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Spontaneous Cytotoxic Earthworm Leukocytes Kill K562 Tumor Cells

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ABSTRACT—Earthworm coelomocytes may act as effector cells which destroy targets *in vitro*. In a ^{51}Cr release assay, *Lumbricus* coelomocyte effectors showed lytic activities of 3–14% against K562 human tumor cells when incubated 1–4 hr at 23°C or 37°C. Cytotoxicity was correlated with effector: target ratio. However, targets were not killed by incubating them in cell-free, 0.2 μm filtered coelomic fluid. The supernatant from coelomocytes cultured alone failed to kill K562 targets but coelomocyte lysates were toxic to target cells in a concentration-dependent manner. Coelomocytes were examined using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). When effectors and targets were examined under TEM, we found close apposition of effector granulocytic coelomocytes and target cell membranes but not with coelomocytes nor eleocytes at up to 15 min incubation. By SEM, effector cells appeared not only to be in close contact with targets, but instances of target lysis were observed. These results suggest that effector cell/target cell contact is essential for cytotoxicity to occur.

INTRODUCTION

In previous experiments we observed no significant spontaneous cytotoxicity against haptenated autogeneic target coelomocytes, but coelomocytes effected significant spontaneous cytotoxicity against haptenated allogeneic targets [Suzuki & Cooper, unpublished data]. In intrafamilial xenogeneic combinations, *Lumbricus* and *Eisenia* coelomocytes cultured together caused significant cytotoxicity. Using mammalian tumor targets YAC-1 and K562 in a ^{51}Cr release assay we observed significant cytotoxicity by earthworm effectors [Suzuki & Cooper, unpublished data]. These results suggest that increasingly greater differences in cell membranes of targets are crucial in the recognition of *non-self*. This approach, i.e. use of mammalian tumor cell targets and the ^{51}Cr release assay, has been employed to analyze spontaneous killer cells from other invertebrates [8, 9, 20, 24, 29]. These results prompted us to work more intensively with a distinct, homogeneous target, using ^{51}Cr-labeled K562 cells as targets. Thus, in contrast to our xenogeneic *Lumbricus-Eisenia* model, there is no confusion in distinguishing between killers and targets during cytotoxicity assays nor in examination of characteristic intracellular responses by electron microscopy. Herein, we describe aspects of spontaneous cytotoxicity when earthworm coelomocytes are used as effectors against K562 mammalian tumor cells and present evidence for the need of close effector: target cell contact by means of transmission and scanning electron microscopy.

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MATERIALS AND METHODS

Earthworms

Earthworms (*Lumbricus terrestris*) were purchased from Carolina Biological Supply Co. (Gladstone, OR) and Allan's Aquarium (Venice, CA). They were maintained at 15°C in moist peat moss and were fed dried baby cereal.

Collecting coelomocytes and coelomic fluid

Lumbricus coelomocytes were harvested by puncturing the body wall at several sites posterior to the clitellum with a sharpened Pasteur pipet. Just before each penetration, the pipet tip was dipped into a solution of L-15 (see below)+caffeine (3 mM)+TAME (2 mM), because the latter two chemicals reduced cell agglutination [1]. Coelomocytes were examined and counted using trypan blue dye exclusion. In other experiments, coelomic fluid was collected by puncturing the body wall with a dry pipet. The resulting crude coelomocyte suspension was pooled from different worms, centrifuged at 100 g for 10 min, the pellet discarded, the supernatant filtered through a 0.2 μm filter membrane, and stored at −20°C until use.

Media

Lumbricus coelomocytes were harvested and washed in Leibovitz L-15 medium (Sigma Chemical Co., catalog no. L4386) with caffeine and TAME (see above). Liquid RPMI-1640 medium was from Sigma Chemical Co. and fetal bovine serum (FBS) was purchased from Atlanta Biologicals. All media were adjusted to pH 7.3.

Maintenance and preparation of mammalian target cells

The K562 cell line was cultured in a 37°C, 5% CO_2 incubator in a solution of RPMI-1640 with 10% FBS. Counting of viable targets was performed by trypan blue dye exclusion in an improved Neubauer hemacytometer.

^{51}Cr release assay

K562 tumor cells were centrifuged and resuspended in fresh RPMI-1640 with 10% FBS to about 2×10^6/ml. Labeled sodium

dichromate in saline ($Na_2{}^{51}CrO_4$, 200 μCi; ICN Biomedicals) was mixed with the targets, which were placed in a 37°C, 5% CO_2 incubator for 1 hr with occasional shaking. Targets were washed twice with L-15 containing 10% FBS, counted, washed again, and suspended at 10^5 cells/ml in either L-15 culture medium with 10% FBS for incubation at 23°C, or in RPMI-1640 with 10% FBS for 37°C incubation. Aliquots of 100 μl were added to each well of a 96-well round-bottom microtiter plate (Costar). Coelomocytes (in 100 μl of either 90% L-15/10% FBS for incubation at 23°C, or in 90% RPMI-1640/10% FBS for 37°C incubation) were added at various effector:target (E:T) ratios. Each well contained effectors from one earthworm. In some experiments, supernatant from 2 hr coelomocyte cultures, or coelomocyte freeze-thawed lysate (prepared by rapid freezing and thawing for several cycles), replaced effector cells. Spontaneous lysis wells contained 100 μl medium instead of coelomocyte suspension. Total lysis wells contained 100 μl of 10% (v/v) Triton X-100 detergent solution instead of coelomocyte suspension. Plates were centrifuged at 50 g for 5 min, and placed in either a dark cabinet at 23°C or a 37°C, 5% CO_2 incubator for various times. Fifty μl of the appropriate medium was added to each well. The plate was centrifuged at 50 g for 5 min. Supernatant (150μl) was separately transferred to individual 4×75 mm glass tubes, which were counted in a Wallac 1470 gamma counter. Specific lysis was determined from the radioactive counts per minute (cpm) by the formula:

$$\text{specific lysis} = \frac{\text{experimental cpm-spontaneous cpm}}{\text{total cpm-spontaneous cpm}}$$

Spontaneous death was always less than 8% of the total lysis.

Preparation of effectors and targets for transmission electron microscopy

Suspensions of effector coelomocytes were combined with K562 target cells in a 1:1 ratio. Cell interaction was allowed for 5, 10, and 15 minutes before the addition of a fixative solution containing ice-cold 1.5–2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 hr, and pelleted by gentle centrifugation at 50 g for 3 min. The pellet was then rinsed with the buffer twice for 10 minutes each and left overnight in the same buffer. Post-fixation was performed in a 1% osmium tetroxide solution in 0.1 M cacodylate buffer for 1 hr at 4°C. The cells were then rinsed twice with the buffer for 10 min each. Dehydration was carried out in a graded series of ethanol solutions. Embedding was in Medcast (Ted Pella). Thin sections were cut on an ultramicrotome Reider T ultracut/E with a diamond knife and placed on a coated grid. Sections were stained with lead citrate for 2 min. Specimens were viewed on a Zeiss 10c electron microscope.

Preparation of effectors and targets for scanning electron microscopy

Suspensions of effector coelomocytes were combined with human tumor target cells in a 1:1 ratio. Cell interaction was allowed for 5, 10, and 15 minutes before the addition of a fixative solution containing glutaraldehyde, paraformaldehyde, and cacodylate buffer. Two controls containing effectors only and targets only were also established at 5 min. All suspensions were maintained in the fixative for 3 hr. The fixed cells were then incubated in 0.2 M cacodylate buffer for two periods of 15 minutes each prior to postfixation for 1 hr in 1% osmium tetroxide followed by another period of incubation in the buffer solution. Finally, the cells were dehydrated through an ethanol series and maintained in a dessicator jar at room temperature for two days. The specimens were then plated with 20 nm gold-palladium by a Hummer I sputter coater and viewed through an E.T.E.C. Autoscan scanning electron microscope at magnifications up to 6000x at 10 kV.

Statistics

We wished to eliminate treatment differences without the confounding effect of the high inter-worm variability which exists in these outbred animals. Therefore each cytotoxicity assay was set up as a randomized complete block design [25] with individual earthworms or groups of earthworms from the same geographic region as the blocking criteria. This experimental design was selected because it allowed for the estimation of the natural variation among earthworms and the exclusion of this variation from experimental error without affecting differences among treatment means. This design has been used successfully in other studies of immunity in invertebrates, specifically in snails [3, 4, 19]. Significance was assessed at $p=0.05$ unless otherwise noted. Stated n values represent the number of earthworms analyzed with each treatment. Standard errors of the means are calculated from ANOVA sums of squares.

RESULTS

Influence of incubation duration at a single (25:1) effector:target cell ratio

We hypothesized that if effector:target cell ratio were held constant, then levels of cytotoxicity would rise with increasing incubation duration. To examine this, coelomocytes were used as effector cells against human K562 cells in a ^{51}Cr release assay during 2 and 3 hr incubations at 37°C. *Lumbricus* coelomocytes lysed K562 cells at levels of 3–5% at an effector:target ratio of 25:1 with no significant difference between 2 and 3 hours (Fig. 1).

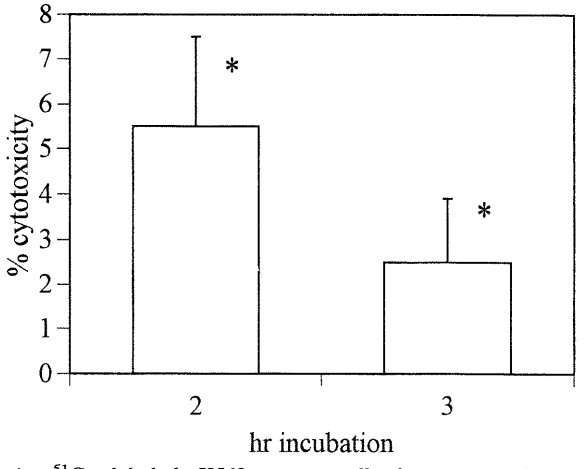


FIG. 1. ^{51}Cr labeled K562 target cells in xenogeneic assay. Effector:target ratio was 25:1. Cells were incubated for 2 hr and 3 hr in 96-well round-bottom microtiter plates at 37°C, 5% CO_2. Incubation medium was RPMI-1640 with 10% FBS. *Lumbricus* coelomocytes lysed K562 cells at levels of 3–5% at an effector:target ratio of 25:1 with no significant difference between 2 and 3 hours ($N=5$, $P=0.42$). Error bars denote standard error of mean. *Significant difference from spontaneous death (zero % cytotoxicity) at $P=0.05$.

Effects of different temperatures and effector:target ratios

With respect to effector:target cell ratios, significant cytotoxicity was observed at 23°C at the ratios of 10:1, 25:1, 50:1, and 100:1 (Fig. 2). The percent cytotoxicity after 2 hr at an E:T ratio of 10:1 was approximately equal to the value obtained after 3 hours at the 25:1 ratio at 37°C (Fig. 1). Levels of cytotoxicity rose as the E:T ratio increased to 100:1. Clearly, percent cytotoxicity at any E:T ratio after 2 hours was 3–5% higher than that after 1 hr. There was no significant difference in cytotoxicity between 2 hr and 4 hr incubation at any E:T ratio (Fig. 2).

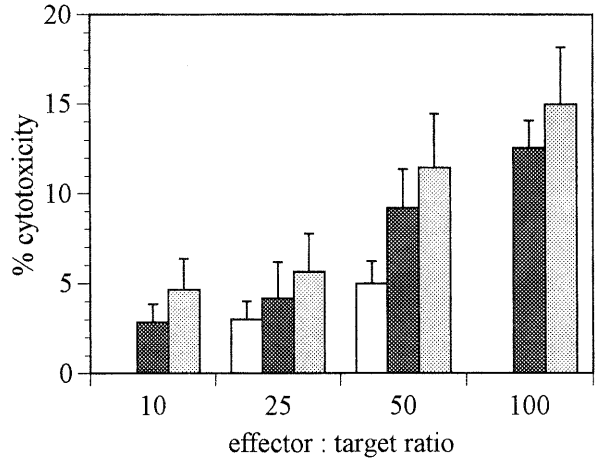


FIG. 2. ^{51}Cr labeled K562 target cells in xenogeneic assay. Effector:target ratios were 10:1, 25:1, 50:1, and 100:1. Incubation times were 1 (□), 2 (▩) and 4 (▨) hr at 23°C. Significant cytotoxicity was observed at all ratios (n=6, for difference from spontaneous death, $P<0.05$ in all cases). Error bars denote standard error of mean.

Requirement for cell-cell contact

To examine the assumption that cell-cell contact was essential for cytotoxicity we incubated K562 targets for 1 hr or 2 hr at both 23°C and 37°C at four concentrations (6.25%, 12.5%, 25%, and 50%) of cell-free, 0.2 μm filtered, coelomic fluid. No significant target cell death was observed at either temperature, suggesting that cell-cell contact was essential for killing to occur (Fig. 3). Additionally, we incubated coelomocytes without target cells, collected supernatants, and tested for any cytotoxic effects of the supernatant on ^{51}Cr-labeled K562 targets. When targets were incubated in supernatant from effector cells cultured alone, there was no significant cytotoxicity (Fig. 4).

Killing of targets by lysates of effector cells

We then performed experiments to test whether soluble cell contents would affect cytotoxicity. Effector cell lysates were prepared by rapidly freeze-thawing coelomocytes several times. The toxicity of the cell-free lysate was tested using K562 cells (Fig. 5). Target cell death ranged from 4–12% in a manner dependent on the concentration of lysate, which ranged from 6.25% to 50% (N=13; for difference between

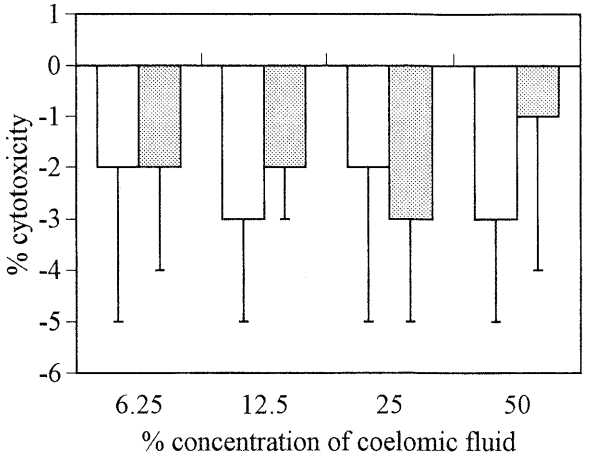


FIG. 3. Percent cytotoxicity of K562 human tumor cells after 2 hr incubation in cell-free, 0.2 μm filtered coelomic fluid. Final concentrations of coelomic fluid were 6.25%, 12.5%, 25% and 50% of native concentration. Two incubation temperatures of 23°C (□) and 37°C (▨) were used. No significant target cell death was observed at either temperature (N=12; $P=0.72$). Error bars denote standard error of mean.

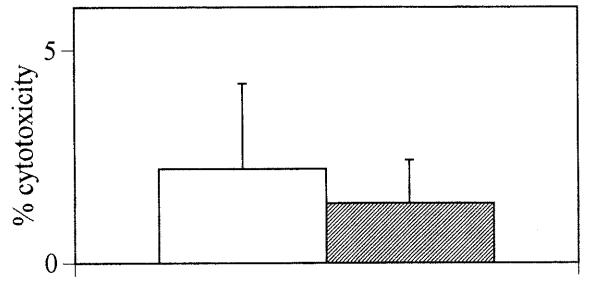


FIG. 4. Percent cytotoxicity of ^{51}Cr labeled K562 target cells in coelomocyte pre-incubation supernatant. Coelomocytes (in L-15 medium without FBS, or RPMI-1640 (90%)+FBS (10%) were cultured for 2 hr at 5×10^5 cells/well in 96-well round bottom microtiter plates at 23°C in room air using L-15, or at 37°C in a 5% CO_2 condition using RPMI-1640 with 10% FBS. Plates were centrifuged, and 100 μl of supernatant was removed. This culture supernatant was added to ^{51}Cr-labeled K562 cells in 96-well round bottom microtiter plates cultured at 23°C (□) in room air using L-15, or at 37°C (▨) in a 5% CO_2 condition using RPMI-1640 with 10% FBS. Targets were cultured with supernatant for 2 hr. There was no significant cytotoxicity (N=14; for difference from spontaneous, $P>0.42$). Error bars denote standard error of mean.

6.25% and 50%, $P=0.005$). There was no difference in cytotoxicity between the incubation temperatures of 23°C and 37°C.

Transmission electron microscopy of effectors and targets

Effector cells: Granulocytic coelomocytes

Although five types of leukocytes were observed in the specimen, only granulocytes appeared to be in contact with targets. Two types of granulocytic coelomocytes were identified, based on a previous classification [18]. Granulocytes named type I can be distinguished by the presence of two cytoplasmic subregions: the endoplasm, which contains most

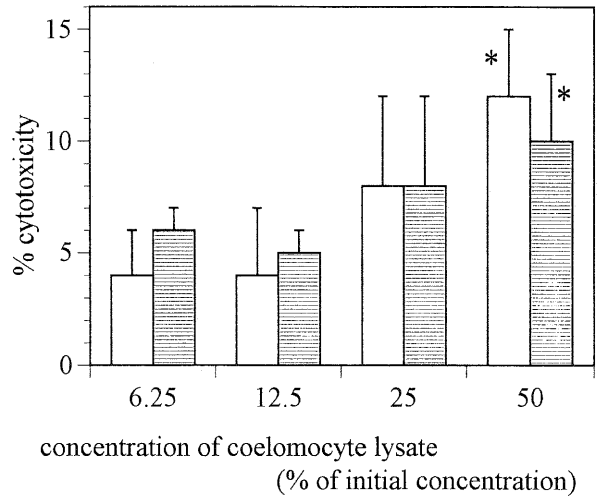


FIG. 5. Percent cytotoxicity of ^{51}Cr labeled K562 target cells in coelomocyte lysate. Coelomocytes (1.5×10^7) were harvested from worms, pooled, lysed by rapid freeze-thawing, and filtrated. Half of the lysate was diluted in 1.5 ml L-15 (90%)+10% FBS, the other half in RPMI-1640 medium (90%)+10% FBS (for the equivalent of 5×10^5 lysed cells/ml). Dilutions of lysate (100 μl) were added to ^{51}Cr-labeled K562 cells (10^4 cells in 100 μl L-15 or 90% RPMI-1640+10% FBS) in 96-well round bottom microtiter plates. Incubation was 2 hr at 23°C in room air (□), or at 37°C in a 5% CO_2 condition (▤). Error bars denote standard error of mean. * Significant difference from cytotoxicity with 6.25% coelomocyte lysate present at $P=0.005$.

of the organelles, and the ectoplasm which had fewer organelles (Fig. 6). In the endoplasm we observed the nucleus, mitochondria, vesicles, small amounts of rough endoplasmic reticulum, Golgi apparatus, microfilaments, as well as dense granules and glycogen granules. The microfilaments are a unique characteristic of the type I granulocyte. The dense granules could be distinguished from the glycogen granules based on size and distribution. The dense granules were spread out more uniformly throughout the cell, while the glycogen granules were clustered together in an asymmetric distribution. Granulocytes named type II had a distinctive feature in that it was somewhat star-shaped in appearance due to its numerous short pseudopodia (not shown). In addition, type II granulocytes had far fewer microfilaments than type I. Another characteristic of this cell was that it had two types of granules: light-staining granules and dense, dark-staining granules. It also had mitochondria, Golgi body, glycogen granules located on its periphery, empty membrane-limited vacuoles, and a nucleus which contained condensed chromatin.

K562 targets

The targets were round or oval, with a high nucleus/cytoplasm ratio (Fig. 7). Nuclei were often indented. Chromatin was dispersed, and there were prominent nucleoli. Target cell membranes had few ruffles or microvilli, in marked contrast to coelomocytes, which had numerous membrane extensions. Many mitochondria and vacuoles were visible.

Coelomocytes and K562 cells in contact

Only a few instances of close granulocytic coelomocyte:K562 contact were observed (Figs. 8, 9). From 15 min assays (1 : 1 effector-target ratio), cell-cell contact over a broad area was evident. Intercellular interdigitation of membranes was observable but not extensive. What is not yet clear are the events which follow the initial contact between the effector and the target cells. For example, in figure 8, there is a viable target cell in contact with a highly granulated effector cell in the upper left; the two can be identified by degrees of surface ruffling. Above that same effector cell there appears to be engulfment of a destroyed target cell.

Scanning electron microscopy of effectors and targets

Effector coelomocytes

Earthworm effector coelomocytes were spheroid in shape (Fig. 10). Their outward appearance was easily distinguishable from the targets because coelomocytes were covered with thin, ruffled projections. These membranous projections were more prevalent on some cells than on others.

K562 targets

The targets were moderately spherical and had slightly wrinkled membranes (Fig. 11). There were several short microvillous protrusions, and several cells showed occasional surface blebs. A moderate number of cells had narrow, ridge-like profiles and small membrane ruffles.

Coelomocytes and K562 cells in contact

After five minutes of interaction, a 1:1 ratio of effectors and targets did not result in significant change in the appearance of either cell type. Slight, yet apparent contact and possible adherence was established only between the peripheral ruffle of the effector and the uneven surface of the target (Fig. 12). No new extensions of any type (i.e. pseudopodia) were visibly protruding from the effectors. After 10 minutes, there were definite changes in the coelomocytes. Whereas only the membranous ruffles of the effectors were in contact with the targets at 5 minutes, the adhesion between the body of the effector cell with the target cell was noticeable at 10 minutes. More distinctive, though, were large cavities forming not only on the surface of the targets, but well into their cytoplasms (Fig. 13). After 15 min, one of the targets had a large hole in its membrane and was clearly destroyed (Fig. 14).

DISCUSSION

Earthworm rejection of allogeneic and xenogeneic body wall grafts suggests that worms possess cells, soluble factors, or both which recognize and destroy foreign *non-self* tissue without previous antigenic exposure [5, 7, 11, 17]. Mammals possess spontaneous cytotoxic cells, which have been studied extensively [28]. Human and murine peripheral

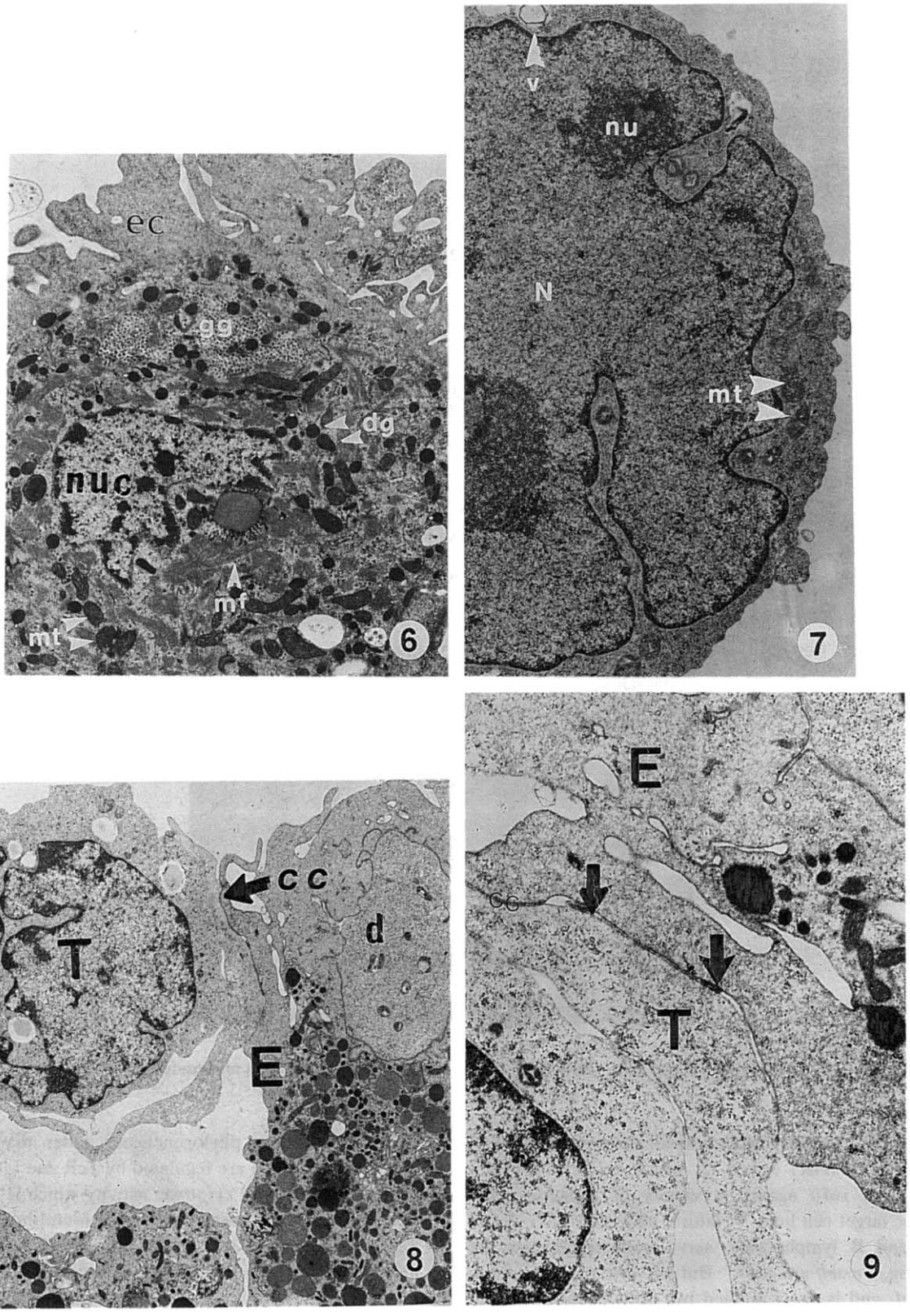
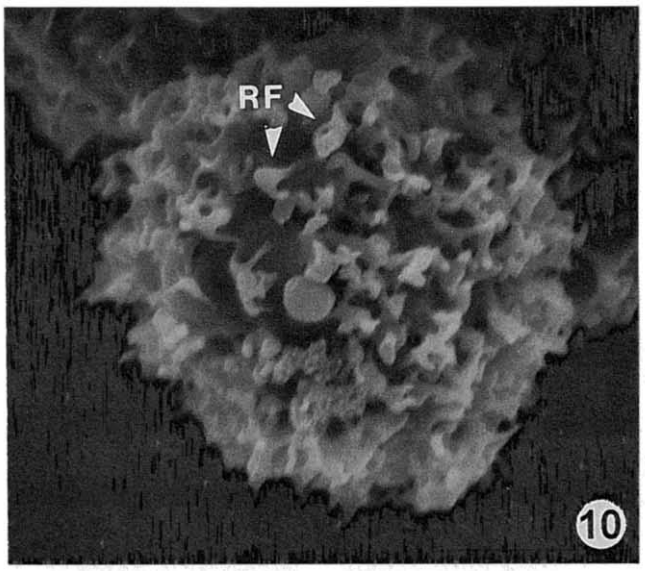


FIG. 6. Type I granulocytic coelomocytes with a distinct division of cytoplasm: endoplasm and an ectoplasm (ec). The endoplasm contains bundles of extensive microfilaments (mf), dense granules (dg), mitochondria (mt), and glycogen granules (gg), as well as small amounts of rough endoplasmic reticulum. nuc=the nucleus. ×7,500.

FIG. 7. K562 human tumor cell line used as targets. The majority of these spherical cells are characterized by a bumpy, wrinkled surface with rounded protrusions; N=nucleus, nu=nucleolus, mt=mitochondria, v=vacuole. ×17,500.

FIG. 8. TEM of *Lumbricus* coelomcyte : K562 conjugate. Note the large number of granules within the effector cell (E). Also note the



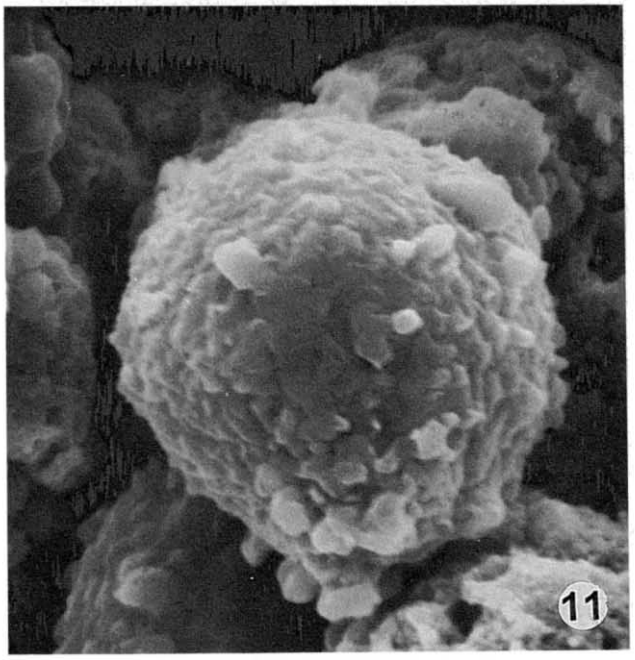


FIG. 10. SEM of effector cells (EC). ×6,000
FIG. 11. SEM of a target cell. Note their wrinkled appearance and rounded protrusions (P). ×6,000.

blood mononuclear cells include a small percentage (5–10%) of natural killer (NK) cells [27], which exhibit significant cytotoxicity *in vitro* against a variety of allogeneic and xenogeneic target cell lines. Natural killer cells, like vertebrate T and B lymphocytes, serve immune function by neutralizing *non-self* antigens. But NK cytotoxicity is independent of, and is not amplified by, prior exposure of the organism to the antigens of those target cells. This suggests that NK cells are phylogenetically older than T and B lymphocytes, which are regulated by TcR and Ig rearranging genes [23]. Natural cytotoxic activity which is functionally similar to that of mammals has been found in annelids [31, 32], sipunculids [2, 30], clams [33], crayfish [24, 29], sea urchins [1], and tunicates [14] [reviewed in 6]. The possibility that certain earthworm coelomocytes can effect natural killer function similar to that observed in mammals has been

presence of cytoplasm of an engulfed target cell (d). There is cell contact (cc) between the target (T) and effector cell. ×6,500.
FIG. 9. TEM of *Lumbricus* coelomcyte : K562 conjugate. An enlargement of figure 8. Note broad area of membrane contact, and interdigitation but not re-orientation of coelomocyte organelles. ×25,000.

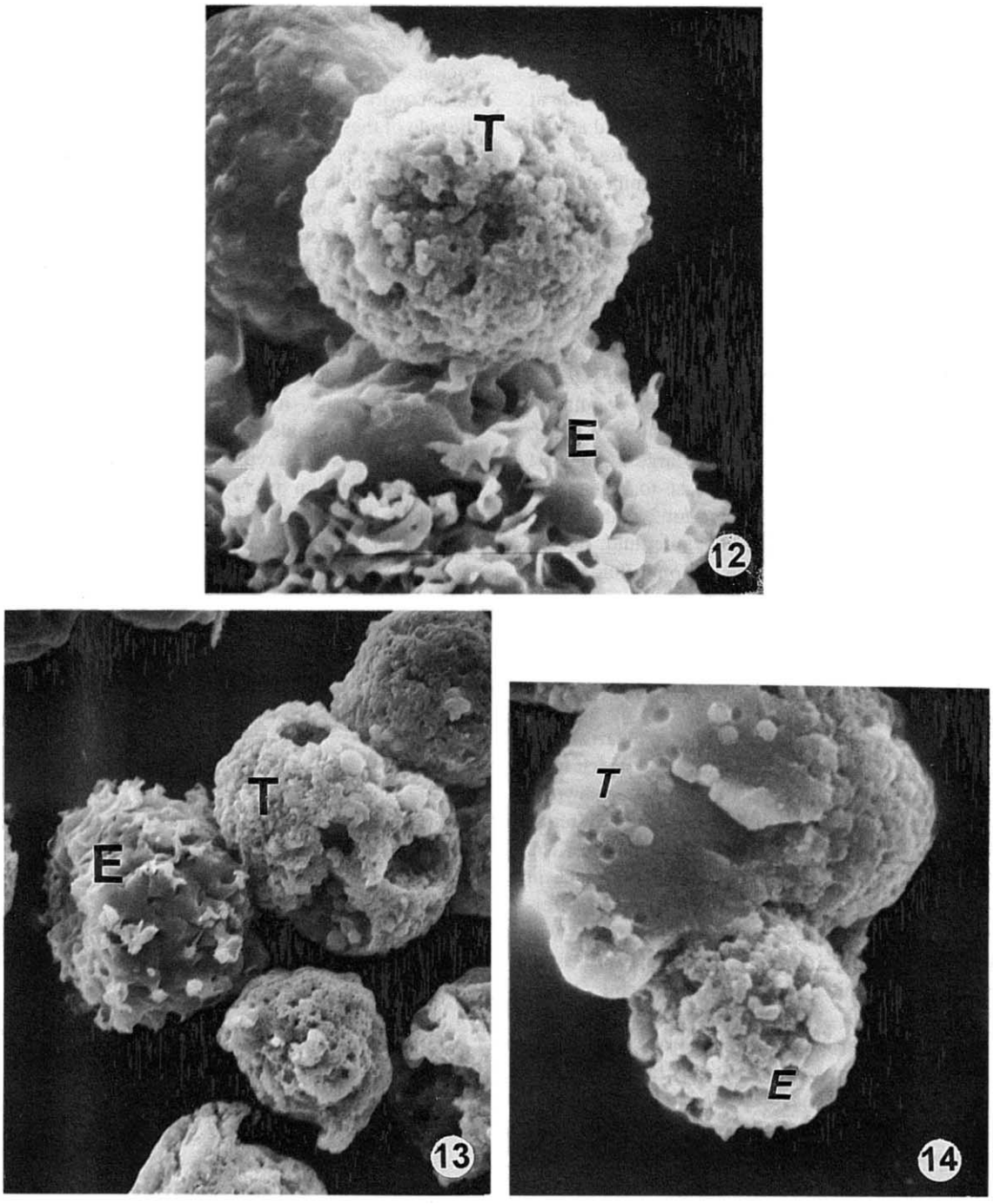


FIG. 12. Interaction between an effector (E) and a target (T) after 5 minutes. Contact has been established, but lytic activity has not commenced. ×6,000.

FIG. 13. Interaction between an effector (EC) and a target cell (T) after 10 minutes. The four large vacant cavities in the target indicate that lytic activity has begun. At least one other target cell (bottom right corner) appears also to have undergone some lytic activity. ×3,000.

FIG. 14. Interaction between an effector (E) and a target (T) after 15 minutes. The effector is embedding itself into the target. ×6,000.

little explored [30].

The purpose of this investigation was to continue our exploration of lytic activities by *Lumbricus* coelomocytes against K562. We addressed several questions and used essentially three different procedures: 1) analysis of per cent cytotoxicity as measured by release of ^{51}Cr; 2) transmission electron microscopy; 3) scanning electron microscopy. In turn we posed several questions concerning effector:target cell interactions with respect to the cytolytic outcome.

The potential similarities in natural killing by mammalian and earthworm effectors deserve further exploration. Our experimental protocol described here using K562 target cells was designed to be as similar as possible to the widely used ^{51}Cr release assay for mammalian NK cells. This work

extends the earlier experiments on spontaneous cytotoxicity of earthworm target and effector cells performed by Valembois et al. [31]. They used a ^{51}Cr release assay to show that spontaneous allogeneic cytotoxicity occurs in *Eisenia fetida* at a E : T ratio of 10 : 1. Significant killing was observed after 1 hour, increasing up to 6 hr incubation. Both *Eisenia* and *Lumbricus* earthworm coelomocytes display significant specific lysis of the mammalian target cell line K562. *Lumbricus* cells effect lysis of YAC-1 cells [Suzuki & Cooper, unpublished data].

In this work, after 1 to 4 hr *in vitro*, incubation of coelomocytes with K562 targets at 23°C, cytotoxicity was 3–14%, and was correlated with effector:target ratio. Even though our SEM results indicate that lysis can occur after 15 min of effector: target incubation, the effector population may be heterogeneous with respect to time of killing. Thus, more than 15 min may be required for a large number of targets to be lysed, and thus for ^{51}Cr release to be measurable. When K562 targets were incubated for 2 hr in various dilutions of cell-free, 0.2 μm filtered coelomic fluid, no cell death was observed. Thus, *Lumbricus* coelomic fluid has no cytotoxic effect on this mammalian tumor cell line at concentrations of up to 50% which supports the view that cytotoxicity is dependent upon contact between effector and target cells. Previous studies have shown that *Lumbricus* fluid contains hemagglutinins [26] which bind to but do not lyse mammalian erythrocytes. Coelomic fluid of the earthworm *Eisenia fetida* possesses bacteriolytic factors [22] which have not been found in *Lumbricus*. Thus, these two earthworm species differ in the repertoire used in their immune systems.

The morphology of effector coelomocytes and the K562 cells in both TEM and SEM were so distinct that even in co-cultures, the two species were easily identified and differentiated. Observations of K562 cells corresponded with those found previously [13]. In our work using transmission electron microscopy, limited evidence of effector: target interaction was observed. Membrane apposition was obvious, but no organelle orientation, membrane interdigitation, or target destruction occurred after a 15 min effector :target contact. This result using *Lumbricus* coelomocytes differs from the results of Porchet-Henneré *et al.* [21], who studied the attack of G3 granulocytes from the polychaete annelid *Nereis diversicolor* against mammalian erythrocyte targets. The Golgi apparatus and centrosome of the G3 cell reoriented toward the target cell. Similar reorientation has been found in mammalian NK cells [12, 16]. Exocytosis of granules from the G3 cells was also observed. These events were apparent in samples of G3 cells and mouse erythrocytes after 20 min incubation. It is possible that similar phenomena can be observed using *Lumbricus* coelomocytes for over 15 min incubation. Coelomocytic granules were observed in TEM and appeared to be similar to those found in mouse NK cells by Frey *et al.* [10]. The outer area of mouse NK granules contained acid phosphatase and inorganic trimetaphosphatase. The K562 target cells matched previous SEM descriptions [13, 15].

This work suggests future directions for investigating spontaneous cell-mediated cytotoxicity by earthworm coelomocytes and confirms the fine structural analysis of earlier work [18]. Our tentative observations also confirm the role of these cells in graft rejection as observed previously [18]. There is need to isolate fractions of coelomocytes to determine which hemocytes are responsible for cytotoxicity. Although we demonstrated contact between granulocytic coelomocyte effectors and K562 targets, we are not certain which of these cells (since there are two types of granulocytes) is the actual effector nor the relative degree of cytotoxicity that each effects. In TEM, we must search for signs of organelle reorientation and degranulation in the effector cell. In SEM, although we observed three dimensional lytic effects (holes in targets), it cannot be determined whether one effector is responsible for complete destruction of a target and then moves on to repeat this lytic process, or causes only partial degradation in many target cells, leading to the hypothesis that the lysis of one target is the result of cooperative action of many effectors. The micrographs display single effectors interacting with single targets only, supporting the notion that one effector is responsible for the complete lysis of one target before proceeding on to repeat its destructive cycle. Obviously the microscopy work needs to be repeated using higher effector:target cell ratios and attention given to the effector's time of exposure and temperature.

ACKNOWLEDGMENTS

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REFERENCES

1 Bertheussen K (1979) The cytotoxic reaction in allogeneic mixtures of echinoid phagocytes. Exper Cell Res 120: 373–381

2 Boiledieu D, Valembois P (1977) Natural cytotoxic activity of sipunculid leukocytes on allogeneic and xenogeneic erythrocytes. Dev Comp Immunol 1: 207–216

3 Connors VA, Lodes MJ, Yoshino TP (1991) Identification of a *Schistosoma mansoni* sporocyst excretory-secretory antioxidant molecule and its effect on superoxide production by *Biomphalaria glabrata* hemocytes. J Invert Pathol 58: 387–395

4 Connors VA, Yoshino TP (1990) In vitro effect of larval *Schistosoma mansoni* excretory-secretory products on phagocytosis-stimulated superoxide production in hemocytes from *Biomphalaria glabrata*. J Parasitol 76: 895–902

5 Cooper EL (1968) Transplantation immunity in Annelids. I. Rejection of xenografts exchanged between *Lumbricus terrestris* and *Eisenia foetida*. Transplant 6: 322–337

6 Cooper EL, Rinkevich B, Uhlenbruck G, Valembois P (1992) Invertebrate immunity: Another viewpoint. Scand J Immunol 35: 247–266

7 Cooper EL, Roch P (1984) Earthworm leukocyte interactions during early stages of graft rejection. J Exp Zool 232: 67–72

8 Decker JM, Elmholt A, Muchmore AV (1981) Spontaneous cytotoxicity mediated by invertebrate mononuclear cells toward normal and malignant vertebrate targets: Inhibition by defined mono- and disaccharides. Cell Immunol 59: 161–170

9 Franceschi C, Cossarizza A, Monti D, Ottaviani E (1991) Cytotoxicity and immunocyte markers in cells from the freshwater snail *Planorbarius corneus* L. (Gastropoda pulmonata): Implication for the evolution of natural killer cells. Eur J Immunol 21: 489–493

10 Frey T, Petty HR, McConnell HM (1982) Electron microscopic study of natural killer cell-tumor cell conjugates. Proc Natl Acad Sci USA 79: 5317–5321

11 Hostetter RK, Cooper EL (1974) Earthworm coelomocyte immunity. In "Contemporary Topics in Immunobiology" Ed by EL Cooper, Plenum Press, New York, pp 91–107

12 Kaneda K, Kataoha M, Kishiye T, Yamamoto H, Wake K (1991) The intracellular distribution of cell organelles in natural killer cells during the cytolysis of bound tumor cells, with special reference to the rod-cored vesicles. Arch Histol Cytol 54: 69–79

13 Kang Y-H, Carl M, Watson LP, Yaffe L (1987) Immunoultrastructural studies of human NK cells. II. Effector-target cell binding and phagocytosis. Anat Rec 217: 290–304

14 Kelly KL, Cooper EL, Raftos DA (1992) In vitro allogeneic cytotoxicity in the solitary urochordate *Styela clava*. J Exp Zool 262: 202–208

15 Klein E (1976) Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. Int J Cancer 18: 421–431

16 Kupfer A, Demert G, Singer SJ (1985) The reorientation of the Golgi apparatus and the microtubule-organizing center in the cytotoxic effector cell is a prerequisite in the lysis of bound target cells. J Mol Cell Immunol 2: 37–49

17 Linthicum DS, Marks DH, Stein EA, Cooper EL (1977) Graft rejection in earthworms: an electron microscopic study Eur J Immunol 7: 871–876

18 Linthicum DS, Stein EA, Marks DS, Cooper EL (1977) Electron-microscopic observations of normal coelomocytes from the earthworm *Lumbricus terrestris*. Cell Tissue Res 185: 315–330

19 Lodes MJ, Connors VA, Yoshino TP (1991) Isolation and functional characterization of snail hemocyte-modulating polypeptide from primary sporocysts of *Schistosoma mansoni*. Molec Biochem Parasit 49: 1–10

20 Luquet G, LeClerc M (1983) Spontaneous and induced cytotoxicity of axial organ cells from *Asterias rubens* (Asteroid-echinoderm). Immunol Lett 6: 339–342

21 Porchet-Henneré E, Dugimont T, Fischer A (1992) Natural killer cells in a lower invertebrate, *Nereis diversicolor*. Eur J Cell Biol 58: 99–107

22 Roch P, Cooper EL (1991) Cellular but not humoral antibacterial activity of earthworms is inhibited by Aroclor 1254. Ecotoxicol Environ Safety 22: 283–290

23 Savary CA, Lotzová E (1986) Phylogeny and ontogeny of NK cells. In: "Immunobiology of Natural Killer Cells Vol 1" Ed by E Lotzová, RB Herberman, CRC Press, Boca Raton, FL, chapter 4, pp 45–61

24 Söderhäll K, Wingren A, Johansson MW, Bertheussen K (1985) The cytotoxic reaction of hemocytes from the freshwater crayfish, *Astacus astacus*. Cell Immunol 94: 326–332

25 Steel RGD, Torrie JH (1980) Principles and Procedures of Statistics. McGraw-Hill, New York, 2nd ed

26 Stein EA, Cooper EL (1988) In vitro agglutinin production by earthworm leukocytes. Dev Comp Immunol 12: 531–547

27 Timonen T, Ortaldo JR, Herberman RB (1981) Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 153: 569–582

28 Trinchieri G (1989) Biology of natural killer cells. Adv Immunol 47: 187–376

29 Tyson CJ, Jenkin CR (1974) The cytotoxic effect of haemocytes from the crayfish (*Parachaeraps bicarinatus*) on tumour cells of vertebrates. Aust J Exp Biol Med Sci 52: 915–923

30 Valembois P, Boiledieu D (1980) Fine structure and functions of haemerythrocytes and leukocytes of *Sipunculus nudus*. J Morph 163: 69–77

31 Valembois P, Roch P, Boiledieu D (1980) Natural and induced cytotoxicities in sipunculids and annelids. In "Phylogeny of Immunological Memory" Ed by MJ Manning, Elsevier/North-Holland Biomedical Press, Amsterdam, pp 47–55

32 Valembois P, Roch P, Boiledieu D (1982) Cellular defense systems of the Platyhelminthes, Nemertea, Sipunculida, and Annelida. In "The Reticuloendothelial System: A Comprehensive Treatise Vol 3" Ed by N Cohen, MM Sigel, Plenum Press, New York, pp 89–139

33 Wittke M, Renwrantz L (1984) Quantification of cytotoxic hemocytes of *Mytilus edulis* using a cytotoxicity assay in agar. J Invert Pathol 43: 248–253