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Target–effector cell interaction in the natural killer cell system

V. ENERGY REQUIREMENTS, MEMBRANE INTEGRITY, AND THE POSSIBLE INVOLVEMENT OF LYSOSOMAL ENZYMES

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Summary. Various inhibitors were used to study further the mechanism of natural killing and to compare it to lympholysis by cytotoxic T lymphocytes (CTL). The respiratory inhibitors DNP and NaN_3 or low temperatures (0°) blocked the cell contact phase of target–effector interaction in the CTL system but not the NK system. The lytic stage was also inhibited by the glycolytic inhibitors, iodoacetate and NaF, in the NK system as previously shown in the CTL system. Dimethylsulphoxide, a dipolar solvent, and cytochalasin B, a microtubule disruptor, inhibited NK target binding. Pre-treatment of NK cells with glutaraldehyde, a protein cross-linking agent, completely prevented lysis, but not the formation of target–effector conjugates. The lytic phase of NK lysis was inhibited by chloroquine which also inhibited lysosomal enzyme function. Lysosome defective, beige mutant mice were also totally deficient in NK lytic function and this defect could not be restored with cGMP. T-cell and macrophage mediated cytolysis was previously shown to be relatively normal in beige mice. These results suggest that (i) the mechanism of NK cytolysis is a complex, multistep process, and (ii) this process is fundamentally different from that occurring in CTL. A 'stimulus-secretion' model of NK cytolysis is presented in which it is postulated that lysosomal enzymes may be the lytic molecules.

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; CTL, cytotoxic T lymphocytes; K cell, lymphocytes mediating ADCC against nucleated tumour cell targets; NK, natural killer lymphocytes; TBC, target binding effector cell.

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INTRODUCTION

The mechanism whereby various lymphocyte subpopulations recognize and destroy target cells in a unidirectional interaction remains a perplexing problem in cellular immunology which has important implications in the destruction of tumours, virus-infected cells and allografts. Most experiments have been performed in the cytotoxic T lymphocyte (CTL) and K cell systems (Cerottini & Brunner, 1977). Although the recognition structure on K cells is known to be acquired antibody and CTL possess intrinsic recognition receptors, analogous to immunoglobulin V_H regions (Binz & Wigzell, 1977), the signal which triggers lethal events within these effector cells subsequent to target cell contact is not known. In addition, none of

the available evidence defines the nature of the actual lytic event. In spite of these gaps, a picture is emerging regarding the genes, metabolic pathways and regulatory molecules involved in the complex series of steps covered under the term 'cytolysis'.

Inhibitor sequencing experiments (Martz, 1977) provide one promising approach to resolving the unknown steps in cell-mediated lysis. In addition, if some inhibitors are found to have differential effects on various effector cell types this would provide a valuable tool for distinguishing the lytic contributions of poorly defined killer cells, such as NK, in a heterogeneous cell mixture.

In the present report, we investigate the mechanism of cytolysis by mouse NK cells, the least studied of all the lymphocytic effectors. The data suggest that contrary to general belief, the mechanism of cytolysis in the NK system is distinct from that in other effector cell types although many similarities exist. A multistep model of NK cytolysis is proposed.

MATERIALS AND METHODS

Mice

Six- to eight-week-old mice were used in all experiments unless specified otherwise and all groups were strictly age and sex matched. A/Sn, C57Bl/6, C57Bl/6J-bg^J/bg/^{J} and CBA mice were maintained in this laboratory by continuous single-line brother–sister mating.

Tumour cell lines

All tumour cell targets were maintained by continuous *in vitro* culture, whereas tumours for alloimmunization were maintained by ascites passage. YAC is a T-cell lymphoma induced by Moloney leukaemia virus in A/Sn mice and P815 is a mastocytoma induced by methylcholanthrene in DBA/2 mice. K562 is a human AML line. Daudi and Raji are Burkitt's lymphoma B cell lines.

Nylon-wool columns

Monodispersed cells from various lymphoid organs were treated briefly (4 s) with H_2O to remove erythrocytes by hypotonic shock and the remaining cells were passed over nylon-wool columns (Julius, Simpson & Herzenberg, 1973) with cell recoveries between 10 and 20% of input.

Cytolytic T cells (CTL)

Spleen cells or peritoneal exudate cells (PEC) were recovered from alloimmunized mice. Phagocytes were removed from the PEC cells by a 45 min incubation with carbonyl iron (0·2 mg/ml) at 37° followed by magnetic removal.

Target binding cell assay

Lymphoid cells, depleted of erythrocytes by hypotonic shock were passed through nylon-wool columns and were labelled with fluorescein isothiocyanate (FITC, BDH, Chemicals Ltd, Poole, England) as described previously, substituting balanced salt solution (BSS) for saline (Roder, Kiessling, Biberfeld & Andersson, 1978). Two million target cells were then mixed with 2×10^5 FITC-labelled lymphoid cells in 0·2 ml culture medium and centrifuged at 200 *g* for 5 min at room temperature (20°) in round-bottomed plastic tubes or microtitre plates. Tubes were then aspirated five to ten times with a Pasteur pipette and stored on ice to avoid lysis of the target cells. The percentage of fluorescing cells (effectors) binding to non-fluorescing cells (targets) was then determined after counting 300–400 effector cells under a UV microscope. Variation between replicate samples was always less than 10%.

Cytolytic assay

Two $\times 10^4$ target cells, labelled with [^{51}Cr]-sodium chromate as previously described (Kiessling, Petranyi, Karre, Jondal, Tracey & Wigzell, 1976), were incubated for 3 h at 37° with varying numbers of effector cells (normal unimmunized lymphocytes) in 0·2 ml microwells (Linbro) in triplicate or quadruplicate samples. The microplates were then centrifuged and 75 μl of supernatant was measured in a gamma counter. Spontaneous release was determined by culturing ^{51}Cr-labelled targets alone and total labelling was determined by counting an aliquot of targets after resuspension in the microwells. The following formula was used to compute % lysis:

$$\% \text{ lysis} = \frac{\text{test c.p.m.} - \text{spontaneous c.p.m.}}{\text{total c.p.m.} - \text{spontaneous c.p.m.}} \times 100$$

Spontaneous release by target cells alone did not exceed 10% of the total label unless stated otherwise.

Lysosomal enzyme assay

Spleen cells were lysed and the extracts were incubated with *p*-nitrophenyl-N-acetyl-β-glucosamine as substrate. The supernatants were measured spectrophotometrically at 400 nm and related to enzymatic activity on the basis of formation of *p*-nitrophenol (Sellinger, Beaufay, Jacques, Doyen & Duve, 1960).

Reagents

Stock solutions consisted of 1·0 M EDTA (ethylenediaminotetraacetic acid, Kebo, Stockholm) in H_2O, 0·1 M DNP (dinitrophenol, Sigma) in 0·1 N NaOH, 1·0 M NaN_3 (Merck, Darstadt) in H_2O, 1·0 M NaF (Baker Chemical Co., Phillipsburg) in H_2O, 1·0 M iodoacetic acid (Sigma) in H_2O, 10% DMSO (dimethylsulphoxide, Sigma) in BSS, 10 mg/ml cytochalasin B (Helminshosporium dematioideum, Sigma) in 100% DMSO, 10^{-1}M chloroquine diphosphate (Sigma) in BSS, 10^{-2} M cGMP (N^2, $O^{2'}$-dibutyryl guanosine 3′:5′; cyclic monophosphoric acid, sodium salt, Sigma) in BSS and glutaraldehyde (Merck, Darmstadt). All reagents were diluted in BSS and the pH was ajusted to 7·0 immediately prior to use.

Statistical analysis

A two-tailed Student's *t* test was used to determine the significance of differences between experimental groups.

RESULTS

Energy metabolism

In previous studies we showed that respiratory inhibitors such as DNP and NaN_3 markedly inhibited NK mediated cytolysis (Roder *et al.*, 1978). As shown in Fig. 1, iodoacetate, an inhibitor of the glycolytic enzyme 3-phosphoglyceraldehyde dehydrogenase also inhibited cytolysis to a large extent and NaF had a small, but significant effect ($P<0·01$) at the highest concentration. Neither agent inhibited the target binding step and the drug concentrations shown were not toxic for target cells (Fig. 1) or spleen cells as revealed by trypan blue exclusion (data not shown). In addition, targets and effectors were treated separately for 2 h with inhibitors, washed and then admixed in a short 20 min ^{51}Cr release assay using cytochalasin B and EDTA which stop further interaction but allow the release of ^{51}Cr from target cells damaged within the first 20 min (Roder *et al.*, 1978). Only pre-treatment of effectors resulted in significant inhibition (data not shown). These results suggest that in the NK system, as previously shown in the CTL and ADCC systems (Cerottini & Brunner, 1977; MacDonald & Koch, 1977), the energy necessary for cytolysis may be derived from both glycolysis and oxidative phosphorylation. However, a clear difference in two of these systems was apparent in the energy requirement for target cell contact. The respiratory inhibitors, DNP and

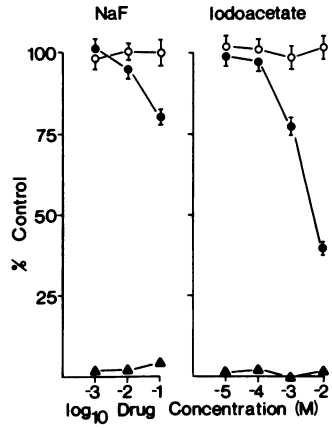

Figure 1. The effect of glycolytic inhibitors on NK function. Nylon-wool column passed spleen cells from CBA mice were pre-incubated 30 min at 20° with the agents shown and ^{51}Cr-labelled YAC target cells were then admixed at a 50 : 1 effector : target cell ratio in a 3 h lytic assay (●). As a toxicity control, ^{51}Cr-labelled YAC cells were also incubated with inhibitors in the absence of effectors (▲). In addition, fluorescein-labelled effectors were mixed with a ten-fold excess of targets and the frequency of TBC was enumerated (○). Values represent the mean ± SEM of triplicate determinations and are expressed as % control. Control values were 45% lysis and 18% TBC. This experiment was repeated four times with similar results.

NaN_3, caused a marked decrease in the frequency of alloimmune T cells binding to the appropriate, NK insensitive target, P815, whereas NK cells binding to YAC were not affected (Fig. 2). As a positive control, the Ca^{2+}/Mg^{2+} chelating agent, EDTA, was shown to inhibit TBC in both the NK and CTL systems.

NK cells in the mouse were also much less temperature dependent than *in vivo* educated CTL (Fig. 3). NK cells bound to targets at 0° and exhibited 50% maximum lysis at 20°, whereas CTL did not bind to targets at 0° and exhibited only 15% maximum lysis at 20° as previously shown (Berke & Gabison, 1975). It should be noted that although NK cells bind to targets at 0°, the rate of attachment is less than at 37° and adhesions may be less stable as shown in depletion type experiments (Roder *et al.*, 1978).

Similar results were also obtained in the human system (unpublished observations).

Membrane integrity

Since it is known that NK-target cell recognition involves broad areas of intimate membrane contact

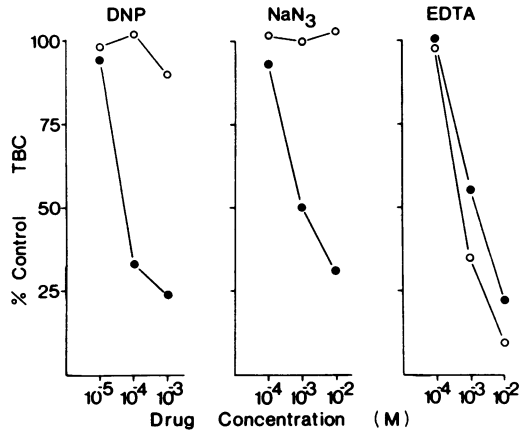

Figure 2. The effect of respiratory inhibitors on target cell contact. Nylon-wool passed spleen cells from normal, unimmunized CBA mice, were treated for 30 min at 20° with inhibitors and then admixed with a ten-fold excess of YAC cells (○). Peritoneal exudate cells (PEC) from CBA mice immunized at day −9 with 30×10^6 P815 cells i.p. were treated twice with carbonyl iron and a magnet and by plastic adherence to remove adherent cells prior to inhibitor treatment and admixing with P815 cells (●). P815 cells are NK insensitive and form <0·5% TBC with NK cells. Values represent % control TBC. Control values were 22% in the CTL system in immunized mice and 0·7% TBC in unimmunized mice. Binding to YAC was 19% in the NK system. The frequency of TBC was reduced 87% and 0% in the CTL and NK systems respectively by pre-treatment with rabbit anti-mouse Thy-1 serum and guinea-pig complement. In addition, 90% and 1% of the lymphocytes in the CTL, and NK TBC respectively stained with mouse anti-theta treatment followed by development with fluorescein-labelled rabbit anti-mouse IgG serum. This experiment was repeated twice with similar results.

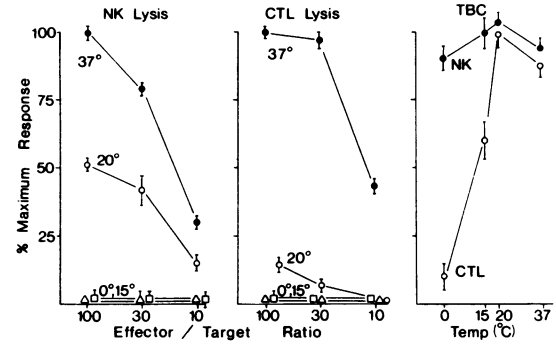

Figure 3. Temperature dependence of target cell binding and lysis in the NK and CTL system. Nylon-wool passed spleen cells from CBA mice were used as effector cells in a 4 h cytolytic assay with YAC targets (left panel). Nylon wool passed spleen cells from CBA mice 10 days after an i.p. injection of 30×10^6 P815 cells, were used as effectors against ^{51}Cr-labelled P815 targets in a 4 h assay (middle panel). Values are expressed as % maximum lysis which was 55% in the NK system and 85% in the CTL system. Nylon-wool passed spleen cells from normal or alloimmunized CBA mice were also enumerated for TBC (right panel) and control values were 18% and 12% TBC respectively. Values represent mean ± SEM. Lysis was reduced 95 and 5% respectively in the CTL and NK system by pre-treatment of the effectors with rabbit anti-mouse Thy-1 serum and guinea-pig complement. This experiment was repeated three times with similar results.

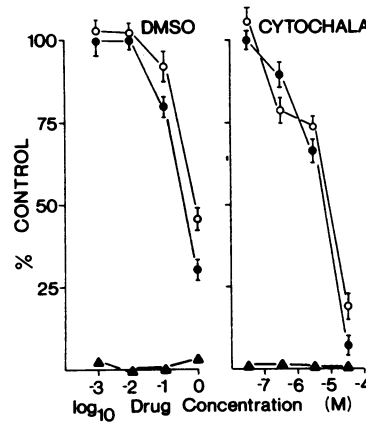

Figure 4. The effect of a dipolar solvent and microtubule disrupter on NK function. The experimental conditions were identical to those described in Fig. 1. This experiment was repeated twice with similar results. (●), % lysis; (○) % TBC; (▲) spontaneous release by YAC cells plus drug. Control values were 60% lysis and 21% TBC.

(Roder *et al.*, 1978), it was expected that agents which interfere with membrane structure might also inhibit cytolysis. As shown in Fig. 4, dimethylsulphoxide (DMSO), a dipolar solvent and a free radical scavenger, inhibited both binding and lysis of YAC targets by murine NK cells. In addition, the fungal metabolite, cytochalasin B, inhibited lysis and TBC, possibly by its disruptive action on microtubules (Kalina & Hollander, 1975). Since these agents are rapidly reversible, it was not possible to determine if they acted at the level of the effector or target cell. These results suggest that the state of the lipid bilayer, and the organization of the cytoskeleton may be of crucial importance for target–effector interaction. However, the free movement of the protein recognition structures may not be necessary. Thus, it was found that pre-treatment of effector cells with glutaraldehyde, a protein cross-linking agent, completely inhibited lytic function, but only partially affected the capacity to bind to the target (Table 1). Although target-binding capacity was reduced in some experiments, the age

Table 1. The effect of glutaraldehyde fixation on NK function

	Buffer control		Glutaraldehyde fixed		
Spleen cell donor*	% TBC	% lysis	% TBC	% lysis	% competition†
Expt 1					
CBA 2 wk	8·6±1·1	22±1·9	1·9±0·7	0·1±0·4	—
CBA 6 wk	17·1±2·0	51±3·7	9·2±0·3	0·0±0·2	—
CBA 30 wk	8·6±0·7	27±1·4	2·1±0·5	0·5±0·4	—
Expt 2					
CBA 6 wk	20·6±1·3	60±3·9	18·2±1·8	0·6±0·5	30
A/Sn 6 wk	7·6±0·6	29±2·6	2·6±0·9	0·0±0·1	8
CBA thymocytes 12 wk	—	—	—	—	0

* Spleen cells from four mice in each group were passed over nylon-wool columns and treated for 30 min at 20° with 0·25% glutaraldehyde or buffer, followed by extensive washing. Spleen cells were then mixed with ^{51}Cr-labelled YAC cells at a 50 : 1 effector : target ratio in a 3 h cytolytic assay. Some spleen cells were labelled with fluorescein and mixed with a ten-fold excess of YAC in order to enumerate the frequency of TBC. Values represent the mean of triplicate determinations ± SEM. The experiment shown is representative of two similar experiments.

† % reduction in CBA spleen cell mediated cytolysis of YAC cells at a 50/1 effector/target ratio by the addition of glutaraldehyde fixed competitor cells at a 0·5 : 1 competitor : target ratio. The same relative differences in competitive effectiveness were maintained at all competitor : target ratios tested (0·25/1, 0·5/1, 1/1, 2/1).

dependence of TBC and the relationship between high (CBA) and low (A/Sn) reactive genotypes was maintained. P815 cells, an NK insensitive target, were not bound by glutaraldehyde fixed or unfixed effector cells (unpublished observation). Furthermore, when glutaraldehyde-fixed spleen cells were used as competitors in a ^{51}Cr-release assay, fixed cells from high reactive genotypes inhibited lysis more ($P < 0·01$) than cells from low reactive genotypes. Glutaraldehyde fixed thymus cells from 12 week old mice did not compete, which suggests that cell crowding effects were not involved.

The lytic moiety

Several different experimental approaches support the notion that lysosome secretion may be involved in the lethal hit phase of NK cytolysis. The beige mutation in the mouse causes a marked impairment in lysosome structure and function (Windhorst & Padgett, 1973; Vassalli, Granelli-Piperno, Griscelli & Reich, 1978; Prieur, David & Padgett, 1972) and as previously shown (Roder & Duwe, 1979) these mice are markedly deficient in their NK function compared to normal littermate controls. This defect could only partially be overcome by interferon or the interferon-inducing agent poly I:C which were previously shown to augment NK activity (Gidlund, Orn, Wigzell, Senik & Gresser, 1978). Target cell binding in the mutant beige mice was normal (Roder & Duwe, 1979) which suggests that the defect lies within the lytic machinery of the cell rather than at the level of the recognition structure or at the level of population size. More direct evidence for the involvement of lysosomal enzymes is shown in Fig. 5. Chloroquine, a selective inhibitor of lysosomal enzymes (de Duve, de Barsy, Poole, Troult, Tulkens & van Hoof, 1974), was shown to decrease markedly NK cytolysis as well as the level of the lysosomal marker enzyme, N-acetyl-β-glucosaminidase, in the effector cell population. In further experiments NK cells were purified to 85–95% purity by (i) separating target–effector conjugates on velocity gradients, (ii) disrupting conjugates with EDTA and (iii) re-separating the small lymphocytes from the larger targets (Roder *et al.*, 1978). When these cells were treated for 1 h with 10^{-4} M chloroquine, washed and

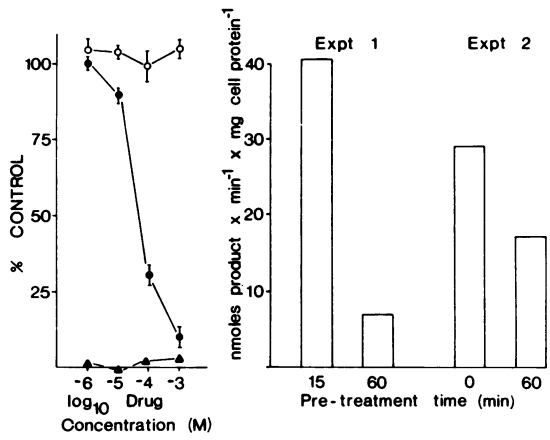

Figure 5. NK function and the level of lysosomal enzyme in chloroquine-treated spleen cells. Spleen cells from C57Bl/6 mice were pre-incubated for 1 h with chloroquine (left panel) and then ^{51}Cr-labelled YAC cells were admixed in a 3 h cytolytic assay at a 100:1 effector:target ratio (•). In addition, ^{51}Cr-labelled YAC cells were cultured with chloroquine in the absence of effectors as a toxicity control (▲). Aliquots of the same spleen cells were passed over nylon wool columns and assayed for target binding cells (○). the data are expressed as % control and control values for lysis and TBC were 45% and 24% respectively. In the right panel, spleen cells from C57Bl/6 mice were assayed for their content of the lysosomal enzyme N-acetyl-β-glucosaminidase after varying pre-treatment periods with 10^{-4} M chloroquine.

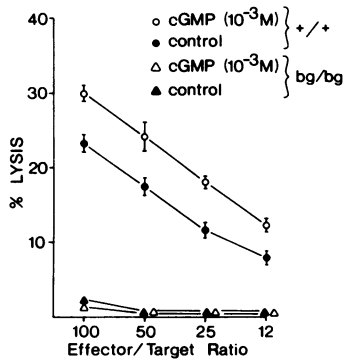

Figure 6. The effect of cGMP on NK mediated lysis in beige mutant mice. Spleen cells from homozygous (bg/bg) mice (triangles) or wild type littermate (+/+) controls (circles) were pre-incubated 2 min at 37° with (open symbols) or without (closed symbols) 10^{-3} M dibutyryl cGMP. ^{51}Cr-labelled YAC cells were added directly to the wells for a 3 h cytolytic assay. Values represent the mean % lysis ± SEM in triplicate wells. This experiment was repeated twice with similar results.

tested in a short 20 min assay, cytolysis was inhibited 70% (data not shown). In longer assays (3 h) the effects of chloroquine were completely reversible.

Part of the lysosomal defect in beige mice appears to involve the failure of the lysosomes to fuse with the plasma membrane and hence they enlarge into the characteristic 'giant granules' (Brandt, Zeigel & Swank, 1974). Since granules return to normal size after treatment of beige cells with the cholinergic

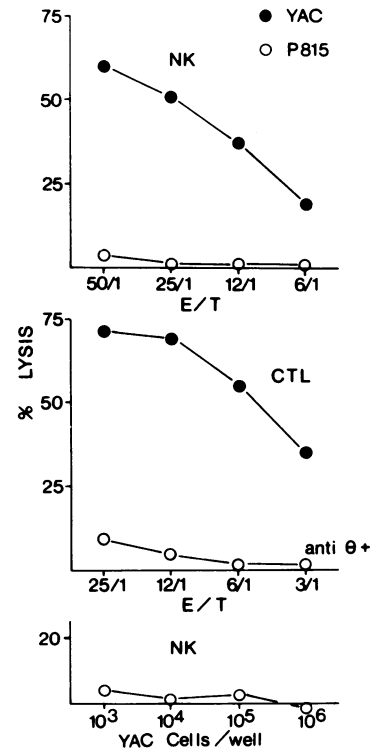

Figure 7. NK cells do not kill 'innocent bystanders'. Top panel: varying numbers of nylon-wool passed spleen cells from normal, unimmunized C57Bl/6 mice were mixed with 10^4, ^{51}Cr-labelled YAC (•) or P815 target cells (○) in a 3 h cytolytic assay. Middle panel: carbonyl iron treated lymphocytes from the peritoneal cavity of alloimmunized C57Bl/6 mice injected i.p. on day −11 with 30×10^6 P815 cells, were tested in a cytolytic assay against 10^4, ^{51}Cr-labelled P815 cells (•). Some effectors were treated with mouse anti-theta serum and complement prior to assay (○). Bottom panel: normal unimmunized C57Bl/6 spleen cells (nylon passed) were mixed with 10^4, ^{51}Cr-labelled P815 cells at a 50 : 1 effector : target ratio. In addition, varying numbers of unlabelled YAC cells were also added and the mixture was centrifuged and incubated 4 h at 37°. This experiment was repeated five times with similar results.

agonist, carbamylcholine (Oliver, Korawiec & Berlin, 1976) an agent which increases endogenous levels of cGMP in lymphocytes (Goldberg, Haddox, Hartle & Haddin, 1973), we attempted to restore NK function by similar treatments. As shown in Fig. 6, dibutyryl cGMP caused a small increase in NK-mediated cytolysis by littermate control cells as previously described (Roder & Klein, 1979), but had not restorative effect on beige cells.

In a final experiment, it is shown that if lysosomes are involved in NK cytolysis their effect must be exerted over a short range since 'innocent bystanders' are not lysed. As shown in Fig. 7, P815 cells are not sensitive to NK cells but are highly susceptible to lysis by CTL in the same experiment. These cells can therefore serve as 'innocent bystanders' if they are included in an NK test. If the NK sensitive YAC cells, which are also included in the test, trigger the NK cells to release some general killing substance, then the P815 cells should also be killed. As shown in the bottom panel of Fig. 7, the P815 cells are not lysed even when centrifuged into the pellet of NK-YAC conjugates. Therefore, the lytic machinery must be associated within the region of intimate membrane contact between NK and YAC.

DISCUSSION

In the present series of investigations, we have aimed to elucidate target–effector interactions in the NK system in terms of (i) the molecular basis of target cell recognition and (ii) the mechanism of cytolysis subsequent to target cell contact. In order to study the recognition step we devised a target-binding assay which was subsequently used both as an isolation procedure for NK cells (Roder *et al.*, 1978) and as a competitive binding assay for the presence of solubilized target molecules (Roder, Rosen, Fenyo & Troy, 1979a). The discovery that the mutant, beige, was defective in NK-mediated lysis but relatively normal in the generation of T killer cells (Roder & Duwe, 1979) demanded a closer comparison of the lytic mechanisms in these two effector cell types.

These results presented here suggest that the cell contact phase of target effector interaction is fundamentally different in NK cells compared to CTL. Hence, the respiratory inhibitors, DNP and NaN_3, as well as low temperatures (0°) completely prevented the formation of target–effector conjugates (TBC) in the CTL system as previously shown (Martz, 1977; Berke *et al.*, 1975; Goldstein & Smith, 1977), but had no significant effect in the NK system (Figs. 2 and 3). The lytic phase of NK cytolysis was also blocked by DNP, NaN_3 or low temperature (Roder *et al.*, 1978) as observed for CTL mediated cytolysis (Berke & Gabison, 1975; Goldstein & Smith, 1977). CTL cytolysis was more temperature dependent than NK cytolysis (Fig. 3) although, as previously shown, CTL responding to a secondary challenge are less temperature dependent than primary CTL (Cerottini & Brunner, 1977). Inhibitors of glycolysis such as iodoacetate and NaF also block the lytic phase (Fig. 1) which suggests that the energy necessary for NK lysis may be derived from both glycolysis and oxidative phosphorylation as in the CTL system (MacDonald & Koch, 1978; Berke & Gabison, 1975; Goldstein & Smith, 1977). These results suggest that, although the metabolic requirements for the lethal hit stage of CTL and NK cytolysis may be similar, the earlier target-binding step is distinct. The underlying basis of this ability of NK cells to bind to targets at low temperatures is not known but may indicate an 'antibody-like' receptor of high avidity or possibly secondary temperature-independent effects such as surface charge or membrane hydrophobicity (Becker, Magnusson & Stendahl, 1978) which may be involved in stabilizing the target–effector conjugate once 'specific' contact has been made via the putative recognition structure. These observations should provide a basis for distinguishing CTL and NK cells in a heterogeneous population.

The importance of membrane and cytoskeleton integrity in the NK-target binding step was also revealed in the present study. Hence, the aprotic dipolar solvent, dimethylsulphoxide (DMSO), inhibited NK-mediated binding and lysis. Although the mechanism of drug action is not entirely clear, this agent would be expected to destabilize the plasma membrane and lead to a disorganization of integral membrane proteins, including recognition receptors (Walberg, Hiemstra, Burge & Singler, 1973). Secondary inhibition of the lytic phase could also occur if free radicals are involved since DMSO is also a free radical scavenger. In the CTL system, DMSO inhibits the CTL-target contact phase (Walberg *et al.*, 1973) but its effects on the lytic step are controversial (Goldstein & Smith., 1977; Walberg *et al.*, 1973). Cytochalasin B also inhibited TBC and lysis in the NK system but it was not possible to assess if the lytic stage was inhibited independently or by virtue of inhibition of the earlier TBC stage. In the CTL system, both target binding and the lytic step are inhibited (Goldstein, Foa & MacLennan, 1978) and it

is unclear whether the relevant drug action involves disruption of microtubules (Kalina & Hollander, 1975) or secondary effects such as inhibition of glucose transport (Goldstein *et al.*, 1978; Bubbers & Henney, 1975b). Since tightly opposed inter-digitating microvilli are observed in the area of NK-target contact (Roder *et al.*, 1978), it is conceivable that such tertiary strengthening of the adhesion is dependent on cell mobility and therefore requires intact microtubules. These results suggest that membrane and cytoskeleton organization may be important for NK-target binding. However, the free movement of integral membrane proteins may not be necessary since the protein cross-linking agent, glutaraldehyde, completely abolished lysis with only partial inhibition of the target binding phase. Lysis may have been inhibited since glutaraldehyde is known to block protein, RNA and DNA synthesis in lymphocytes (Bubbers & Henney, 1975a).

Previous studies have shown that protein synthesis is necessary to maintain the NK effector in a 'killer-ready' state (Roder *et al.*, 1978). Pre-treatment of the effector with proteolytic enzymes such as trypsin inhibits subsequent target–effector binding and consequently the lytic phase is also blocked. In the absence of inhibitors of protein synthesis both binding and lytic functions regenerate in an overlapping time course in 2–4 h (Roder *et al.*, 1978). It is conceivable that protein synthesis is necessary to (i) compensate for the turnover of protein-like recognition structures on the cell surface, and (ii) maintain a preformed pool of lytic proteins. It is of interest that the entire lytic cycle in the CTL system appears to be independent of protein synthesis as shown by recent experiments using pactamycin and emetine (Thorn & Henney, 1976).

Fewer points of comparison are available in the ADCC system. As shown earlier (Kiessling *et al.*, 1976) K cells are much more trypsin resistant than NK cells. We have observed (unpublished results) that NK and K cells are equally sensitive to metabolic inhibitors and when the shape of kinetic curves for cytolysis are compared, NK resembles ADCC but not CTL (i.e. concave as opposed to sigmoidal). Recent studies suggest that NK cells have relatively weak but detectable receptors for the Fc portion of antibody molecules (Ojo & Wigzell, 1978; Herberman, Bartram, Haskill, Nunn, Holden & West, 1977) and a point mutation in the mouse which completely blocks a step in the lytic machinery of NK cells also blocks lymphocyte-mediated ADCC of tumour cell targets although macrophage ADCC remains normal (Roder, Lohmann-Matthes, Domzig & Wigzell, 1979b). Therefore, the effector cells themselves may be identical in the NK and ADCC systems and a single NK cell may possess two recognition systems (antibody-dependent and antibody-independent) but one lytic pathway.

The target cell requirements for target–effector interaction in the NK system are less well studied. However, biochemical isolation procedures have established that the NK-target 'antigen' on the surface of target cells consists of up to three glycoprotein molecules of mol. wt 120 K-240 K (Roder *et al.*, 1979a) These structures may also be cleaved by trypsin and pronase (unpublished observation) and may turn over at a slow rate since 3 h pre-treatments with inhibitors of protein synthesis had no effect. Energy metabolism does not appear to be important and in other systems it has been shown that neither protein syntheis, RNA nor DNA synthesis is required and indeed enucleated targets are lysed equally well by NK cells (Siliciano & Henney, 1978).

We favour a 'stimulus-secretion' model of NK cytolysis and postulate that lysosomal enzymes may be the actual lytic moiety since (i) chloroquine inhibited NK cytolysis as well as lysosomal enzymes (Fig. 5). (ii) the mutant mouse, beige, is defective in lysosomal enzyme function (Vassalii *et al.*, 1978; Prieur *et al.*, 1972; Brandt *et al.*, 1974) and has a profound impairment in the NK lytic mechanism (Fig. 6), whereas the levels of NK-TBC; and T cell and macrophage cytolysis are normal (Roder & Duwe, 1979) and (iii) the same concentrations and variety of agents which inhibit the lytic phase of NK cytolysis (cAMP, PGE_1, theophylline, and histamine) also inhibit lysosome secretion in cells (Roder & Klein, 1979; Weissmann, Goldstein, Hoffstern & Tsung, 1975) and conversely agents which increase lysosomal discharge (cGMP, carbamylcholine) significantly augment NK lysis. If lysosomes are involved in target destruction, then a special localized mode of delivery is implicated since 'innocent bystanders' were not killed in NK cytolytic experiments (Fig. 7). These observations suggest that a lysosome-dependent, 'stimulus-secretion' model of NK cytolysis is worthy of further investigation.

Note added in proof

Unlike the situation in the beige mouse, we have recently found that pre-incubation of human, Chediak-Higashi lymphocytes with cGMP completely restores the defective NK response (Katz, Roder, Fauci &

Herberman, submitted for publication) recently described in these patients (Roder, J.C., Haliotis, T., Klein, M., Korec, S., Jett, J., Ortaldo, J., Herberman, R.B., Katz, P. and Fauci, A.S. *Nature*, in press).

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