isotopes were counted simultaneously in Aquasol (New England Nuclear Corp., Boston, Mass.) with a two-channel liquid scintillation spectrometer (Beckman Instruments, Fullerton, Calif.). Counts were corrected for spillover and specific release was computed with a Wang 700A-701-703 electronic calculator system (Wang Laboratories, Inc., Waltham, Mass.).

A control culture containing unsensitized spleen cells was made for each experimental culture containing sensitized spleen cells, and both were treated identically. Release was calculated for experimental (e) and control (c) cultures as a percentage of the total releasable isotope in each culture as described previously (4). Specific release was computed as $(e - c)/(100 - c)$.

Complement-Mediated Lysis of P815 Cells. Cultures were made in L15S in 10 × 75-mm glass test tubes. Approximately 5×10^4 ^{51}Cr-labeled P815 cells in 0.05 ml were mixed with 0.05 ml of 1:25 hyperimmune alloantiserum (C57BL/6J mouse antiserum against P815 cells) and incubated 5 min at 37°, after which 0.25 ml of 25% polyvinylpyrrolidinone K-30 (PVP) of average molecular weight 40,000 (Matheson, Coleman, and Bell, Norwood, Ohio) was added and mixed, followed by 0.15 ml of neat reconstituted guinea-pig complement (BBL Division of Bioquest, Cockeysville, Md. 21031). After 20 min incubation at 37°, some cultures were transferred to 0°. PBES (2 ml) was then added to all cultures in order to dilute the PVP concentration and initiate colloid osmotic lysis. For sampling, cultures were centrifuged at 800 rcf for 5 min at 4°. Supernatants were decanted and counted in a gamma spectrometer (Packard Instruments, Downer's Grove, Ill.). Release was computed as a percentage of the isotope released by freezing and thawing replicate samples of the same batch of labeled target cells.

RESULTS

Comparison of ^{14}C and ^{51}Cr Release. P815 ascitic mastocytoma cells from DBA/2J mice were incubated overnight with [^{14}C]nicotinamide in MEMS, as detailed in *Materials and Methods.* ^{51}Cr was added during the last 2 hr of incubation before experimental use as target cells. Upon further incubation in MEMS after washing, 50% of the ^{14}C taken up by these cells was spontaneously released in about 7 hr. This rate of release permitted kinetic studies of TSL. Only about 15% of ^{51}Cr was spontaneously released during the same time. Virtually all of both isotopes was released immediately upon freezing and thawing the cells.

The specific release of ^{14}C and ^{51}Cr during TSL is shown in Fig. 1. ^{14}C is specifically released much sooner than ^{51}Cr. At 2 hr of incubation with specific killer cells, when 20% of the ^{51}Cr had been specifically released, 52% of the ^{14}C had been specifically released. The ^{14}C is presumably released primarily in the form of nicotinamide adenine dinucleotide (molecular weight 664), although verification of this is only recently being undertaken in our laboratory. Hence, this result corroborates the findings of Henney (7) with ^{86}Rb and adenosine 5′-triphosphate.

Effect of Killing the Killer Cells. Also depicted in Fig. 1 is the effect of inactivation of the effector cells by specific antibody (DBA/2J mouse antiserum against C57BL/6J mouse cells, see ref. 4) and complement (Ab + C). As reported previously (4), ^{51}Cr-specific release, which had reached only 4% at the time of addition of Ab + C, continued to occur, finally reaching 62% of the maximum specific release reached in untreated cultures. Controls (ref. 4, see also Fig. 1) demonstrate that all or most of this ^{51}Cr release occurs after the effector cells are rendered unable to initiate new cytolytic events, and hence, is independent of intact effector cells.

^{14}C-specific release also continues to increase after inactivation of the effector cells (from 23% to 64% of the maximum value reached in untreated cultures). Specific release of both isotopes plateaus at the same level in cultures treated with Ab + C, and hence the fraction of the target cells committed to die before treatment with Ab + C is the same whether measured by specific release of ^{14}C or of ^{51}Cr.

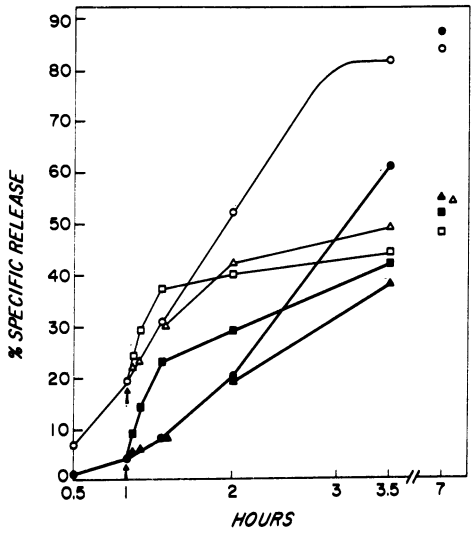

FIG. 2. Rapid, specific release of ^{14}C and ^{51}Cr in EDTA-treated cultures (additional data from the experiment described in Fig. 1): *Open symbols* and *thin lines* represent ^{14}C-specific release; *filled symbols* and *heavy lines* represent ^{51}Cr-specific release. *Circles* represent untreated cell-cultures, *triangles* represent addition of Ab + C at 1 hr (*arrow*) of incubation, and *squares* represent addition of EDTA at 1 hr (*arrow*).

Effect of EDTA. In a previous study, we showed that addition of EDTA at any given time during culture had approximately the same effect as addition of Ab + C (4). Fig. 1 shows that ^{14}C-specific release, as well as ^{51}Cr-specific release, plateaus at the same level whether EDTA or Ab + C is added. Immediately after addition, however, EDTA causes a more rapid specific isotope release than Ab + C, as shown in Fig. 2. After Ab + C addition, specific release of both isotopes coincides with that in untreated cultures until the plateau is approached in the cultures treated with Ab + C. In contrast, immediately after EDTA addition, specific release of both isotopes considerably exceeds that in the cultures treated with Ab + C or in the untreated cultures (Fig. 2).

Effect of Low Temperature. We have previously shown that cooling cultures from 37° to 0° immediately stops specific release of ^{51}Cr (5). As shown in Fig. 3, this is also true for specific release of ^{14}C. Remarkably, the ^{14}C-specific release can be stopped at a high level while the ^{51}Cr-specific release remains at a much lower level for many hours in the cold (45% versus 15% in Fig. 3). The cessation of ^{51}Cr release at 0° was previously shown to be reversible at 37° (5), and, hence, the cessation of ^{14}C-specific release can be assumed also to be reversible.

^{14}C Release During the Killer-Cell-Independent Phase. It was pointed out above that ^{14}C-specific release increased significantly after the addition of Ab + C (i.e., during the killer-cell-independent phase). We have also just seen that ^{14}C-specific release does not increase after cooling to 0°. Comparison of the two treatments (Fig. 4) dramatizes the fact that ^{14}C-specific release continues to occur after inactivation of the killer cells.

^{14}C-specific release similarly increases after addition of EDTA (Fig. 2). The possibility that this increment of ^{14}C-specific release might be an artifact caused by EDTA was eliminated by adding EDTA to cultures which had been cooled to 0°. This did not cause a significant increase in either ^{14}C-specific release or ^{51}Cr-specific release. Warming such cultures back to 37° caused a rapid increase in isotope-specific release to the same level reached in cultures to which EDTA had been added without cooling. Thus, there is a temperature-sensitive, divalent-cation-independent, killer-cell-independent phase for both ^{14}C-specific release and ^{51}Cr-specific

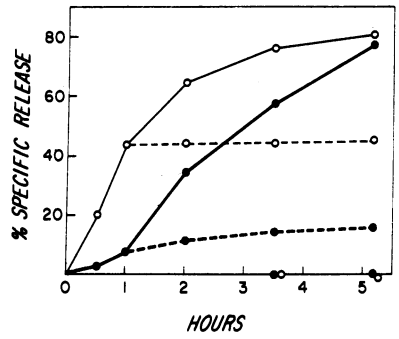

FIG. 3. Cessation of specific release of ^{14}C and ^{51}Cr at 0°: P815 cells were labeled with [^{14}C]nicotinamide and $Na_2{}^{51}CrO_4$. *Open symbols* and *thin lines* represent ^{14}C-specific release. *Filled symbols* and *heavy lines* represent ^{51}Cr-specific release. C57BL/6J mouse-spleen cells were incubated with 8.3×10^4 P815 cells per culture at a ratio of 18:1 in 0.2 ml of MEMS. After 1 hr of incubation, some cultures were transferred from 37° to 0° (*broken lines*). Some control cultures were incubated at 0° from the beginning (points on the abscissa at 3.5 and 5.2 hr). Each point represents the average from duplicate cultures; four comparable experiments yielded similar results. Release of isotope at 5.2 hr from cultures containing unsensitized spleen was 8% for ^{51}Cr and 35% for ^{14}C.

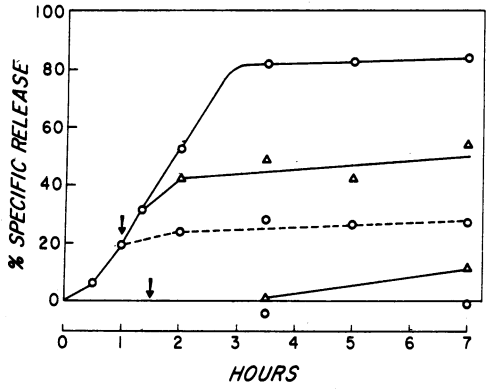

FIG. 4. Temperature sensitivity of ^{14}C-specific release during the killer-cell-independent phase (additional data from the experiment described in Fig. 1): Specific release of ^{14}C is shown at 37° (*circles, solid line*). Effector cells were lysed in some cultures by addition of Ab + C (see legend to Fig. 1) at 1 hr (*triangles*). To some of these cultures, which had been initiated with unlabeled target cells, labeled target cells were added 30 min later (*arrow* at 1.5 hr) to verify the efficacy of the Ab + C treatment (*triangles* near the abscissa). Other cultures were transferred from 37° to 0° at 1 hr (*broken line*). A few control cultures were incubated at 0° from the beginning (*circles on abscissa*). The additional specific release in the cultures treated with Ab + C, above the specific release in the cultures transferred to 0°, was also seen in two other similar experiments.

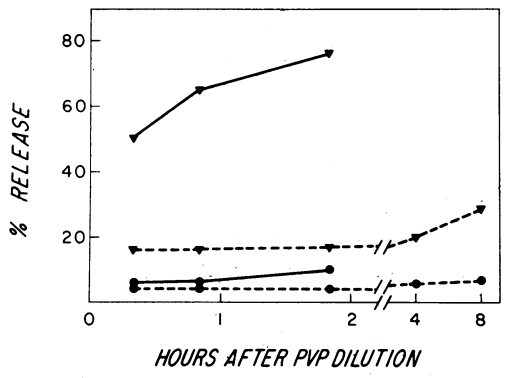

FIG. 5. Cessation of ^{51}Cr release from complement-damaged tumor cells cooled to 0°: Cultures of ^{51}Cr-labeled P815 cells containing alloantiserum against P815 cells, complement, and 12.5% PVP (for osmotic protection) were incubated for 20 min at 37°. (Other experiments demonstrated that under the conditions used, virtually all cells were damaged by Ab + C within 5 min.) Some cultures were transferred to 0° after the 20 min incubation. Then, after 10 min for temperature equilibration, sufficient PBES was added to all cultures to dilute the PVP to 2.5% in order to remove osmotic protection. ^{51}Cr release was monitored after dilution (*inverted triangles*). Antiserum was omitted from control cultures (*circles*). Release of ^{51}Cr at 37° is represented by *unbroken lines* and at 0° by *broken lines*. Data shown for 4 and 8 hr after dilution are from a separate but comparable experiment. (Data for 37° are not shown after 2 hr because PVP induced ^{51}Cr release in the control cultures.) The results of five comparable experiments sampled prior to 2 hr after dilution and one experiment sampled at later times were similar to the results shown.

release, although that for ^{14}C is of a shorter duration and a smaller extent than that for ^{51}Cr.

Cessation of Complement-Induced Colloid Osmotic Lysis of P815 Cells at 0°. The cessation of T-lymphocyte-induced lysis of P815 cells at 0° seemed difficult to understand if the lysis were colloid osmotic lysis due to increased membrane permeability. Therefore, we turned to the complement system where the mechanism of lysis of both nucleated cells and red cells is believed to be better understood (9, 10). Complement lesions produce increased membrane permeability to molecules of less than about molecular weight 20,000 (11), and the consequent equilibration of ions produces a subsequent colloid osmotic swelling and eventually lysis, i.e., release of the largest soluble cytoplasmic constituents (9).

The colloid osmotic phase of complement-induced lysis can be prevented, however, by a high concentration of macromolecules in the external medium (9). Thus, if complement lesions are produced in the presence of osmotically protective macromolecules, the cells can be cooled to 0° and the effect of temperature on colloid osmotic lysis investigated. Using this technique, Frank (12) and Mayer (personal communication) have shown that the colloid osmotic phase of complement-induced lysis of erythrocytes proceeds very rapidly, even in the cold, a fact subsequently verified in our laboratory (unpublished results). This turned out not to be true for P815 cells, however, as shown in Fig. 5. P815 cells in medium containing 12.5% PVP can be protected from antibody and complement-induced colloid osmotic lysis for at least 30 min at 37°, as judged by ^{51}Cr release. When the PVP is diluted 5-fold with culture medium at 37°, rapid ^{51}Cr release ensues, reaching 50% of plateau value in less than 30 min (Fig. 5). If, however, the cells are cooled to 0° before PVP dilution, little ^{51}Cr release occurs even during 8 hr (Fig. 5).

DISCUSSION

Using target cells labeled with both [^{14}C]nicotinamide and $Na_2{}^{51}CrO_4$, we have confirmed and extended Henney's finding (7) that, during thymus-derived, lymphocyte-mediated cytolysis of P815 mastocytoma target cells, small molecules are released earlier than larger molecules. Specific release of ^{14}C (presumably primarily as nicotinamide adenine dinucleotide, molecular weight 664) was much greater, at any given time, than was specific release of ^{51}Cr [previously shown by Henney to be bound to macromolecules (7)]. The nature of the ^{14}C-labeled material released in these experiments has not yet been investigated. For convenience, we shall designate this material, which may include several derivatives of nicotinamide, as [^{14}C]Nic.

We have previously resolved specific T-lymphocyte-mediated lysis into two phases. The first phase is rapid and requires intact effector cells, divalent cations, and, despite little, if any, ^{51}Cr release, commits the target cell to eventual lysis. The second phase is slower, requiring up to several hours, proceeds in the absence of intact effector cells, is largely or entirely independent of divalent cations, and ends with release of ^{51}Cr. For convenience, we refer to these phases as the killer-cell-dependent phase and the killer-cell-independent phase, respectively.

The process of T-lymphocyte-mediated cytolysis can be interrupted either by antibody and complement mediated specific lysis of the *effector* cells (Ab + C) or by the addition of EDTA. After interruption by either method, specific release of ^{51}Cr or of [^{14}C]Nic eventually reaches identical plateau levels, the values of which signify the percentage of target cells which had been committed to die at the time the killing process was interrupted. We previously demonstrated that this plateau reaches approximately the same level whether Ab + C or EDTA is added (4). In the present study, it was shown as well that the plateau level of [^{14}C]Nic coincides with the plateau level of ^{51}Cr.

Our present results also show that a substantial amount of [^{14}C]Nic was specifically released during the killer-cell-independent phase. This could be due to either (*i*) slow leakage from preexisting lesions already large enough to pass [^{14}C]-Nic, (*ii*) leakage from preexisting lesions which first become large enough to pass [^{14}C]Nic during the killer-cell-independent phase, or (*iii*) production of entirely new lesions. We have shown that EDTA actually speeds up the specific release of [^{14}C]Nic during the killer-cell-independent phase. Since EDTA can also prevent the initiation of target-cell damage by killer cells, it seems most probable that EDTA causes this speed-up of isotope release by enlarging preexisting lesions. Thus, (*iii*) above seems improbable, and the killer-cell-independent phase may, therefore, consist entirely of the transition of lesions initially too small to pass [^{14}C]Nic to larger leaks first capable of releasing [^{14}C]Nic and later ^{51}Cr, as in (*ii*) above. Alternatively, lesions of adequate size to release [^{14}C]Nic may already exist at the beginning of the killer-cell-independent phase, and the observation that [^{14}C]Nic is released more rapidly than ^{51}Cr could then be explained by a more rapid diffusion of the smaller [^{14}C]Nic than of the larger ^{51}Cr-labeled material through lesions of fixed size, as in (*i*) above.

We have also shown that specific release of molecules of both smaller ([^{14}C]Nic) and larger (^{51}Cr) sizes is brought to a virtual standstill immediately upon cooling P815 target cells to 0°. If (*i*) above is the case, this would suggest that the leaks of fixed size are resealed in the cold. If (*ii*) above is the case, this would imply that the transition from small to large leaks is prevented in the cold. Since ^{51}Cr release continues on rewarming to 37°, the events occurring in the cold are reversible.

We have found that low temperature can arrest the specific release of ^{51}Cr at a low level (15%) even though specific release of [^{14}C]Nic had already reached a much higher level (45%). Thus, P815 cells which must already have suffered equilibration of small ions (especially sodium and potassium) and the consequent colloid osmotic pressure do not proceed to lyse at 0°.

In order to gain a better understanding of this phenomenon, we studied the effect of low temperature on complement-induced colloid osmotic lysis. It had earlier been shown by Frank (12) and Mayer (personal communication) that complement-induced colloid osmotic lysis of erythrocytes occurs rapidly in the cold. To our surprise, however, we found this not to be true for the P815 mastocytoma cell. Hence, in both complement and T-effector-cell-mediated damage, the lysis following equilibration of small ions can be prevented at 0°.

The explanation for the failure of colloid osmotic lysis of P815 mastocytoma cells to proceed in the cold in both systems depends on whether or not the membrane leaks reseal. If they reseal, then water influx through the remaining intact membrane structure may be too slow to lyse the cell within a few hours. If, alternatively, the leaks do not reseal in the cold, then some property of the membrane of the nucleated cell, in contrast to the erythrocyte, must enable it to withstand the colloid osmotic pressure (stiffening of the membrane) or resultant swelling (expansion of the membrane surface without it becoming leaky).

It is noteworthy that no present evidence is contrary to the possibility that the initial membrane leakiness produced by T-effector cells is transient, and reseals even at 37°. Indeed, the fact that lysis does not occur for several hours after small ion equilibration takes place could be explained if the initial leaks reseal, permitting a temporary return to an intact membrane structure, which would slow the rate of water influx. Quite possibly, the later collapse of the membrane may then be initiated simply as a result of a failure of cell metabolism to keep the membrane in repair, due to the earlier massive loss of small metabolites. Eventually, the cell would succumb to colloid osmotic lysis, particularly if the ion pumps are also rendered inoperative by the loss of small molecules.

In conclusion, EDTA or specific complement-mediated lysis of the killer cells interrupts TSL by preventing the initiation of any new damage to target cells, without preventing the completion of the lytic process in those target cells already damaged. Cooling to 0°, on the other hand, stops the lytic process in already-damaged target cells as well as preventing any new damage.

Lastly, without making any inference that the initial lesions in TSL and complement-mediated lysis need be similar, it is nevertheless interesting that the data demonstrate some operational similarities between these two lytic phenomena: (*a*) in both lytic phenomena, the loss of small molecules preceeds the loss of larger molecules, and (*b*) in both lytic phenomena, loss of small and large molecules from nucleated cells stops at 0°.

Note Added in Proof. Experiments similar to that shown in Fig. 5 have now been conducted using ^{86}Rb as an indicator of initial damage by specific antibody and complement in the presence of 13% albumin for osmotic protection. After P815 cells specifically released all of their ^{86}Rb under these conditions, dilution of the albumin caused rapid specific release of ^{51}Cr in cultures incubated at 37°, but little or no specific release for periods of up to eight hours in cultures cooled to 0°.

We are grateful to Ms. Ann Strand for excellent technical assistance. Thanks are due Dr. Mark Poznansky for stimulating discussions, Dr. Eugene Kennedy for pointing out that nicotinamide meets our theoretical criteria for a marker of early membrane damage, and Dr. Manfred Mayer for helpful discussion and unpublished data. This work was supported by a grant from the New York Cancer Research Institue, Inc., and a Grant (CA 14723-01) from the National Institutes of Health.

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