LYMPHOCYTE-LIKE FUNCTIONS IN THE SOLITARY ASCIDIAN 'CIONA INTESTINALIS'

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A Thesis Submitted for the Degree of PhD
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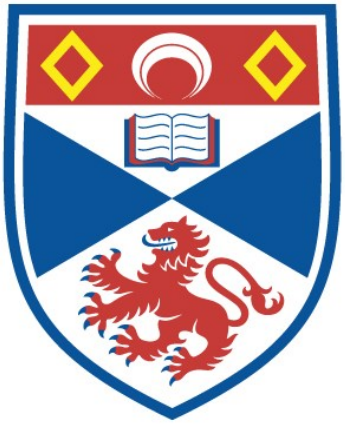
University of St Andrews



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Lymphocyte-like functions

in the Solitary Ascidian,

Ciona intestinalis.

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Submitted for the Degree of Doctor of Philosophy in

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School of Biological and Medical Sciences

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Dedication

To Donald, Nicola and James.

Declaration

I, Clare Peddie, hereby certify that this thesis which is approximately 45,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 9th March, 1995 Signature of Candidate

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Abstract

The blood cells of the solitary ascidian, *Ciona intestinalis*, were examined for lymphocyte-like functions with a view to clarifying the phylogenetic origin of lymphocytes in invertebrates. It was found that cells, present in the circulating blood, mediate the haemolysis of sheep red blood cells, and that a different cell type mediates cytotoxic activity against a range of mammalian tumour cell lines *in vitro*. The blood cells, cytotoxic to mammalian cell lines, were enriched by continuous density gradient centrifugation, and their activity was ameliorated by heat-treatment. Parameters of cytotoxic activity against the target cell line, WEHI, a mouse myelomonocytic leukemic cell (strain 3B), were ascertained by fluorochromasia and the phenomenon was found to be rapid, temperature dependent and sensitive to osmotic conditions. Cytotoxicity was also found to be dependent upon the presence of magnesium and calcium ions, effector to target cell binding, and active metabolic, cytoskeletal and secretory processes in the effector cells. The cytotoxic cells were non-adherent to glass or nylon wool and transmission electron microscope studies of the target-binding cells showed that they were undifferentiated, with a high nucleus to cytoplasm ratio, containing a few large mitochondria, some profiles of rough endoplasmic reticulum and many free ribosomes. In addition, TEM studies revealed close inter-digitation of the effector and target cell membranes and evidence of secretory activity within the effector cell, in the area of target cell binding. The effector cell population was cultured *in vitro* and proliferation in response to concanavalin A, phytohaemagglutinin-B, lipopolysaccharide, or allogeneic leucocytes, measured by the uptake of tritiated thymidine, showed that these cells respond to both T and B cell mitogens and exhibit a mixed leucocyte reaction. In addition, the culture of pharyngeal explants and the measurement of cytotoxic activity by cells migrating from the explants indicates that the cytotoxic cells originate in a thymus-like haemopoietic tissue. Therefore, the undifferentiated blood cells of *C. intestinalis*, possess functional and morphological properties consistent with phylogenetic precursors of vertebrate lymphocytes.

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List of Abbreviations

^{3}H-TdR	Tritiated thymidine
CCM	Cell culture medium
CFDA	5-carboxyfluorescein diacetate
Con A	Concanavalin A
CPM	Counts per minute
DMSO	Dimethyl sulfoxide
E:T	Effector to target cell
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneguanyltetraacetic acid
LPS	Lipopolysaccharide
LTSEM	Low temperature scanning electron microscopy
MAC	Marine anticoagulent
MS	Marine saline
MSD	Marine saline (calcium and magnesium depleted)
MSI	Marine saline (low salt)
NK	Natural killer cell
PHA-B	Phytohaemagglutinin
SI	Stimulation Index
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
WEHI (3B)	Walter and Eliza Hall Institiute, cell line 3B

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Chapter Five

UC	Undifferentiated cell
GA	Granular amoebocyte
HA	Hyaline amoebocyte
VA	Vacuolar amoebocyte
MC	Morula cell
SC	Signet ring cell
VGC	Violet granular cell
VG	Violet granule
AHA	Aggregating hyaline amoebocytes
PC	Pigment cell

Chapter Six

E	Cytotoxic effector cell
T	Target cell (WEHI 3B)

Chapter Seven

A	Undifferentiated blood cell
B	Hyaline amoebocyte
C	Undifferentiated cell with mitotic figure
D	Cell in active DNA synthesis

Chapter Eight

TB	Transverse bar
PC	Pigment cell
HN	Haemopoietic nodule
A	Atypical haemocytes
B	Undifferentiated cell
C	Signet ring cell
UC	Undifferentiated cell

Chapter One
General Introduction

1.1. Introduction

In the absence of a fossil record, the evolution of the immune system can be investigated only by studying mechanisms from the comparative viewpoint. Through the examination of extant species, we may learn the missing links in the evolutionary development of vertebrate defence systems. Broadly, this thesis is concerned with the question of where the evolutionary origins of lymphocytic function lie and examines this question from within the discipline of comparative immunology.

In 1989, Anderson *et al.* suggested that the specific recognition systems of vertebrates may be evolutionary recent additions to pre-existing, non-clonal systems of host defence such as the natural killer cell system. Previously, it had often been suggested that T-cell mediated immune function might have preceded the emergence of the vertebrates (Hildemann and Reddy, 1973; Cooper, 1980), and the lower deuterostomes have often been considered as ideal experimental systems in which functional T-cell/MHC recognition could be studied in the absence of B-cells and immunoglobulin (Marchalonis, 1977).

The idea that T-cell immunity arose in deuterostome invertebrates (echinoderms and tunicates), based on cellular rejection phenomena, has recently become the target of severe criticism (Klein, 1989; Smith and Davidson, 1992). Klein (1989) considers that invertebrate cells referred to as 'lymphocytes' are completely unrelated to true vertebrate lymphocytes, and believes that true lymphocytes will be shown to be a vertebrate (chordate?) invention. Correspondingly, invertebrate immune responses will prove to be based completely upon induction of protein synthesis through feedback pathways (Klein, 1989). In a recent review, Smith and Davidson (1992) suggested that invertebrate deuterostomes do not use primitive versions of the allorecognition systems

of fish and tetrapods, but rely instead upon completely unrelated recognition of foreign cellular adhesion molecules. In reply, Raftos and Raison (1992) partly support the views of Smith and Davidson (1992), but point out that such theories do not preclude the possible existence of building blocks for adaptive immunity, such as allorecognition, cell mediated cytotoxicity, and proliferative mixed leucocyte reactions, in invertebrate systems.

It is generally thought that all vertebrates have the capacity to generate diverse sets of immunoglobulins in response to foreign antigen (Marchalonis and Schulter, 1994). Whilst many invertebrates show quasi-inducible cellular or humoral responses following stimulation, these reactions are considered by some to be unrelated to vertebrate-type immunity (Marchalonis and Schulter, 1994). The echinoid axial organ cells have been reported to produce membrane-bound and secreted molecules whose specificity and size resembled vertebrate immunoglobulin (Delmotte *et al.*, 1986). However, a recent detailed study by Larson and Bayne (1994), revealed evidence that immunoglobulin did not evolve until after the echinoderm and chordate ancestors diverged. To date, no true antibody response has been detected in the invertebrates and, although molecules containing Ig domains are found in insects, e.g. fasciclin II and amalgam (Harrelson and Goodman, 1988: Seeger *et al.*, 1988) and in other invertebrates (Schulter *et al.*, 1994), they do not act as functional true immunoglobulins (Schulter *et al.*, 1994). Thus, the rearranging immunoglobulin system seems to have arisen rapidly at the time of the emergence of the vertebrates from the protochordates (Schulter *et al.*, 1994). Some authors have claimed that the definitive solutions to the question of the origin of adaptive immune responses must come from the characterisation of recognition molecules and the analysis of gene arrangements (Marchalonis and Schulter, 1994). These authors suggest that, because of the extremely low protein levels for cyclostomes and tunicates, this task has to be performed using a combination of protein immunochemistry and

recombinant DNA technology (Marchalonis and Schulter, 1994). Certainly, a molecular phylogenetic approach has value, however, a more comprehensive insight into the origins of the vertebrate immune system may be achieved by combining it with observations of cellular immune function.

Functionally, lymphocytes are the primary effector cells of vertebrate immunity. They are cytotoxic (cytotoxic T-cells, NK cells), proliferate in response to certain stimuli (e.g. mitogens or allogeneic leucocytes) (T- and B- cells), produce immunoglobulins (B-cells only) in response to antigen, and interact with macrophages in the regulation of immunity (Roitt *et al.*, 1993). Primitive effector systems may have consisted of NK-like cells with multiple, non-clonally distributed recognition mechanisms (Janeway, 1989). Although functional and morphological characteristics of the lymphocyte may have evolved before antibody-production and be significant in terms of the evolutionary development of the immune system, very little is known about lymphocyte-like function in invertebrates. The present study is concerned with the evolutionary development of two aspects of lymphocyte function, cytotoxicity and cell proliferation.

1.2. Cell-mediated cytotoxicity.

1.2.1 Definition of cell-mediated cytotoxicity.

Cytotoxicity, the killing of foreign cells by immune cells, is an important process in resistance against viruses, certain other pathogens, grafts and neoplastic growths (Roitt *et al.*, 1993). Non-specific antibody-independent cytotoxic activity (i.e. requiring no prior sensitization), against mammalian tumour cells *in vitro*, is well documented for many vertebrate species (see review by Evans and Cooper, 1990) and recently research

has been directed towards determining the phylogenetic origins of this fundamental immune response (see review by Evans and Cooper, 1990).

1.2.2. Techniques for measuring cell-mediated cytotoxicity

The ability to assay and quantify cytotoxicity has been one of the essential tools in the study of effector cell function of the immune system. It is measured *in vitro* by presenting possible effector cells with targets for lysis. Various methods have been developed to assess cellular mediated cytotoxicity; those relating to target cells include DNA fragmentation by ^{125}IUdR release (Hughes *et al.*, 1964) and the ^{51}Cr release assay (Brunner *et al.*, 1968). This latter, frequently used, assay entails the incubation of the target cells with ^{51}Cr, which is taken up into the cells and binds to protein (Brunner *et al.*, 1968). The target cells are then washed and co-cultured with the effector cells for 4-16 hours, before removing the supernatant and determining ^{51}Cr released from the lysed cells in a liquid scintillation counter (Brunner *et al.*, 1968).

A number of alternative methods, based on the use of fluorescent dyes, are available (Provinciali *et al.*, 1992). In particular, carboxyfluorescein and its derivatives have been thoroughly investigated as target cell labels in quantifying cell mediated cytotoxicity (Bruning *et al.*, 1980, Provinciali *et al.*, 1992). Carboxyfluorescein diacetate (CFDA) passively crosses the target cell membrane and is converted by intracellular esterases to a polar fluorescent product that is retained only by cells with intact plasma membranes (Bruning *et al.*, 1980). Dead or dying cells with compromised membranes rapidly leak the dye allowing quantitative assays of cell viability (Bruning *et al.*, 1980). The use of CFDA allows for rapid labelling of the target cells, fast measurement of activity, direct visual quantification of labelling and enables the use of small sample volumes (Wierda *et*

al., 1989). In addition to these significant advantages of economy, safety and processing time, results have demonstrated that the measurement of CFDA retained by target cells (fluorochromasia) represents a method comparable to the ^{51}Cr release assay (Provinciali *et al.*, 1992).

Clonogenic potential/ colony forming efficiency provides unequivocal quantitative measurement of target cell survival in terms of replicative ability (Kluin-Nelemans *et al.*, 1989). Soft agar in cell culture medium immobilises proliferating cells, thereby promoting the formation of discrete colonies which may be visualised by inverted microscopy (Kluin-Nelemans *et al.*, 1989). The cloning efficiency of a given cell line is reduced by toxic or inhibitory factors, or enhanced by the presence of cytokines or colony promoting factors (Kluin-Nelemans *et al.*, 1989). Clonogenicity assays are therefore a measure of not only cytolysis, but also, inhibited proliferation (Kluin-Nelemans *et al.*, 1989).

Some studies of the invertebrate cytolytic system have been based on the ability of the effector cells to lyse erythrocytes *in vitro* (Ratcliffe *et al.*, 1985). This haemolytic activity is measured by incubating the effector cells with either xenogeneic or allogeneic erythrocytes and measuring the amount of released haemoglobin spectrophotometrically (Parrinello *et al.*, 1993). Haemolytic activity is expressed as the percentage of released pigment of the maximum possible release measured by freeze/thaw cycles or incubation in distilled water. In vertebrates, haemolytic activity is not considered a measure of contact dependent cell-mediated cytotoxic activity but of the humoral complement system (Roitt *et al.*, 1993).

1.2.3. Cytotoxicity and cytotoxic cells in mammals.

Mammals possess a number of naturally occurring blood cell types that have cytotoxic properties. These include cytotoxic T-cells, natural killer (NK) cells and lymphokine-activated killer cells (Roitt *et al.*, 1993). Antibody-independent spontaneous extracellular killing is primarily a function of the NK cells (Roitt *et al.*, 1993). Other mechanisms of nonphagocytic killing require stimulation either by antigen for antibody-dependent cell-mediated cytotoxicity, or by lymphokines for non-specific macrophage activation (Roitt *et al.*, 1993). The precise mechanism of cytotoxic killing is still the subject of great debate (see review by Taylor and Cohen, 1992), although research has revealed dependence upon effector to target cell binding, intact secretory apparatus, divalent cations, and a functional cytoskeleton (Carpén *et al.*, 1981). Studies on human neutrophil mediated cytotoxicity have linked the generation of reactive oxygen metabolites with antibody-dependent target cell destruction (Dongrong *et al.*, 1993). However, antibody-independent natural killer cell activity does not appear to be mediated by superoxide radicals or hydrogen peroxide (Duwe *et al.*, 1985).

In phylogenetic terms, the most primitive cellular mediator of cytotoxic activity found in the higher vertebrates is the NK cell (Anderson *et al.*, 1989). These cells do not require activation by antibody-dependent processes and are the first line of defence against tumour development *in vivo* (Roitt *et al.*, 1993).

1.2.4. Cytotoxicity and cytotoxic cells in lower vertebrates

Lymphocytes develop in the thymus and spleen of amphibians and reptiles, and additionally in the head kidney of fish, which are homologous to their mammalian

counterparts (Warr, 1981). Distinguishable populations of cytotoxic T-cells have been described in all lower invertebrates, including fish, although little information is available for hagfish and lampreys (Manning, 1994).

Non-specific cytotoxic activity against xenogeneic tumour targets by small NK-like cells has been described for several species of anuran amphibians (Ghoneum *et al.*, 1990). As in higher vertebrates, activity in anurans requires effector to target cell binding and is dependent upon the presence of divalent cations (Ghoneum and Cooper, 1987; Ghoneum *et al.*, 1987). Furthermore, TEM studies have revealed similarities between frog and mammalian NK with respect to cell structure and intracellular changes following target cell binding (Smith *et al.*, 1988).

In teleost fish, cytotoxic activity towards a range of normal and transformed cell lines from both fish and mammals is mediated by lymphoid-type cells, called non-specific cytotoxic cells (NCC) (Graves *et al.*, 1984). As with mammalian NK cells, NCC have requirements for electron transport systems, effector cell motility, Mg^{2+} and Ca^{2+} (for binding and cytolysis respectively) and intact secretory apparatus (Carlson *et al.*, 1985). Fish NCC are small, with a high nuclear/cytoplasmic ratio, margination of nuclear chromatin, a prominent polarized Golgi apparatus, and no cytoplasmic granularity (Evans and McKinney, 1990). Salmonid NCCs have been shown to be non-adherent to nylon wool (Hayden and Laux, 1985; Moody *et al.*, 1985), as have those of catfish NCCs (Evans *et al.*, 1984a).

By contrast, elasmobranchs have two cytotoxic cell populations; macrophage-like cells, which exhibit spontaneous cytotoxicity requiring no *in vitro* activation, and the effectors of antibody dependent cell-mediated cytotoxicity which are probably non-phagocytic granulocytes (see review by Evans and McKinney, 1990). The cellular mediators of

spontaneous cytotoxicity in sharks are glass adherent, macrophage-type cells (McKinney *et al.*, 1986).

Unfortunately, little is known about cytotoxic activity in lampreys and hagfish, although, both these animals possess circulating lymphocytes, produce antibody, develop delayed hypersensitivity reactions and reject skin homografts (Finstad and Good, 1964; Manning, 1994)

Thus it appears that non-specific cytotoxic activity is probably a functional feature of lymphocyte populations for all vertebrate groups. Analysis of this response in invertebrates could be used to ascertain the phylogenetic emergence of cytotoxic lymphocytes.

1.2.5. Cytotoxicity and cytotoxic cells in non-chordates

Because non-specific cytotoxic activity has been detected in virtually all species of vertebrates examined, the phylogenetic emergence of the cytotoxic cells from the invertebrates has attracted much interest (see review by Cooper, 1980). In colonial animals (e.g coelenterates or compound ascidians) cytotoxic reactions serve to maintain the integrity of adjacent colonies (Rinkevich, 1992; Rinkevich *et al.*, 1994), while in solitary invertebrates, cytotoxic processes by circulating haemocytes have been likened to vertebrate immune surveillance systems (Valembois *et al.*, 1980; Cooper, 1981).

Table 1.1. Studies of cytotoxic activity in invertebrates

Effector cells	Target cells	Assay system	Reference
Non-marine invertebrates			
Eisenia f. andrei & *Eisenia f. foetida*	Allogeneic leucocytes	^{51}Cr release	Valembois *et al.*, 1980
Parachaeraps bicarinatus	Erlich ascites, Krebs II ascites, & HeLa cells	^{51}Cr release	Tyson & Jenkin, 1974
Astacus astacus	P815, K562, YAC-1, RAJI	^{51}Cr release	Söderhäll *et al.*, 1985
Planorbarius corneus	K562	^{51}Cr release	Francheschi *et al.*, 1991
Biomphalaria	Sporocysts	TEM	Bayne *et al.*, 1980
Marine invertebrates			
Strongylocentrotus droebachiensis	Allogeneic and xenogeneic phagocytes	^{51}Cr release	Bertheussen, 1979.
Styela calva	Allogeneic and	Eosin-Y staining	Kelly *et al.*, 1992
Halocynthia roretzi	xenogeneic haemocytes	LM of cell lysis	Fuke & Numakunai, 1982
Ciona intestinalis	Rabbit, human, guinea pig and sheep erythrocytes	Spectrophotometry of hemolysis	Parrinello *et al.*, 1993
Asterias rubens	MBL2 & SP2 mouse tumour cells	^{51}Cr release	Luquet & Leclerc, 1983
Megathura crenulata, *Pisaster giganteus* & *Glycera* sp.	P815 & PA3B tumour cells	^{51}Cr release	Decker *et al.*, 1981
Nereis diversicolor	Human, mouse and sheep erythrocytes	LM, TEM, SEM	Porchet-Henneré *et al*, 1992
Siphonostoma arcassanense & *Sipunculus nudus*	Allo & xenogeneic sipunculid erythrocytes	Trypan blue exclusion	Boiledieu & Valembois, 1977
Corbicula fluminea	Human erythrocytes	Plaque assay	Yoshino, 1988
Mytilus edulis			Wittke & Renwrantz, 1984

Blood cell mediated cytotoxic responses *in vitro* have been reported in many phyla and sub-phyla : Sipunculoidea (Valembois *et al.*, 1980; Boiledeau and Valembois, 1977a), Annelida (Valembois *et al.*, 1980; Decker *et al.*, 1981; Porchet-Hennére *et al.*, 1992) Mollusca (Wittke and Renwrantz, 1984; Decker *et al*, 1981; Francheschi *et al.*, 1991; Bayne *et al.*, 1980) Arthropoda (Tyson and Jenkin, 1974; Söderhäll *et al.*, 1985), Echinodermata (Bertheussen, 1979; Decker *et al.*, 1981), and Urochordata (Kelly *et al.* 1992; Parrinello *et al.*, 1993) (Table 1.1). However, it is not known whether invertebrate haemocytes recognise and destroy foreign cells in a manner similar to the cytotoxic effects exerted by vertebrate NK cells.

Most spontaneous cytotoxic cells in invertebrates, where characterised, have been shown to be amoebocytic, and sometimes phagocytic. For example, Bertheussen (1979) found that the effector cells in the echinoid, *Strongylocentrotus droebachiensis,* were phagocytic amoebocytes, while Söderhäll *et al.* (1985) demonstrated cytotoxic activity towards mammalian target cells by both the phagocytic semi-granular cells and the non-phagocytic granular cells from the freshwater crayfish, *Astacus astacus*.

Several studies have described the existence of non-specific cytotoxic cells in various invertebrate groups (Table 1.1), but few have investigated killing mechanisms. Certainly, little is known about possible cytotoxic factors in invertebrates, although observations that cytotoxic activity may be inhibited by a variety of carbohydrates in annelids, molluscs and echinoderms, indicate that lectins may be involved (Decker *et al.*, 1981). The most detailed study by Boiledieu and Valembois (1977b), reports that the cytotoxic activity of sipunculid leucocytes *in vitro* requires effector to target cell contact, divalent calcium ions and intact microtubule assemblages. With molluscs, there is also limited evidence that haemocyte-mediated cytotoxicity involves reactive oxygen intermediates and/or the action of lysosomal enzymes (see review by Adema *et al.*,

1991). Additionally, in sipunculids, a phospholipase may be responsible for target cell lysis (Valembois *et al.*, 1980), and a similar enzyme has been implicated in the cytotoxic activity of the haemocytes of the Asian clam, *Corbicula fluminea* (Yoshino, 1988). Unusually, in *Mytilus edulis*, contact between the effector and target cells does not appear to be required, as haemolytic killing seems to be mediated by factors which diffuse into the area of the targets (Wittke and Renwrantz, 1984).

Attempts to enhance cytotoxic reactions in invertebrates by immunization have produced variable results. In sipunculids, tolerance may be induced by previous exposure to the antigen (Valembois *et al.*, 1980), and, in molluscs, it is possible to take non-cytotoxic haemocytes from a strain of *Biomphalaria glabrata*, susceptible towards *Schistosoma mansoni* sporocysts, and to transform them into cytotoxic cells by incubation in cell-free plasma from resistant strains of *B. glabrata* (Bayne *et al.*, 1980). Since the cytotoxic response in *B. glabrata* occurs in the absence of plasma it is likely that the factor involved is cytophilic (Bayne *et al.*, 1980). Luquet and Leclerc (1983) claimed to have induced cytotoxicity against mouse tumour cells in the axial organ cells of *Asterias rubens* after 4 days of culture. However, their results were derived from only 2 out of 20 animals because of high cell culture mortalities (Luquet and Leclerc, 1983).

Many different cell types have been used as targets in studies of cytotoxic activity in invertebrates (Table 1.1). In non-marine invertebrates, the targets normally employed are highly xenogeneic mammalian cell lines and cytolytic activity is measured by ^{51}Cr release (Söderhäll *et al.*, 1985; Tyson and Jenkin, 1974) (Table 1.1). Occasionally, for improved mimicry of the *in vivo* responses, pathogens to the host have been used (Bayne *et al.*, 1980) (Table 1.1). For marine animals, however, it is necessary to avoid problems of osmotic incompatibility between target and effector cells. There are no blood cell lines cultured from marine invertebrates, so researchers have resorted to using

haemolytic activity of xenogeneic or allogeneic erythrocytes as a measure of cytotoxic activity (Boiledeau and Valembois, 1997a) (Table 1.1). Unfortunately, haemolytic activity is not only a measure of cell-mediated activity but also of humoral lytic potential (see section 1.2.2). Others have used simple vital staining or the more complicated ^{51}Cr release from allogeneic and xenogeneic phagocytes (Kelly *et al.*, 1992; Bertheussen, 1979) (Table 1.1). However, full investigation of the mechanisms underlying the cytotoxic activity using allogeneic or xenogeneic phagocytes as targets is prohibitory because of the mutual lysis of both effector and target cells (Fuke and Numakunai, 1982).

Ideal targets for the measurement of cytotoxic activity are thus immunologically inert and highly xenogeneic mammalian cell lines. Two studies have used mammalian target cell lines as targets for marine invertebrate effector cells but have measured activity in medium sub-optimal for effector cell function (Decker *et al.*, 1981, Luquet and Leclerc, 1983). The axial organ cells of the echinoderm, *Asterias rubens*, were found to have cytotoxic activity against a mouse tumour cell line (MBL2) but only at 37°C and after 6 hours co-incubation (Luquet and Leclerc, 1983). Decker *et al.* (1981) also used mammalian target cell lines as targets, although the low osmolality medium was considered by the authors not to be ideal for the marine effector cells. The ^{51}Cr release assay has the drawback of requiring long incubation periods of the target and effector cells in a mammalian saline (see section 1.2.2). Under these conditions, undue stress must inevitably either reduce the optimal functioning of the effector cells or cause the release of cytotoxic metabolites from lysed effector cells not normally involved in cell-mediated lysis.

Due to the lack of suitable assays, the mechanisms and cellular mediators of cytotoxic activity in marine non-chordates is poorly understood. An alternative assay technique is

required for the measurement of cytotoxic activity by marine effectors against mammalian target cells. This must firstly, enable the effectors to function normally and secondly, involve much shorter incubation of the targets in a high osmolality medium.

1.3. Mitogen-induced proliferation and mixed leucocyte reactions

1.3.1. Definition of mitogen-induced proliferation and mixed leucocyte reactions.

In addition to their cytotoxic properties, lymphocytes proliferate in response to antigenic stimulation (Roitt *et al.*, 1993). Lymphocyte activation by antigens or mitogens results in intracellular changes and which leads to either cell proliferation or differentiation into effector or memory cells (Roitt *et al.*, 1993). Antigen-induced lymphocyte proliferation can be visualised *in vitro* by cultivating lymphoid cells with plant or bacterial lectins (phytohaemagglutinin (PHA), concanavalin A (Con A) or lipopolysaccharide (LPS)) or allogeneic cells (Roitt *et al.*, 1993).

1.3.2. Measurement of mitogen-induced proliferation and mixed leucocyte reactions.

Lymphocytes are enriched from peripheral blood, washed, and cultured with a suspension of mitogen or allogeneic cells (Roitt *et al.*, 1993). In response, the lymphocytes enter S-phase and incorporate precursors of DNA synthesis (Roitt *et al.*, 1993). Tritiated thymidine (^{3}H-TdR) is added to the cultures and, after 16 hours, the cells are harvested onto glass fibre filter discs (Roitt *et al.*, 1993). The ^{3}H-TdR,

incorporated into the synthesized DNA of the nucleus of the replicating cell, is measured by liquid scintillation counting (Roitt *et al.*, 1993). A high count indicates that the lymphocytes have undergone transformation and confirms their sensitivity to the mitogen (Roitt *et al.*, 1993).

1.3.3. Mitogen-induced proliferation and mixed leucocyte reactions in mammals.

Both T- and B- lymphocytes are activated when they bind antigen or mitogens in the presence of accessory cells (Roitt *et al.*, 1993). These activated lymphocytes then proliferate and mature to give rise to expanded specific cell clones which either differentiate terminally into effectors, or give rise to memory cells (Roitt *et al.*, 1993). Thus, proliferation serves to expand the cytotoxic cell population *in vivo* (Roitt *et al.*, 1993). T-cells are also stimulated to proliferate in response to cells carrying foreign histocompatibility class II antigens (mixed lymphocyte reaction - MLR) (Roitt *et al.*, 1993). For this to occur, the two types of cells must differ within the major histocompatibility complex (MHC) namely the I region in mouse or the HLA-D region in humans (Roitt *et al.*, 1993). Analogous MHC systems have been found in all mammalian species studied so far (Roitt *et al.*, 1993).

1.3.4. Mitogen-induced proliferation and mixed leucocyte reactions.in lower vertebrates.

Proliferation of lymphocyte populations in response to mitogens and mixed leucocyte reactions has been described in all lower vertebrates including reptiles (see Jurd, 1994),

amphibians (see Horton, 1994) and teleost fish (see Manning, 1994). No evidence has been presented for proliferation of the NCC of teleost fish, nor to indicate that proliferation of the lymphocytes produces functionally cytotoxic cells.

1.3.5. Mitogen-induced proliferation and mixed leucocyte reactions in non-chordates.

Mitogen or mixed leucocyte induced proliferative responses have only been found in a few non-chordate species. Proliferation of earthworm (*Lumbricus terrestris*) coelomocytes *in vitro* has been demonstrated following treatment with PHA or Con A (Roch *et al.*, 1975; Toupin and Lamoureaux, 1976). This phenomenon has been linked to *in vivo* proliferation of coelomocytes in response to wounding or grafting (Valembois and Roch, 1977). A similar mixed leucocyte-like response has been demonstrated in *Eisenia fetida* (Valembois *et al.*, 1980). This response was shown for coelomocytes that are also involved in graft rejections, cytotoxic reactions or mitogenic stimulations (Valembois *et al.*, 1980).

Cells found in the axial organ of the starfish, *Asterias rubens*, proliferate when treated with Con A, pokeweed mitogen, or lipopolysaccharide extract from *Salmonella typhimurium* (Brillouet *et al.*, 1981). These authors further found that the proliferative population could be sub-divided, in terms of adherence to nylon wool, into adherent cells stimulated only by LPS (B-like) and non-adherent cells stimulated only by Con A (T-like) (Brillouet *et al.*, 1981). This is the only report of a heterogeneous proliferative population in an invertebrate.

1.3.6. Lymphopoiesis in vertebrates.

Primary lymphoid organs are the major sites of lymphopoiesis (Roitt *et al.*, 1993). Here, lymphocytes differentiate from lymphoid stem cells, proliferate and mature into functional cells (Roitt *et al.*, 1993). Secondary lymphoid organs, such as the spleen and lymph nodes, are areas where lymphocytes interact with each other and with antigens (Roitt *et al.*, 1993). In mammals, T-lymphocytes are produced in the thymus, and B-lymphocytes in the bone marrow and foetal liver (Roitt *et al.*, 1993). Teleost fish lack bone marrow and lymph nodes but possess a well-developed thymus (Roitt *et al.*, 1993). However, in lampreys and hagfish the lymphopoeitic tissue has not yet been located..

1.4. Ascidians

1.4.1. The phylogenetic significance of ascidians

The present study looks for precursors of vertebrate lymphocytic function in ascidians, because anatomical, embryological and molecular phylogenetic studies place these invertebrates close to the origin of the vertebrate line (Bone, 1979, Crowther and Whittaker, 1992, Field *et al.*, 1988, Conway-Morris, 1993). As the larvae of ascidians have certain features, (e.g. a notochord, a dorsal nerve cord and gill slits) in common with chordates, they were initially classified as members of the phylum Chordata (Drach, 1948; Katz, 1983). The ascidian tadpole was first described histologically by Kowalevsky (1866), and subsequent workers recognise it as preserving the body plan from which the vertebrates may have evolved by paedomorphis (Garstang, 1928; Berrill, 1955; Bone, 1960; 1981). By contrast, recent studies of the sequences of 18s

rDNA from invertebrates and vertebrates by Wada and Satoh (1994) support the proposal by Tokioka (1971) that the ancestors of chordates were free-living animals from which cephalochordates and vertebrates evolved directly. In the chordate lineage, larvaceans, with a lower tendency towards pelagic life, emerged first, followed by the emergence of sessile ascidians and pelagic salps (Wada and Satoh, 1994). Whilst the exact sequence in which the vertebrates evolved remains in some doubt, there is no debate that the ascidians are phylogenetically closer to the origin of the vertebrates than any other major invertebrate group.

1.4.2. Biology of Ascidians

The class Ascidiacea is placed within the phylum Chordata. Ascidians are all marine. The adult is sessile and may take either a compound or solitary form. The larvae are motile and may exist for a few hours to several days in the water column (Svane and Havenhand, 1993). In the adult form, movement is restricted to the opening and closing of apertures and retraction of the body (Millar, 1971).

The adult has no coelom, shows no segmentation and possesses no bony tissues (Millar, 1971). The external surface comprises a tunic, which contains cellulose, protein and inorganic compounds which varies in consistency from gelatinous to fibrous (Millar, 1971). Within the tunic there are two distinct body regions: the abdominal region containing the heart, guts and gonads, and the anterior pharyngeal region containing the pharyngeal basket (Millar, 1971). The central nervous system is degenerate, being reduced to a solid ganglion with radiating nerves located between branchial and atrial apertures (Millar, 1971).

Most ascidians feed by filtering plankton from the seawater into the pharyngeal basket (Hayward and Ryland, 1990). Water enters through the oral siphon, passes across the pharynx to the atrium, and then exits through the atrial siphon (Hayward and Ryland, 1990). There are several subdivided gill clefts made up of external longitudinal bars in the pharyngeal region (Laverack and Dando, 1987). The heart is composed of a single layer of myoepithelial cells that propels blood through two large vessels exiting from each end of the heart and branching throughout the body and tunic (Laverack and Dando, 1987). The circulation is not continuous since the heart periodically reverses the direction of flow (Laverack and Dando, 1987). The beat is peristaltic and is governed by pacemakers positioned at each end of the heart (Laverack and Dando, 1987). The blood circulates through a network of vessels which are seldom walled, and therefore are more properly considered as open channels and lacunae which run through the connective tissue (Hayward and Ryland, 1990).

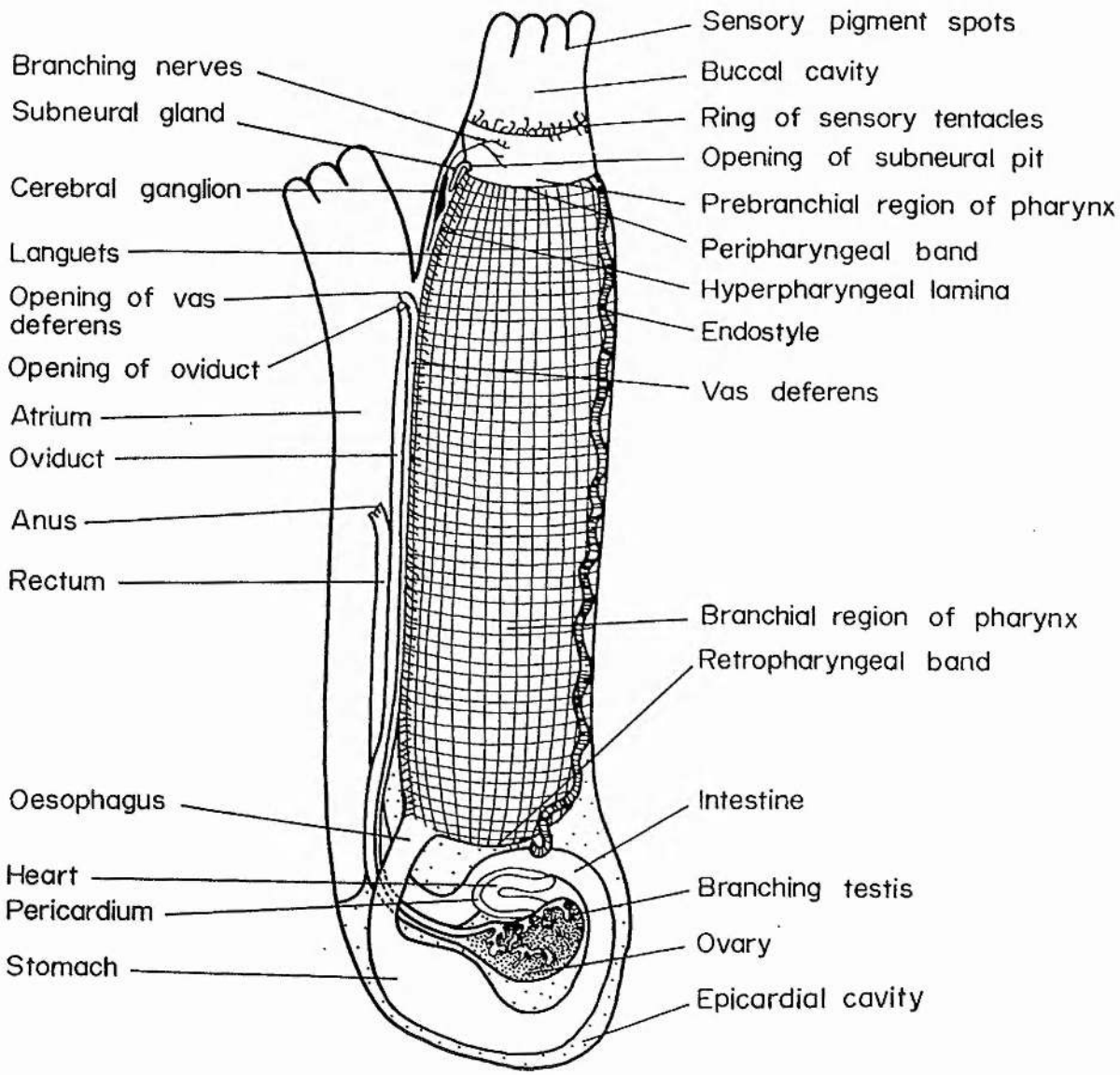
1.4.3. The biology of the solitary ascidian, *Ciona intestinalis*

The solitary ascidian,*C. intestinalis,* is a member of the family Cionidae, sub-order Aplousobranchia, order Enterogona, class Ascidiacea, subphylum Tunicata (=Urochordata), phylum Chordata (Hayward and Ryland, 1990). The test is soft and translucent and the cylindrical body reaches 100-150 mm long (Hayward and Ryland, 1990). Specimens are normally pale yellow-green, gelatinous and contractile (Hayward and Ryland, 1990). Five longitudinal muscle bands are clearly visible in the mantle in each side (Hayward and Ryland, 1990). The apertures of the siphons often have bright yellow margins, ca 70 oral tentacles and small red pigment spots between the lobes; eight marginal lobes on the oral siphon and six on the atrial siphon (Hayward and Ryland, 1990). *C. intestinalis* is oviparous and generally spawns either freely into the water column or in mucus strings, which are particularly adhesive (Svane and

Figure 1.1. Underwater photograph of the solitary ascidian, *Ciona intestinalis*.

This photograph was taken in February, 1995. *C. intestinalis* is the most prevalent species amongst the epifouling community found on floating pontoons at Croab Haven. Other species present include *Ascidiella aspersa*, *Antedon bifida*, *Sabellid* worms, bryozoans, encrusting sponges and algae.

Figure 1.2. The anatomy of *Ciona intestinalis* (after Laverack and Dando, 1987).



Havenhand, 1993). The eggs are fertilised externally and develop into free-swimming non-feeding larvae (Svane and Havenhand, 1993).

C. intestinalis is widely distributed around British coasts and is found in many parts of the world (Millar, 1970). It may be present in densities of several thousand individuals per m^2 (Svane and Havenhand, 1993), occupying vertical or overhanging underwater surfaces between the lower shore and down to 500 metres (Millar, 1970). Individuals flourish in areas of moderate tidal flow with a good supply of plankton (Svane and Havenhand, 1993). In the shallows, they tend to prefer settlement in shady places, and animals living on substratum exposed to bright sunlight tend to develop orange pigmentation (personal observations). The filtration rate of *C. intestinalis* is very high (approx. 2 litres per hour for a 2 g animal, dry weight), and ecological studies have estimated that dense populations of *C. intestinalis* have important grazing impacts upon phytoplankton blooms in late summer-early autumn in shallow seas (Petersent and Riisgård, 1992).

The family Cionidae are thought to be the most 'primitive' surviving group of ascidians (Berrill, 1936) and are, therefore, especially well suited to the subject of the present thesis. Moreover, *C. intestinalis* is an ideal model for immunological studies amongst the solitary ascidians because it easy to keep in aquaria and it yields large volumes of cell-rich blood . The circulating blood cells have been characterised by light and transmission electron microscopy (Rowley, 1981, 1982a,b), and separated blood cell populations can been obtained by continuous density gradient centrifugation (Smith and Söderhäll, 1991; Smith and Peddie, 1992).

1.4.4. Blood cell classification in ascidians.

Literature describing the morphology and function of ascidian blood cells is extensive and, at times, confusing. Since the publication of 'Invertebrate Blood Cells' (Ratcliffe and Rowley, 1981) in which Wright (1981) gave a detailed classification of ascidian blood cells and their function, many papers have appeared offering alternative comprehensive classifications (for example De Leo, 1992; Sawada *et al.*, 1993; Fuke and Fukumoto, 1993). So many differing classifications may have been proposed because of differences in blood cell profile between species and, also, because of differences in maturation, season and reproductive state within species. In addition, researchers have differed in their choice of fixation methods and examination techniques (light microscopy of fixed and stained cells, phase contrast microscopy of living cells, SEM and TEM). Consequently, the situation is highly confused and a comprehensive classification of the blood cells of ascidians has yet to be achieved. Table 1.2, lists all the papers published since Wright's review in 1981, together with a few earlier significant papers. In the present study, where suitable, terminology used in Wright's review (1981) has been maintained.

Table 1.2. Blood cell classification in ascidians.

Characteristics	Terminology	Genus/species	Reference
Cell type 1	*Undifferentiated cell*		
Size 5-7 μm in diameter	Lymphocyte-like cell	*Botryllus schlosseri*	Ballarin *et al.*, 1993
Large nucleus central,		*Ascidia malaca*	Scippa *et al.*, 1987
with large amounts of		*Ascidia malaca*	Scippa *et al.*, 1982
heterochromatin, often a		*Halocynthia roretzi*	Fuke & Fukumoto, 1993
prominent nucleolus.		*Botryllus schlosseri*	Ballarin *et al.*, 1993
Cytoplasm basophilic		*Phallusia mamillata*	Cammarata *et al.*, 1993
and homogeneous poorly		*Pyura stolonifera*	Warr *et al.*, 1977
differentiated, free	Lymphocyte	*H. roretzi*	Overton, 1966
ribosomes, Golgi		Review	Wright & Ermak, 1982
cisternae present, few		Review	Wright, 1981
large mitochondria,	Haemoblast	Review	Wright, 1981
vesicles rarely visible,		*Perophora viridis*	Milansei & Burighel, 1978
short profiles of rough	LLC hemoblast	*B. schlosseri*	Sawada *et al.*, 1993
endoplasmic reticulum.	Haemocytoblast	*Styela clava*	Ermak, 1975
No granules or lysosome-		*C. intestinalis*	Mukai & Watanabe, 1976
like bodies. High nuclear	Stem cell	*C. intestinalis*	De Leo, 1992
: cytoplasmic ratio		*C. intestinalis*	Rowley, 1982b
		C. intestinalis	Rowley *et al.*, 1984
	Lymphoid cells	*H. roretzi*	Zhang *et al.*, 1992
		H. roretzi	Ohatke *et al.*, 1994

Cell type 2	*Hyaline amoebocyte*		
Size 6-12 μm in diameter.	Hyaline amoebocytes		
Nucleus central and round,	& small amoebocytes	*H. roretzi*	Fuke & Fukumoto, 1993
possible nucleolus	Hyaline amoebocyte	*B. schlosseri*	Ballarin *et al.*, 1993
Abundant cytoplasm		*C. intestinalis*	Rowley, 1981
divided into two zones,	Non-vacuolar hyaline	'	
inner zone containing	amoebocytes	*C. intestinalis*	Rowley, 1982b
rough E. R., a few small	Hyaline cells	*S. clava*	Sawada *et al.*, 1993
vesicles and/or small		*P. mamillata*	Cammarata *et al.*, 1993
granules, Golgi complex,	Amoebocyte-like cell	*A. malaca*	Scippa *et al.*, 1982
few mitochondria,	Clear granulocyte	*C. intestinalis*	De Leo *et al.*, 1987
ectoplasm devoid of	Hyaline leucocytes	Review	Wright & Ermak, 1982
organelles but containing			Wright, 1981
a few microtubules and	Phagocytes	*H. roretzi*	Zhang *et al.*, 1992
microfilaments, amoeboid	Small granular		
characteristics,phagocytic.	amoebocyte	*H. roretzi*	Ohatke *et al.*, 1994
Cell type 3	*Granular amoebocyte*		
Size 5-10 μm in diameter	Granular amoebocytes	*H. roretzi*	Fuke & Fukumoto, 1993
Nucleus central or		*P. viridis*	Overton, 1966
eccentric. Granules round		*C. intestinalis*	Rowley, 1982b
or rod-shaped. Widely			Rowley, 1981
distributed vacuoles and		*B. schlosseri*	Ballarin *et al.*, 1993
small smooth vesicles	(large/dense)	*H. roretzi*	Ohatke *et al.*, 1994
both filled with loose or	Microgranular	*B. schlosseri*	Milansei & Burighel, 1978
fibrular material. Scarce	amoebocyte	*P. mamillata*	Cammarata *et al.*, 1993
mitochondria. Occasional	Microgranulocye	*C. intestinalis*	De Leo *et al.*, 1987
cytoplasmic extensions.	Basophilic		
Vanadium accumulating	granulocytes	*S. clava*	Sawada *et al.*, 1993
stored in the vacuoles..	Granulocytes	*P. stolonifera*	Warr *et al.*, 1977
Amoeboid and phagocytic	Granular leucocytes	*H. roretzi*	Zhang *et al.*, 1992
		Review	Wright & Ermak, 1982
		Review	Wright, 1981

Cell type 4	*Vacuolar amoebocyte*		
Size 5-12 μm in diameter	Phagocyte	*P. viridis*	Overton, 1966
Central.nucleus.	Vacuolated cells	*H. roretzi*	Fuke & Fukumoto, 1993
Cytoplasm containing			Ohatke *et al.*, 1994
several large & small	Vacuolar hyaline	*C. intestinalis*	Rowley, 1982b
vacuoles, empty or	amoebocytes	*B. schlosseri*	Ballarin *et al.*, 1993
containing diffuse fibrous	Macrophage	*Ascidia syndeienis*	Michibata *et al.*, 1990
material, small amounts	Compartment cell	*P. mamillata*	Cammarata *et al.*, 1993
of rough endoplasmic		*C. intestinalis*	De Leo *et al.*, 1987
reticulum, occasional	Compartment cell (B)	*H. roretzi*	Zhang *et al.*, 1992
Golgi complexes & a few	Vesicle containing		
vesicles. Phagocytic and	cells	Review	Wright & Ermak, 1982
amoeboid			
Cell type 5	***Morula cell***		
Size 5.-11 μm in	Macrogranular &		
diameter Berry-like cells	globular cells	*H. roretzi*	Fuke & Fukumoto, 1993
(LM). Phase dark	v2	*H. roretzi*	Ohatke *et al.*, 1994
pleomorphic, electron	Morula cells	*B. schlosseri*	Milansei & Burighel, 1978
dense cytoplasmic		*B. schlosseri*	Ballarin *et al.*, 1993
inclusions. Cell		*P. viridis*	Overton, 1966.
subdivision by globule,		*A. malaca*	Scippa *et al.*, 1982
vesicles and vacuole		*A.syndeienis*	Michibata *et al.*, 1990.
confluence. Variable		*P. mamillata*	Cammarata *et al.*, 1993
nucleus, rare nucleolus,		*C. intestinalis*	Rowley, 1981
with a few patches of		Review	Wright, 1981
chromatin in the	Refractile amoebocyte	*C. intestinalis*	Rowley, 1982b
periphery.	Eosinophilic		
	granulocytes	*S. clava*	Sawada *et al.*, 1993
	Globular granulocyte	*C. intestinalis*	De Leo *et al.*, 1987
	Vacuolated cells	*C. intestinalis*	Rowley *et al.*, 1984
		H. roretzi	Zhang *et al.*, 1992
	Compartment cells	*P. viridis*	Overton, 1966
		Review	Wright, 1981

Cell type 6	*Signet ring cell*		
Size 5-8 μm in diameter Highly eccentric nucleus, one round vacuole often containing fibrogranular material, and a thin rim of cytoplasm surrounding the vacuole, mitochondria and ribosomes very rare.	Macrogranular amoebocyte	*B. schlosseri*	Milansei & Burighel, 1978
	Unilocular granulocyte	*C. intestinalis*	De Leo *et al.*, 1987
		P. viridis	Overton, 1966
	Signet ring cells	*B. schlosseri*	Ballarin *et al.*, 1993
		A. syndeienis	Michibata *et al.*, 1990-
		P. stolonifera	Warr *et al.*, 1977
		C. intestinalis	Rowley, 1981
		Review	Wright, 1981
		H. roretzi	Fuke & Fukumoto, 1993
	Vacuolated cells	*H. roretzi*	Ohatke *et al.*, 1994

Cell type 7	*Nephrocyte*		
Size 15-40 μm in diameter Packed with large vacuoles containing numerous small granules, peripheral small nucleus with dense chromatin.	Nephrocyte	*B. schlosseri*	Milanesi & Burighel, 1978
		Review	Wright, 1981
	Large basophilic cells	*H. roretzi*	Fuke & Fukumoto, 1993
	Giant cells	*A. syndeienis*	Michibata *et al.*, 1990.

Cell type 8	*Pigment cell*		
Size 6-13 μm in diameter Highly pigmented (orange/yellow/red/green) cytoplasmic inclusions occupying most/all of the cytoplasm. Nucleus small and condensed often obscured by pigment granules.	Pigment cell	*A. syndeienis*	Michibata *et al.*, 1990.
		P. mamillata	Cammarata *et al.*, 1993
		Review	Wright, 1981
	Orange cell	*P. viridis*	Overton, 1966
		C. intestinalis	Rowley, 1981
	Large granular cells	*H. roretzi*	Fuke & Fukumoto, 1993.

Type 1. Undifferentiated cell.

Undifferentiated cells are found within the haemopoietic tissue as well as in the circulating blood (Ermak, 1982). Morphologically, they are poorly differentiated spherical cells, about 5-7 μm in diameter, with a large central nucleus (Rowley, 1982b). Usually, in the nucleus, a defined nucleolus is present and dense peripheral chromatin (Rowley, 1982b). Characteristically, they have a high nuclear : cytoplasmic ratio, with basophilic, homogeneous cytoplasm (Rowley, 1982b). Vesicles are rarely visible in the cytoplasm, and granules or lysosome-like bodies are absent (Wright, 1981). Instead, the cytoplasm contains numerous free polyribosomes, a few cisternae of rough E.R., short profiles of rough endoplasmic reticulum and a few large elongate mitochondria (Wright, 1981). Occasionally small dense granules are present in the cytoplasm and a pair of centrioles are sometimes located near the Golgi complex (Wright, 1981). Within the haemopoietic tissue, some undifferentiated cells are reported to increase in size, lose the prominent nucleolus and free polyribosomes, develop a granular cytoplasm, a larger Golgi complex, elongate mitochondria, and longer cisternae of rough endoplasmic reticulum (R.E.R.) (Ermak, 1976). Wright (1981) suggested that there are two morphologically different undifferentiated cell types (lymphocytes and haemoblasts) (Table 1.2), one of which lacks a prominent nucleolus. However, Rowley *et al.* (1984), were unable to distinguish between these two groups of cells in *C. intestinalis*.

Type 2. Hyaline amoebocytes.

In the hyaline amoebocytes, the round nucleus is also central and the nucleolus is occasionally present (Rowley, 1982b). The abundant cytoplasm is divided into two zones (Rowley, 1982b). The inner zone contains R.E.R., and is filled with a few small vesicles and/or small granules, a well developed Golgi complex and mitochondria

(Rowley, 1982b). The outer zone is devoid of organelles but contains a few microtubules and microfilaments (Rowley, 1982b). In *Halocynthia roretzi*, mitochondria are seen to be located near the nucleus (Fuke and Fukumoto, 1993). Under phase contrast microscopy, the cytoplasm appears reticulated or clumped (Smith, 1970) and the cell has amoeboid characteristics (Rowley, 1982) (Table 1.2).

Type 3. Granular amoebocyte.

Granular amoebocytes are similar in size to the hyaline amoebocytes but are the only amoebocytic cell which contains cytoplasmic granules (Rowley, 1982b). These granules are round or rod-shaped, and measure 0.2 to 3.5 μm in diameter (Wright, 1981). They have a small central or eccentric nucleus with no nucleolus, and the chromatin is condensed and clumped around the inner margin of the nuclear membrane (Wright, 1981). The cytoplasm contains numerous vacuoles and small, smooth vesicles, both of which are filled with loose or fibrular material (Wright, 1981). Occasionally cytoplasmic extensions are located at the periphery of the cell (Rowley, 1982b). These cells are amoeboid and phagocytic (Smith and Peddie, 1992) (Table 1.2).

Type 4. Vacuolar amoebocytes

The cytoplasm of vacuolar amoebocytes contains several large and small vacuoles which may or may not contain diffuse fibrous material (Rowley, 1982b). In addition, the cytoplasm contains small amounts of R.E.R., occasional Golgi complexes and a few vesicles (Rowley, 1982b). Like the other amoebocytic cells in ascidians, these cells are actively phagocytic (Rowley, 1982b; Smith and Peddie, 1992) (Table 1.2).

Type 5. Morula cells

The morula cells are 6-16 μm in diameter and are distinctive, under phase contrast microscopy, due to the phase dark pleomorphic cytoplasmic inclusions which produce a berry-like appearance (Wright, 1981). The position of the nucleus is variable, it is usually central but it is sometimes eccentric (Wright, 1981). A visible nucleolus is rare, and a few patches of chromatin are often located at the periphery (Wright, 1981). Compartment cells have often been considered as a separate cell type, distinct from the morula cell (Overton, 1966; Wright, 1981), but in the present they have been grouped together (see Table 1.2), as they appear to be structurally similar, differing only in the degree of vacuolation (Rowley, 1982a).

Type 6. Signet ring cells

Signet ring cells are ca. 6 -12 μm in diameter and easily recognised because of their highly eccentric nucleus and the presence of a single, large, round vacuole which often contains fibrogranular material (Wright, 1981). The cytoplasm is reduced to a thin rim surrounding the vacuole (Wright, 1981). Mitochondria or ribosomes are rare (Wright, 1981) (Table 1.2).

Type 7. Nephrocytes

The nephrocytes, have been recorded only in a few species. They are large spherical cells, up to 40 μm in diameter (Wright, 1981). The cytoplasm is packed with large membrane bound vacuoles containing numerous small granules or microfilaments (Fuke and Fukumoto, 1993), and the nucleus is small and peripheral with dense chromatin (Wright, 1981) (Table 1.2).

Type 8. Pigment cells

These cells occur in the blood of some species of ascidians (Table 1.2). They are highly pigmented (blue/orange/yellow/red/green), and ca 6-13 μm in diameter (Wright, 1981). The colour of the cells is due to pigmented cytoplasmic inclusions occupying most, if not all, of the cytoplasm (Wright, 1981). These inclusions are not held within a vacuolar membrane but are embedded directly in the cytoplasm (Wright, 1981). The nucleus is small and condensed and is often obscured by pigment granules (Wright, 1981) (Table 1.2).

The blood cells of C. intestinalis

All of these blood cell types, with the exception of the nephrocyte, have been identified and characterised in *C. intestinalis* (Rowley, 1981, 1982a,b) (see Table 1.2).

1.4.5. Blood cell functions in ascidians.

In Table 1.3., the function of the blood cells in ascidians, as reported by researchers since, and including, the review by Wright (1981) are summarised.

Phagocytosis

Recent investigations have revealed that, in ascidians, phagocytosis is mediated only by the vacuolar and granular amoebocytes (Rowley, 1981; Smith and Peddie, 1992) (Table 1.3). Phagocytic rates are enhanced by the presence of cellular opsonic factors (Smith and Peddie, 1992; Raftos, 1994), which, in *C. intestinalis*, are released by the morula cells *in vitro* (Smith and Peddie, 1992) (Table 1.3). The *in vitro* opsonic activity of the morula cells is reduced by the inclusion of protease inhibitors (Smith and Peddie, 1992).

Other studies have shown that LPS stimulates the release of proteases from the blood cells (of undefined type) of *Halocynthia roretzi*. (Azumi *et al.*, 1991) and that morula cells are the main repositories of phenoloxidase and an associated LPS-sensitive serine protease (Jackson *et al.*, 1993; Jackson and Smith, 1993; Ballarin *et al.*, 1993, 1994) (Table 1.3).

Lectins

Lectins are a class of multivalent carbohydrate-binding proteins, mostly Ca^{2+} dependent, and which are generally assayed as haemagglutinins. The presence of lectins in the haemolymph of ascidians was first reported by Cantacuzène (1919) and, subsequently, many lectins and their properties have been described in the literature with considerable variation reported between species (Taneda *et al.*, 1985; Vasta and Marchalonis, 1987). Examples include the galactose-specific lectins which have been found in *Didemnum candidum* (Vasta *et al.*, 1986), *Ascidia malaca* (Parrinello and Arizza, 1988) and *Halocynthia roretzi* (Yokosawa *et al.*, 1986), while three different lectins have been isolated from the haemolymph of *Botrylloides leachii* (Schulter and Ey, 1989). In addition, the 'compartment cells' of *Phallusia mamillata* have been shown to store alpha-lactose specific cellular lectins in the vacuoles, which are released under *in vitro* conditions (Arizza *et al.*, 1993, Cammarata *et al.*, 1993) (Table 1.3). The evidence for the immunological functions of lectins as agglutinins, opsonins, complement activators and cell-associated recognition molecules in vertebrates is extensive (Vasta *et al.*, 1994). The experimental evidence supporting the immunological role of invertebrate lectins is summarized by Vasta *et al.* (1994), but their homology to vertebrate lectins in terms of function, remains controversial.

Encapsulation

Encapsulation occurs when the foreign material is too large to be phagocytosed. Such responses have been demonstrated experimentally *in vivo* by the insertion into the tunic of glass fragments (Anderson, 1971), or splinters (Metchnikoff, 1892) and by the injection of bacteria (Thomas, 1931) or vertebrate erythrocytes (Wright, 1974, Wright and Cooper, 1975; Parrinello *et al.*, 1977, Parrinello *et al.*, 1984). The process of encapsulation initially involves the signet ring cells and the morula cells, but after two days only the morula cells remain (Anderson, 1971) (Table 1.3). The morula cells form aggregates with strands of tunicin to make up multi-layered structures surrounding the foreign article (Anderson, 1971) (Table 1.3). With injected erythrocytes or bacteria, those that are not phagocytosed within 24 hours are agglutinated into a mass and then encapsulated within 6-10 days (Wright, 1974). A lesion in the tunic eventually forms directly over the capsule through which the capsule and its contents are ejected (Parrinello *et al.*, 1977). Following capsule release, the tunic normally heals (Parrinello *et al.*, 1977). Heightened secondary responses to erythrocyte injection have been recorded, although this response may be due to rapid infiltration of cells or increased morula cell production in response to the primary injection (Parrinello *et al.*, 1977) (Table 1.3).

Vanadium accumulation

Many ascidians are able to concentrate vanadium to several million times the level in sea water (Martoja *et al.*, 1994). Vanadium is accumulated in the reduced state in specialised vacuolar cells which have been termed vanadocytes (Martoja *et al.*, 1994). The term 'vanadocyte' has been loosely associated with any cell type that has been found to accumulate vanadium (Martoja *et al.*, 1994). Most authors agree that vanadium

is stored in three cell types; signet ring cells, compartment cells and morula cells (Robinson *et al.*, 1984) (Table 1.3). However, vanadium-free morula cells have also been reported (Michibata and Uyama, 1990; Hawkins *et al.*, 1980, Rowley, 1982a; Scippa *et al.*, 1985). Vanadium has also been reported in other cell types, e.g. the granular amoebocytes of *C. intestinalis* (Rowley, 1982a) and the pigmented cells of *Ascidia ceratodes* (Anderson and Swinehart, 1991) (Table 1.3). Rowley (1983) observed the entry of vanadium into the cytoplasmic vesicles and the phagosome during the phagocytosis of bacteria and suggested that it may play a role in antimicrobial defence (Table 1.3). However, the biological significance of vanadium-accumulating blood cells in ascidians remains enigmatic, since various other roles, (chemical defence against predation, excretion, tunic formation, or pigmentation) have been proposed (Martoja *et al.*, 1994) (Table 1.3).

Antibacterial activity

Over twenty antimicrobial substances have been extracted from the whole animal bodies of various species of ascidians (see review by Davidson, 1993). However, blood cell mediated antibacterial activity has been detected only in *C. intestinalis* (Johnson and Chapman, 1970; Findlay and Smith, 1995) and *Halocynthia roretzi* (Azumi *et al.*, 1990). The antibacterial activity in *C. intestinalis*, has recently been characterised in more detail. It was found to be effective against both Gram positive and Gram negative organisms, to be present at very low concentrations, to be calcium but not magnesium dependent, temperature stable and to be located predominantly within the morula cells (Findlay and Smith, 1995) (Table 1.3). Azumi *et al.* (1990) also found that the morula cells of *H. roretzi* contained antimicrobial tetrapeptide-like substances, Halocyamine A and B (Table 1.3). Part of the broad spectrum of *in vivo* non-specific inflammatory processes that ascidians use in response to wounding or grafting and during non-fusion

during non-fusion reactions (Reddy *et al.* 1975, Scofield and Nagashima, 1983) (Table 1.3) may involve these antibacterial agents present in the morula cells.

Haemostasis

In the absence of a true clotting mechanism in ascidians, the haemocytes aggregate in response to wounding, thereby arresting bleeding (Takahashi *et al.*, 1994). *In vitro* studies have demonstrated that this aggregation is inhibited in the absence of plasma and in the presence of the divalent cation chelator EDTA or cytochalasin B (Takahashi *et al.*, 1994). Preliminary studies on the haemocyte-aggregate inducing factor indicate that a bioactive low-molecular weight peptide may be released by cells which induces the further aggregation of cells (Takahashi *et al.*, 1994). In *C. intestinalis*, the non-vacuolar hyaline amoebocytes are the aggregating cells (Rowley *et al.*, 1984) (Table 1.3).

Graft rejection

Tunic allograft rejections have been studied by Reddy *et al.* (1975) and Raftos *et al.* (1987a,b). In *C. intestinalis*, rejection is expressed 6-8 weeks after transplantation, and at the end of the eight week, the frequency of undifferentiated cells is significantly higher in the allografts over the autografts (Reddy *et al.*, 1975). Similarly, in *Styela plicata*, the lymphocyte-like cells are responsible for the recognition and destruction of allogeneic tissue (Raftos *et al.*, 1987b) (Table 1.3). Morula cells are present at higher but equal frequencies in both autografts and allografts alike (Raftos *et al.*, 1987a,b; Reddy *et al.*, 1975) (Table 1.3). The genetic control of allograft rejections is discussed in section 1.4.7.

Non-fusion reactions

In all species of colonial ascidians, blood cells have been shown to infiltrate ampullae at areas of non-fusion reactions (Sabbadin *et al.*, 1992) and it has frequently been suggested that the blood cells play a crucial part in non-fusion reactions (Taneda and Watanabe, 1982a). In addition, the factors controlling fusibility have been shown to be sensitive to X-irradiation (Taneda and Watanbe, 1982c); a treatment which is known to kill the undifferentiated cells (Sabbadin *et al.*, 1992)

There have been several histological examinations of non-fusion reaction areas to determine the morphological characteristics of the blood cells involved. The morula cells were most commonly implicated (Table 1.3) (Taneda and Watanbe, 1982a; Scofield and Nagashima, 1983). Rinkevich *et al.* (1992), on the other hand, noticed a higher concentration of 'yellow blood cells' at the site of non-fusion reactions. The genetic control of non-fusion reactions is discussed in section 1.4.7.

Cytotoxic activity

In the course of the search for pharmacologically useful compounds, over sixty metabolites, cytotoxic to mammalian tumour cell lines, have been isolated from homogenates of the whole bodies of various species of ascidians since 1987 (see review by Davidson, 1993). However, the tissue of origin is unknown and there is no information on their function in relation to the immune system.

In vivo solitary ascidians mount rejection responses to allografts (Reddy *et al.*, 1975, Raftos, 1987a) while compound ascidians exhibit non-fusion reactions between allogeneic individuals (see review by Rinkevich 1992). Taneda and Watanabe (1982a) have demonstrated the involvement of a variety of blood cells in colonial ascidian

histocompatibility reaction, and Raftos *et al.* (1987b) has shown that 'lymphocyte-like' cells specifically accumulate around allografts (but not autografts) immediately prior to rejection (Table 1.3). Both tissue graft rejection and non-fusion reactions are associated with infiltration by the blood cells and subsequent cytotoxic events (Raftos, 1987a; Rinkevich, 1992).

However, there have been a few detailed *in vitro* analyses of cytotoxic responses by ascidian haemocytes. Cytotoxic responses occur between some, but not all, allogeneic combinations of haemocytes *in vitro* from the tunicate, *Halocynthia roretzi* (Fuke and Numakunai, 1982). However, because of the mutual death of apposed cells (Fuke and Numakunai, 1982), there are limited possibilities for quantitative assessment of the mechanism and kinetics behind these responses. Kelly *et al.* (1992) also observed *in vitro* cytotoxic responses between allogeneic cells and considered that these *in vivo* allogeneic responses and allogeneic transplantation rejection reactions may be mediated by the same cellular and molecular recognition mechanisms. However, the cellular mechanisms underlying both of these cytotoxic functions have yet to be fully investigated, and characterisation of the effector cells involved requires clarification.

Blood cell proliferation and renewal

In ascidians, haemopoietic tissue occurs in the pharyngeal wall, around the digestive tract and in advanced species in the body wall (Ermak, 1976). In *Styela* spp. the haemopoietic nodules consist of clusters of dividing haemoblasts (stem cells) surrounded by non-dividing maturing blood cells (Ermak, 1976) (Table 1.3). These dividing cells are reported to have increased proliferation within haemopoietic crypts of animals challenged with allogeneic tissue (Raftos and Cooper, 1991), and to proliferate in cultures of haemopoietic tissue in response to mitogens or human recombinant interleukin-2 (Raftos *et al.*, 1991). In addition, autoradiographic preparations of

haemopoietic tissue have revealed labelled undifferentiated cells in the circulating blood, indicating that cell proliferation outside the nodule can also occur (Ermak, 1975) (Table 1.3). However, Raftos *et al.* (1990) were unable to record proliferation in culture by the circulating blood cells from *S. clava.*

Studies aimed at the stimulation of proliferation by the circulating ascidian blood cells *in vitro* using classical T or B cell mitogens (see section 1.3.2) have produced varied results (De Leo, 1993). For example, Tam *et al.* (1976) reported that the circulatory blood cells from *C. intestinalis* were able to synthesize DNA but only showed increased proliferation in response to very high concentrations of PHA and over long culture periods. An *in vitro* study of the blood cells of *Pyura stolonifera* by Warr *et al.* (1977) looked for other functions comparable with those of mammalian lymphocytes. They were able to demonstrate receptors on almost all the cell types for Con A, wheat germ agglutinin and soy bean agglutinin by binding of radioionated lectins (Warr *et al.*, 1977). However, these authors were unable to demonstrate mitogen-induced proliferation or mixed leucocyte reactions in culture (Warr *et al.*, 1977).

Table 1.3. Blood cell function in ascidians.

Cell type	Cell function	Reference
Cell type 1 (undifferentiated cell)	Haemopoiesis	Ermak, 1976
		Mukai & Watanabe, 1976
		Milansei & Burighel, 1978
		Wright, 1981
		De Leo *et al.*, 1987
		Scippa *et al.*, 1987
	Antigen recognition	Wright, 1981.
	Allo but not auto graft rejection	Reddy *et al.*, 1975
		Raftos *et al.*, 1987a,b
	Non-fusion reactions	Taneda & Watanabe, 1982
	Bud formation	Wright, 1981
	Gonad and germ cell formation	Wright, 1981
Cell type 2 (Hyaline amoebocyte)	Phagocytosis	Sawada *et al.*, 1993
		Zhang *et al.*, 1992
		Ohatke *et al.*, 1994
		Wright, 1981
		Wright & Ermak, 1982
	Haemostasis	Rowley *et al.*, 1984
		Ohatke *et al.*, 1994
		Takahashi *et al.*, 1994
	Progenitor of vacuolated cells?	Wright, 1981
Cell type 3 (granular amoebocyte)	Phagocytosis	Sawada *et al.*, 1993
		Ohatke *et al.*, 1994
		Wright, 1981
		Wright & Ermak, 1982
	Allo & autograft rejection	Azumi *et al.*, 1990
	Protease release	Ohatke *et al.*, 1994
	Haemostasis	Wright, 1981
	Non-fusion reactions	Wright, 1981
		Wright & Ermak, 1982
	Vanadium accumulation	Rowley, 1982a

Cell type 4. (vacuolar amoebocyte)	Phagocytosis	Fuke , 1979
		Smith & Peddie, 1992
	Encapsulation	Anderson, 1971
	Non-self recognition	Fuke, 1980
	Antibacterial	Azumi *et al.*, 1990
	In vitro cellular lectin release	Cammarata *et al.*, 1993
Cell type 5. (morula cell)	Haemostasis	Wright, 1981
	Tunic formation	Wright, 1981
		Smith, 1970
		Zaniolo, 1981
		De Leo *et al.*, 1981
	Vanadium accumulation	Wright, 1981
		Martoja *et al.*, 1994
	Cell communication	Smith & Peddie, 1992
	Phagocytosis	Sawada *et al.*, 1993
	In vitro cellular lectin release	Cammarata *et al.*, 1993
	Haemagglutinin synthesis	Azumi *et al.*, 1993
	Antibacterial activity	Azumi *et al.*, 1990
		Findlay & Smith, 1995
	Encapsulation	Anderson, 1971.
		Parrinello *et al.*, 1977
	Allo & autograft rejection	Wright, 1981
		Wright & Ermak, 1982
		Wright & Ermak, 1982
		Raftos *et al.*, 1987a,b
		Reddy *et al.*, 1975
	Non-fusion reactions	Taneda & Watanbe, 1982c
		Scofield &Nagashima,1983
	Releasers of opsonins	Smith & Peddie, 1992
	Repositories of phenoloxidase	Jackson *et al.*, 1993
	Repositories of proteases	Azumi *et al.*, 1991
		Jackson & Smith, 1993

Cell type 6. (signet ring cell)	Vanadium accumulation	Martoja *et al.*, 1994
	Encapsulation	Wright, 1981
		Anderson, 1971
	Allo & autograft rejection	Wright & Ermak, 1982
Cell type 7 (nephrocyte)	Excretion	Wright, 1981
		Ratcliffe *et al.*, 1985
Cell type 8 (pigment cell)	Vanadium accumulation	Anderson & Swinehart, 1991
	Pigmentation	Wright, 1981
	Photoreceptors?	Wright, 1981
	Protection against UV light?	Wright, 1981

1.4.6. Genetic control of allorecognition.

In the styelids, the genetic rules which govern the recognition of allogeneic tissue are identical to those of vertebrates (Kingsley *et al.*, 1989). Rejection follows the specific recognition of cellular determinants that are encoded by discrete, polymorphic histocompatibility loci (Raftos, 1994). Raftos and Briscoe (1990) identified five discrete haplotypes within the tested population, and found that only tunicates of identical tissue type are mutually compatable. A single disparate haplotype , therefore, must be sufficient to stimulate rejection. In *H. roretzi,* four alleles on each individual recognition system are present, so this species probably carries two genes that control alloreactivity (Fuke and Nakamura, 1985).

Within all colonial botryllid ascidians tested, the genetics for fusibility are essentially the same, whereas the responses that occur upon non-self recognition differ from species to species (see review by Rinkevich, 1992). In contrast to solitary ascidians, allorecognition is controlled by a single, highly polymorphic gene locus with multiple co-dominantly expressed alleles (Scofield *et al.*, 1982). This haplotype was recently termed the tunicate Fu/HC locus (Weissman *et al.*, 1990). The rules for histocompatibility differ from vertebrates and solitary ascidians, in that rejection occurs between colonies that share no alleles, and that fusion will occur between colonies sharing one or both alleles (Oka and Watanabe, 1960, Scofield *et al.*, 1982).

1.4.7. Lymphocyte-like functions in ascidians - evidence to date.

Assuming that the evolutionary origin of immunoglobulin-producing cells lies in lower vertebrates, the primary functional attributes required of a candidate for the primordial

lymphocyte precursor in ascidians, are morphological similarities, cytotoxic activity and proliferation in response to mitogens and allogeneic cells.

The morphological similarities between the undifferentiated cells (see section 1.4.5 and Table 1.2) and vertebrate lymphocytes have been noted by several authors (for example Fuke, 1979; Wright, 1981; Scippa *et al.*, 1982). In addition, circumstantial *in vivo* evidence exists for lymphocyte-like functions by the blood cells in ascidians. This includes the cytotoxic mechanisms of allograft rejection (Raftos *et al.*, 1987a,b), and proliferation in response to allogeneic stimulation (Raftos and Cooper, 1991). Cytotoxic activity *in vitro* between allogeneic and xenogeneic mixed blood cells has also been recorded (Kelly *et al.*, 1992) but there is no information on the morphology of the mediating blood cell nor of the mechanism of activity. Other workers have been unable to find experimental evidence *in vitro* for mitogen or allogeneic cell stimulated proliferation to support these hypotheses (Warr *et al.*, 1977). In the absence of conclusive evidence for a lymphocytic function by the undifferentiated blood cells (see section 1.4.5) researchers have classified these cells as stem cells, haemocytoblasts, haemoblasts or progenitor cells (see review by De Leo, 1993) (Table 1.2). Evidence for the stem cell function of the undifferentiated blood cells is provided by the studies of Freeman (1970) who showed the complete reconstitution of the circulating haemocytes of lethally irradiated colonial tunicates solely from this cell type. Unarguably, this supports the hypothesis that undifferentiated blood cells of ascidians fulfil a haemopoietic role within the circulation (Rowley, 1982b). From all the available evidence, it is concluded by most authors that the undifferentiated blood cells are not directly homologous to vertebrate lymphocytes but serve as haemopoietic cells (De Leo, 1993) and that to date conclusive, direct evidence for lymphocyte precursors in ascidians does not exist.

1.5. Aims of the thesis.

The morpho-functional analogies already evidenced between ascidian and vertebrate lymphocytes, at present, cannot point to a common phylogenetic origin. However, there is not yet sufficient evidence to disprove the hypothesis that the ancestor of the vertebrate lymphocyte can be found in the ascidians. The broad aim of this thesis is to investigate the blood cells of the solitary ascidian *C. intestinalis* for lymphocyte-like functions which may indicate the presence of a evolutionary precursor of the vertebrate lymphocyte. This broad aim will be achieved by examining experimentally the following questions.

1. Do the blood cells of *C. intestinalis* mediate cytotoxic activity. What are the mechanisms underlying the cytotoxic activity, and are they similar to those which mediate cytotoxic activity by vertebrate cytotoxic cells? Is effector to target cell contact necessary for cytolysis to occur? What are the morphological characteristics and adherence properties of the cytotoxic effector cells?
2. Are the blood cells capable of *in vitro* proliferation in response to mitogens and/or allogeneic cells?
3. Where are the cytotoxic cells produced?

Figure 1.3. Underwater photograph of the solitary ascidian, *Ciona intestinalis*.

This photograph was taken in February, 1995.

Chapter Two
Haemolytic activity of the blood cells of *Ciona intestinalis*

2.1. Introduction.

Haemolysins are humoral factors which mediate the lysis of erythrocytes. They naturally occur in the blood of a range of invertebrates including molluscs, sipunculids, crustaceans, echinoderms and annelids (see review by Ratcliffe *et al.*, 1985). Haemolysins bind to the erythrocyte target through an interaction with membrane lipids or membrane sugars resulting in some cases in circular holes on the erythrocyte membrane (Roch *et al.*, 1989). In general, haemolysins tend to be thermolabile factors which are sensitive to proteolytic enzymes and dependent upon calcium ions for optimal activity. Haemolytic factors may also function as antibacterial agents. For example, Valembois *et al.* (1982) found that in the earthworm, *Eisenia foetida* , the same lipoprotein is responsible for both haemolytic and antibacterial activities.

Previously, haemolytic capability of invertebrate haemocytes has been utilised as a model for measuring cell mediated cytotoxic activity (Parrinello *et al.*, 1993). However it is important to distinguish between haemolysis mediated by soluble humoral factors and that mediated by cell to cell contact often described as cytotoxic activity. In this chapter, the haemolytic capability of the blood cells from *C. intestinalis* is investigated as a possible means of exploring cell-mediated cytotoxic activity.

2.2. Materials and Methods.

2.2.1. Collection and maintenance of animals

Specimens of *C. intestinalis* , measuring between 5-10 cm long, were collected by SCUBA from the marina at Croab Haven, Argyll, Scotland. They were kept in aquaria with constantly circulating seawater (32 ± 2‰; 10°C) and fed twice weekly

with either a laboratory grown culture of marine algae (*Rhodomonas sp.*) or live plankton samples collected with a fine net plankton tow from St. Andrews Bay, Scotland.

2.2.2. Collection of blood cells.

The inner and outer tests of the animals were removed to expose the mantle. An incision was made into the mantle to reveal the heart. The animals were then bled directly from the heart by lancing it with a sterile scalpel blade and collecting the released blood in a sterile petri dish using ice cold marine anticoagulant (MAC) (985 mOsm kg^{-1}) (0.1 M glucose; 15 mM trisodium citrate; 13 mM citric acid; 10 mM EDTA; 0.45 M NaCl; pH 7.0) as diluent.

2.2.3. Preparation of target cells

Sheep blood erythrocytes (SRBC) stored in a 50% v/v Alsevers solution (Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland) (SAPU) were used as targets. Aliquots of 0.5 ml of 1% vol/vol SRBC were washed three times in marine saline (MS) (940 mOsm kg^{-1}) (12 mM $CaCl_2.6H_2O$; 11 mM KCl; 26 mM $MgCl_2.6H_2O$; 45 mM tris; 38 mM HCl; 0.4 M NaCl; pH 7.4) by centrifugation at 200 g and then resuspended in MS to give a concentration of 10^7 ml^{-1}.

2.2.4. Preparation, viability and counting of effector cells

Mixed blood cell populations of *C. intestinalis* diluted in MAC, were washed twice in MS at 800 g for 10 min, resuspended in MS at 1 x 10^7 cells ml^{-1} and kept on ice until needed.

The number and viability of the cells was checked by eosin-Y dye exclusion assay. Ten μl aliquots of cells were mixed with 10 μl of 0.5% wt/vl eosin-Y in MS. The cell count (cells ml^{-1}), and the percentage of eosin-Y stained cells, was determined by light microscopy using new improved Neubauer counting chambers.

2.2.5 Preparation of the effector cell lysate

Effector cells, prepared as in section 2.2.4., were homogenised on ice using a glass piston homogeniser for 10 minutes. The homogenate was spun (40 000 g, 20 min, 4°C) and the supernatant used as lysate.

2.2.6. Preparation of plasma

Plasma was prepared by bleeding the animals into ice cold centrifuge tubes and centrifuging immediately at 250 g for 10 min at 4°C. The resulting supernatant (plasma) was then sterilised by passing it through three 0.22 μ sterile Millipore filters at 4°C. All plasma samples were then stored at -20°C until use.

2.2.7. Haemolytic assay

Cytotoxic activity was detected by incubating the SRBC with the effector cells from *C. intestinalis*. The SRBC (100 μl at a concentration of 10^6 cells ml^{-1}) and effector cell suspensions (100 μl at a concentration of 10^7 cells ml^{-1}) were added to each well of U-bottomed 96 well transparent microtitre plates. Other wells contained 100 μl of plasma, or 100 μl of cell lysate with the SRBC (100 μl at a concentration of 10^6 ml^{-1}). For controls, 100 μl of heat inactivated effector cells (15 min, 46°C in water bath), or 100 μl of MS were substituted for the effector cell suspensions. The plates were covered and incubated for 120 min at 20°C. The maximum possible release was measured by freeze/thawing a plate containing 8 wells of 100 μl SRBC and 100 μl MS three times. At the end of the incubation period, all the microtitre plates were centrifuged (250 g, 5 min) and the 100 μl of supernatant carefully pipetted into a fresh 96-well microtitre plate. The absorbance of each supernatant was measured at 405 nm using a multiplate reading spectrophotometer (Dynatech, Billinghurst, Essex).

2.2.8. Analysis of results

Absorbances from all experiments and/or treatments were compared with the freeze/thaw positive control using the Student's t-test for paired variants (Sokal and Rohlf, 1981). If differences were considered significant ($p \geq 0.05$) then the percentage specific release (%SR) for each assay was calculated from the absorbances of the maximum release wells (A_m) and experimental wells (A_e) with each of the eight well replicates as follows :-

$$\%SR = (A_e / A_m) \times 100$$

2.3. Results

The results shown in Table 2.1 are those obtained, with eight animals, using the same preparation of SRBC. The absorbances of all the experimental wells differed significantly from the positive control wells ($p<0.05$). The haemolytic activity of the *C. intestinalis* effector cells was 25.80 ± 4.29 %, and this activity was ameliorated upon heat-treatment of the effector cells to 0.59 ± 0.36% (Table 2.1). The haemolytic activity of the effector cell lysate was 15.50 ± 1.48 %, which is less than with whole effector cells (25.80 ± 4.29 %) (Table 2.1), but was greater than the activity found in plasma (10.65 ± 3.75%) (Table 2.1). When using different preparations of SRBC, it was found that the variation in susceptibility to haemolysis was too great to obtain statistically comparable results.

Table 2.1. The haemolysis of sheep red blood cells by intact blood cells, heat treated blood cells, plasma, or blood cell lysate supernatant from *Ciona intestinalis*.

Treatment	Absorbance at 405 nm	% Specific Release
Saline (control)	0.001 ± 0.001	0.12 ± 0.11
Intact blood cells from *C. intestinalis*	0.218 ± 0.016	25.80 ± 4.29
Plasma from *C. intestinalis*	0.090 ± 0.033	10.65 ± 3.75
Blood cell lysate supernatant from *C. intestinalis*	0.131 ± 0.014	15.50 ± 1.48
Heat treated blood cells from *C. intestinalis*	0.005 ± 0.003	0.59 ± 0.36
Freeze/thawed erythrocytes	0.845 ± 0.009	100

Haemolytic activity was measured by incubating SRBC (100 μl at a concentration of 10^6 cells ml^{-1}) with effector cell suspension (100 μl at a concentration of 10^7 cells ml^{-1}), or 100 μl of plasma, or 100 μl of cell lysate for 120 min at 20°C. Controls consisted of SRBC incubated with 100 μl of heat treated effector cells (15 min, 46°C in water bath) or 100 μl of MS. The maximum possible release was measured by freeze/thawing a plate containing 8 wells of 100 μl SRBC (10^6 cells ml^{-1}) and 100 μl MS three times. The microtitre plates were then centrifuged (250 g, 5 min). The percentage specific release (%SR) for each assay was calculated from the absorbances (405 nm) of the supernatants from the maximum release wells (A_m) and experimental wells (A_e)

: $\%SR = (A_e / A_m) \times 100$

2.4. Discussion.

The haemolytic activity of haemocytes from the solitary ascidian *C. intestinalis* was examined using sheep red blood cells as targets. The activity was found to be present in intact haemocytes but was inactivated by the heat treatment of the effector cells. In addition, some activity was present in the cell lysate supernatant obtained by homogenising the effector cells.

Haemolysis by the blood cells of *C. intestinalis* has been investigated by Parrinello *et al.*, (1993). Rabbit, human, guinea pig and sheep erythrocytes were lysed by mixed haemocytes from *C. intestinalis* to a variable extent (Parrinello *et al.*, 1993). These authors also experienced high degree of variability between experiments with haemolytic values varying from 58.2 to 96.7% on sheep erythrocytes (Parrinello *et al.*, 1993). Reduction of the variability can be achieved by the use of SRBC collected from one individual, and to store the erythrocytes for exactly the same numbers of days in Alsevers for every experiment (Parrinello, personal communication). During the present study it was found that the erythrocytes, as well as being lysed, were internalised by phagocytic cells. This phagocytic activity had the potential to compound future analysis of the mechanism of cytotoxicity.

The identity of the effector cell population was not investigated in this study, although subsequent separation of the effector cell population by discontinuous Percoll density gradient centrifugation, indicated that the effector cell is located on the lower bands of the gradient and could be morula cells (Parrinello, personal communication) or granulocytes (Parrinello *et al.*, 1994).

The results presented by Parrinello *et al.* (1993), indicate that haemolysis is calcium dependent and occurs within 30 minutes of co-incubation with effectors. However, he made no account of effector : target cell contact during haemolysis or the role, if any of phagocytosis (Parrinello *et al.*, 1993). Cell-mediated killing differs from

haemolysis in that it usually requires surface contact between effector and target cells which is probably mediated by receptors on the effector cells (Parrinello, 1992). To date no evidence has been presented that indicates that the process of haemolysis by the haemocytes of *C. intestinalis* described in this chapter and by Parrinello and co-workers (1992, 1993, 1994) is comparable with the cell-mediated cytotoxic activity investigated in this thesis. A method for measuring cytotoxic activity of blood cells from *C. intestinalis* without complication by phagocytosis or haemolysis needs to be developed.

Chapter Three
Parameters of cytotoxic activity by the blood cells of *Ciona intestinalis in vitro*

3.1. Introduction

Taneda and Watanabe (1982a,b,c) demonstrated the involvement of a variety of blood cells in colonial ascidian histoincompatibility reactions *in vivo*, and Raftos (1987b) has shown that 'lymphocyte-like cells' specifically accumulate around allografts (but not autografts) immediately prior to rejection. Kelly *et al.* (1992) considered that both these *in vivo* rejection reactions and *in vitro* allogeneic cytotoxic responses may be mediated by the same cellular and molecular recognition mechanisms.

Some studies have been directed at further understanding these cytotoxic mechanisms by various *in vitro* techniques. For example, using simple cell lysis observations by under phase contrast microscopy, Fuke and Numakunai (1982), showed that *in vitro* cytotoxic responses occurred between some, but not all, allogeneic combinations of haemocytes from the tunicate, *H. roretzi*. This assay, however, provides limited possibilities for the quantitative assessment of the mechanism and kinetics (see section 1.2.2). In chapter two, and in a study by Parrinello *et al.* (1993), cytotoxicity was investigated by measuring the haemolytic activity of ascidian haemocytes but there were problems of variability, osmotic incompatibility between the effector and target cells and confounding phagocytic activity.

Studies on vertebrate cytotoxic activity have frequently used mammalian tumour cell lines, such as P815 or YAC-1, as targets (Doherty, 1993). The use of such targets has enhanced reproducibility of the assay between experimental runs and between studies carried out in different laboratories. The only study to investigate cytotoxic activity against mammalian tumour cell lines by the blood cells of marine invertebrates was by Decker *et al.* (1981) (see section 1.2.5). However, the assay was performed in a medium which was considered by the authors as not ideal for the effector cells (Decker *et al.*, 1981). This chapter examines *in vitro* cell-mediated cytotoxic activity in *C. intestinalis*, towards mammalian cells by modifying the fluorochromasia assay

described by Bruning *et al.* (1980) to overcome the problems of osmotic incompatibility (see section 1.2.2). This modification entails salt conditioning the target cells in a 740 mOsm kg^{-1} buffer prior to labelling, thereby permitting the subsequent assay of cytotoxic activity in a high salt medium (940 mOsm kg^{-1}) optimal for the effectors.

3.2. Materials and Methods

3.2.1. Collection and maintenance of animals.

The collection and maintenance of animals was as described in section 2.2.1.

3.2.2. Preparation of target cells

The following cell lines were used as targets : WEHI, a mouse myelomonocytic leukemic cell (strain 3B); K562, a human erythromyeloid leukemia cell; L929, an adherent murine fibroblast; YAC-1, a murine lymphoma cell induced by Moloney virus in A/Sn mouse; and P815, a methylcholantrene-induced mastocytoma cell from DBA/2 mouse. The WEHI 3B, L929, and P815 cells were cultured in Dulbecco Eagle's medium supplemented with 10 % fetal bovine serum, while the YAC-1 and K562 cells were cultured in RPMI 1640, also with 10 % fetal calf serum. Preliminary trials showed an increased subsequent rate of labelling in target cells which were allowed to stabilise in a higher osmolality saline.

3.2.3. Target cell labelling

Prior to labelling, each target cell line at logarithmic growth phase, was washed from the culture medium by centrifugation for 5 min at 400 g at 4°C, resuspended in 10 ml low salt marine saline (MS I) (740 mOsm kg^{-1}) (12 mM $CaCl_2.6H_2O$; 11 mM KCl; 26 mM $MgCl_2.6H_2O$; 45 mM tris; 38 mM HCl; 0.3 M NaCl; pH 7.4) and incubated

for 30 mins at 20°C. The target cells were labelled with 5-carboxyfluorescein diacetate (CFDA) (Sigma, Poole, Dorset, England). A stock solution of 10 mg CFDA in 1 ml AnalaR acetone (BDH, Poole, Dorset) was prepared and stored in the dark at -20°C. Preliminary experiments showed that the background leakage from the mammalian target cells was minimised by conditioning in a medium of 735 mOsm kg^{-1} (MS 1) for 30 min at 20°C prior to labelling (Fig 3.1). The target cells in MS I were centrifuged at 400 g for 5 min, resuspended in 5 ml of labelling solution (15 μl of the CFDA stock solution in 5 ml MS I), and incubated for 15 min at 37°C in the dark. The labelled targets were then washed twice in MS I and resuspended in a marine saline of higher osmolality (MS) (940 mOsm kg^{-1}) (12 mM $CaCl_2 \cdot 6H_2O$; 11 mM KCl; 26 mM $MgCl_2.6H_2O$; 45 mM tris; 38 mM HCl; 0.4 M NaCl; pH 7.4) at a concentration of 2×10^6 ml^{-1}. Staining of the target cells was assessed using the fluorescent attachment of a Leitz Diaplan phase contrast microscope. Any cultures with less than 90 % positively staining cells were rejected.

3.2.4. Preparation of the effector cells

The blood cell populations of *C. intestinalis* were separated using the continuous density gradient centrifugation method (Smith and Söderhäll, 1991; Smith and Peddie, 1992). To pre-form the continuous gradients, 10 ml of 60% Percoll (6 ml sterile 32% NaCl solution, 60 ml sterile 3.2% NaCl, 54 ml Percoll (Pharmacia, Uppsala, Sweden) was added to each of ten 15 ml polycarbonate centrifuge tubes (Beckman) and then spun at 47 000 g in a high speed centrifuge with a fixed angle rotor centrifuge (Beckman) for 20 min at 4°C. The pre-formed gradients were covered and stored at 4°C for up to 1 week. To separate the cell populations, *C. intestinalis* blood diluted in MAC (see section 2.2.2) was drawn into a sterile plastic syringe and then gently layered onto the preformed gradient. The gradient was then spun at 2000 g in a refrigerated bench top centrifuge with swing out buckets to carry 15 ml tubes for 10 minutes at 4°C. Each visible band of cells (see Fig. 3.2) was then

carefully harvested from the gradient using a sterile plastic pasteur pipette. The enriched cell populations, or for some experiments (see below) mixed blood cells in MAC, were washed twice in MS at 800 g for 10 min, resuspended in MS at the required concentration and kept on ice until needed.

3.2.5. Cytotoxicity assay

Cytotoxic activity was detected by incubating selected mammalian target cells with the haemocytes from *C. intestinalis*. Labelled target cells (25 μl at a concentration of 2 x 10^6 ml^{-1}) and effector cell suspensions (25 μl, usually at a concentration of 2.10^7 ml^{-1} depending on the effector to target cell (E:T) ratio required) were added to each well of U-bottomed 96 well microtitre plates for fluorometric use (Dynatech, Billinghurst, Sussex). The trays were then covered, and unless otherwise stated, incubated in the dark for 60 min at 20°C. Control conditions were designed to match those of the positive assay as closely as possible to avoid the effects of quenching by the effector cells (see Bruning *et al.*, 1980). For controls, therefore, 25 μl of heat inactivated effector cells (15 min, 46°C in water bath) were substituted for the effector cells kept on ice (Bruning *et al.*, 1980). At the end of the incubation period, the microtitre trays were centrifuged (250 g, 5 min) and the supernatant decanted. The cell pellets were then gently resuspended in 50 μl MS and the fluorescence measured using a microplate attachment to a luminescence spectrometer (Perkin Elmer LS50) in fluorescence mode (excitation wavelength 490 nm, emission wavelength 518 nm). Typically, the control and experimental wells yielded fluorescent intensities of ca. 80 units and ca. 58 units respectively when measured against a blank of unstained targets in MS. Each assay consisted of at least four well replicates of each treatment and was repeated at least three times.

3.2.6. Clonogenic assays.

To confirm that incubation in the high salt media in itself was not lethal to the target cells, clonogenic assays were performed to show that the target cells were still capable of growth when returned to their original culture medium (see section 1.2.2). For this, 8 ml of Dulbecco Eagle's medium supplemented with 10 % horse serum and antibiotics was mixed with 1 ml 3 % agar and 1 ml target cell suspension (approx 5 x 10^5 cells per ml). The target cells had previously been incubated in MS for the 40 minutes at 20°C. One ml of this mixture was plated out into eight 30 mm non tissue culture petri dishes and these were allowed to set. The plates were then incubated for one week at 37°C in air with 5% CO_2. The colonies are counted and the cloning efficiency expressed as colonies per 10^3 cells. In addition, clonogenic assays were performed upon target cells incubated in marine saline, or marine saline with effector cells (E:T 10:1) or marine saline with heat treated effector cells (E:T 10:1).

The cloning efficiency is 140 per 10^3 WEHI (3B) cells, when kept in Dulbecco's medium, but after incubation in MS and then returning to normal culture medium the number of colonies formed per 1000 cells is reduced to ca 30. In the cytotoxicity assay, the targets cells are not subjected to the second centrifugation and osmolality transfer necessary for this clonogenic assay, which probably accounts for a large proportion of the reduction in proliferative capabilities. The cytotoxic activity due to live effector cells is not detectable at these low cloning efficiencies. Interestingly, there does however appear to be a factor that inhibits almost completely the proliferation of WEHI (3B) present in the heat-treated effector cells from *C. intestinalis*.

3.2.7. Experimental assays

Initially, to establish the presence of cytotoxic activity by the blood cells of *C. intestinalis*, mixed blood cells were assayed for activity at an effector to target cell

ratio of 35:1 against WEHI 3B. Subsequent assays were performed with separated cell populations enriched by density gradient centrifugation (see section 3.2.4.). Each cell population was removed from the gradient, washed and resuspended in MS to give a final effector to target cell (WEHI 3B) ratio in the wells of 5:1 and assayed for cytotoxic activity as above.

Using the enriched effector cell preparation (see section 3.2.4, bands 2 & 3) the conditions for target cell lysis were examined. The optimal osmolality of the incubation saline was found by running the assay in salines of 0.05, 0.1, 0.2, 0.3, or 0.4 M NaCl to give osmolarities of 325, 370, 560, 735, and 940 mOsm kg^{-1} respectively (all other components of the saline were prepared as above). Osmolality was measured using a freezing point osmometer (Roebling). Using the optimal saline, the assay was performed firstly with effector to target cell ratios of 5, 10, 15, 20, 25 or 35:1, secondly at a range of incubation temperatures (5, 10, 20, 25, 30 or 35°C) and thirdly over incubation periods of 15, 45, 75, 90 or 105 min. Finally, the cytotoxic activity of the effector cells against a range of target cells typically used in NK assays was screened.

3.2.8. Analysis of results

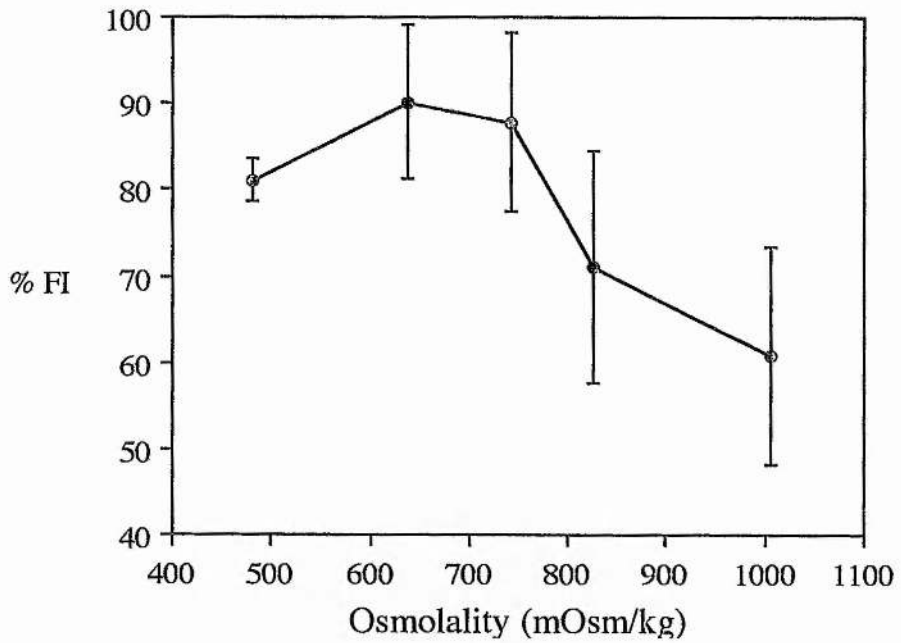
The percentage specific release of CFDA (%SR) for each assay was calculated from the fluorescent intensities of the control (F_c) and experimental wells (F_e) with each of the four well replicates (Bruning *et al.*, 1980) as follows :-

$$\%SR = (1 - F_e / F_c) \times 100$$

Data from all experiments and/or treatments were compared using the Student's *t*-test. In the experiments designed to determine the cell population responsible for the cytotoxic activity and to examine the effect of osmolality, temperature or incubation time, effector cells from the same animal were assayed at each variable; therefore the test for paired variants was used (Sokal and Rohlf, 1981). In the assays to determine

the importance of the effector to target cell ratio, effector cells from different animals were used for each assay and the *t*-test for unpaired variants was used (Sokal and Rohlf, 1981). Differences were considered significant if $p \geq 0.05$.

Figure 3.1. The percentage fluorescent intensity (%FI) of WEHI (3B) target cells after pre-incubation in salines of differing osmolality.



Target cells were conditioned in a medium of various osmolalities (MS I) (measured with a freezing point osmometer) for 30 min at 20°C prior to labelling. Staining of 50 μl of each suspension of target cells was assessed using a luminescence spectrometer (Perkin Elmer LS50) in fluorescence mode (excitation wavelength 490 nm, emission wavelength 518 nm). Values are means ± SE. n = 4.

Table 3.1. The cloning efficiency per 10^3 WEHI (3B) cells plated after test incubations in marine saline with or without effector cells from *Ciona intestinalis*.

	Colonies per 1000 cells
WEHI (3B) in Dulbecco's (control)	143 ± 3.10
WEHI (3B) in MS	31.5 ± 2.96
WEHI (3B) in MS (control)	20.63 ± 1.51
WEHI (3B) in MS + effector cells	21.15 ± 0.98
WEHI (3B) in MS + heated effector cells	7.21 ± 0.45

Dulbecco Eagle's medium (8 ml) supplemented with 10 % horse serum and antibiotics was mixed with 1 ml 3 % agar and 1 ml target cell suspension (approx 5 x 10^5 cells per ml). The target cells had previously been incubated in MS for the 40 minutes at 20°C. One ml of this mixture was plated onto eight 30 mm non tissue culture petri dishes and incubated for one week at 37°C in air with 5% CO_2. The colonies are counted and the cloning efficiency expressed as colonies per 10^3 cells.

3.3. Results

3.3.1. Cytotoxic activity by mixed and separated haemocytes

The percentage specific release (%SR) from the WEHI (3B) cells following incubation with mixed haemocytes from *C. intestinalis* (2 h, 20°C) in a saline of 940 mOsm kg^{-1} was 42.66 ± 8.67 (E:T 35:1) (n=8). This activity was ameliorated by heat treating (46°C, 15 min) the effector cells. Comparison of the activity by each of the separated cell bands (Fig. 3.2) showed that the cytotoxicity resides predominantly in the cells enriched on bands 2 & 3 (Fig. 3.3). Statistical analysis by paired *t*-test revealed that the %SR with effectors from band 2 was significantly larger than from band 3 (p=0.007) (Fig. 3.3). The lower bands of cells, 4, 5,and 6, did not exhibit any significant cytotoxic activity (Fig. 3.2). The cells in band 1 were seldom present in sufficient numbers to achieve an equivalent E:T ratio, although the few experiments completed failed to show cytotoxic activity by these cells. In all further experiments, activity was examined using the enriched effector cell population, in bands 2 & 3.

3.3.2. Cytotoxic activity in media of increasing osmolality

Experiments to determine the optimum osmolality for activity showed that the cytotoxic effector cells functioned significantly better in a medium of 940 mOsm kg^{-1} (0.4 M Na Cl) than in media of 735, 560, 370 or 325 mOsm kg^{-1} (p=0.05, 0.006, 0.023 and 0.027 respectively) (Fig. 3.4).

3.3.3. Cytotoxic activity and effector:target cell ratio

By measuring the percentage specific release of CFDA from the targets following incubation with the effectors at E:T ratios of 5, 10, 15, 20, 25 or 35:1, the cytotoxic activity was shown to increase with E:T cell ratio (Fig. 3.5). The %SR increased significantly from 11.36 ± 1.47 to 19.81 ± 1.80 when the E:T ratio was increased from 5:1 to 10:1 (p=0.001) (Fig. 3.5). Likewise when the E:T ratio was increased

from 10:1 to 20:1, the %SR significantly rose from 19.81 ± 1.80 to 28.94 ± 4.03 (p=0.030) (Fig. 3.5). The %SR for the E:T ratio of 35:1 increased from 28.94 to 36.69 ± 6.65, but this increase was not statistically significant at the 5% level (Fig. 3.5). Thereafter, an effector to target cell ratio of 10:1 was used. This ratio was more manageable in terms of effector cell number and consistently gave significant results.

3.3.4. Effect of incubation temperature on cytotoxic activity

The measurement of the cytotoxic response of *C. intestinalis* at different incubation temperatures revealed that the %SR of CFDA was significantly higher at 20°C (23.50 ± 1.10) than at 15°C, 10°C or 5°C (p=0.001, 0.013 and 0.004 respectively) (Fig 3.6). At 25°C and 35°C, the %SR declined to 20.02 ± 1.88 and 18.92 ± 2.81, respectively, although these values were not significantly lower than the %SR at 20°C due to the larger standard errors at 25°C and 30°C (p>0.05) (Fig. 3.6). An incubation temperature of 20°C was used in all subsequent experiments.

3.3.5. Optimal time for cytotoxic activity.

The time study of the killing by the cytotoxic effector cells showed that the cytotoxic activity by the haemocytes of *C. intestinalis* was rapid; the percentage specific release of CFDA was 19.94 ± 4.26 after 15 min incubation (Fig. 3.7). After 45 min the %SR had significantly increased from 19.94 ± 4.26 to 28.44 ± 4.09 (p=0.028), and at 75 min reached a maximum of 36.22 ± 4.33. No further increase in activity was recorded after this time (Fig. 3.7).

3.3.6. Activity against different target cells

Finally, in experiments to determine the specificity of the cytotoxic reaction, the effector cells from *C. intestinalis* were found to exhibit cytotoxic activity against all the cell lines tested (Table 3.2). The values shown are not statistically comparable because the experiments were performed on separate occasions with different effector

cell preparations (Table 3.2). However, the activity against the adherent cell line, L929, was apparently noticeably lower than against the other non-adherent cell lines (Table 3.2).

Figure 3.2. Diagram to show the cell bands achieved upon density gradient centrifugation of blood cells from *Ciona intestinalis*.

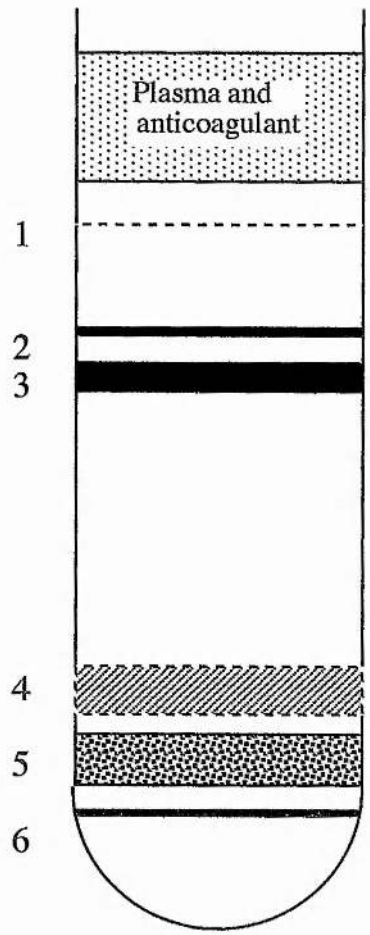
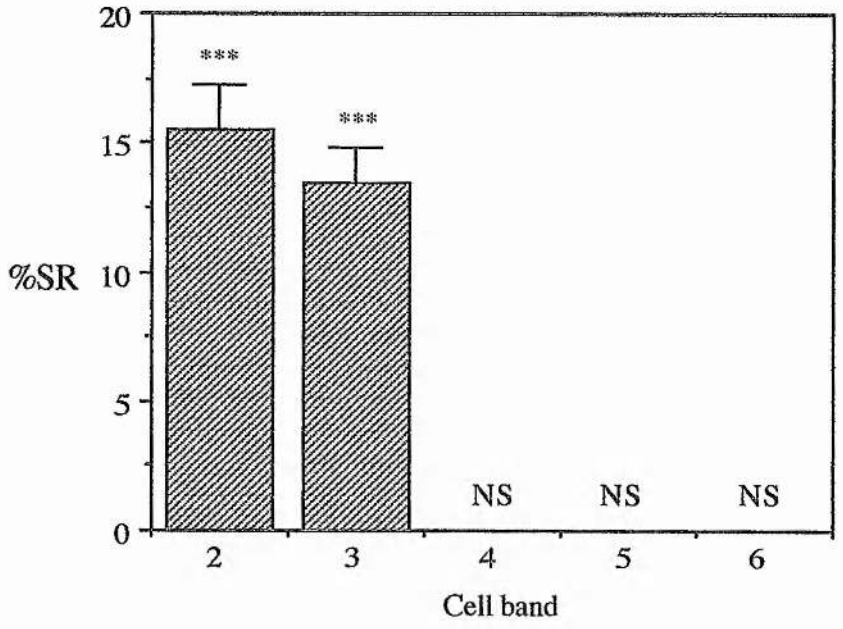
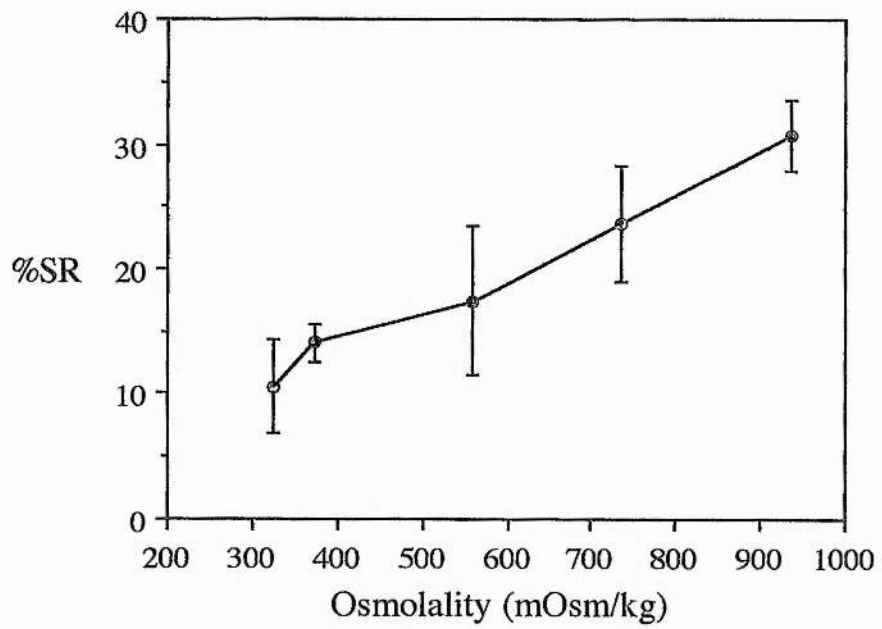


Figure 3.3. Percentage specific release of carboxyfluorescein diacetate (CFDA) (%SR) from the target cells (WEHI 3B) after incubation with separated blood cell populations from *Ciona intestinalis*.



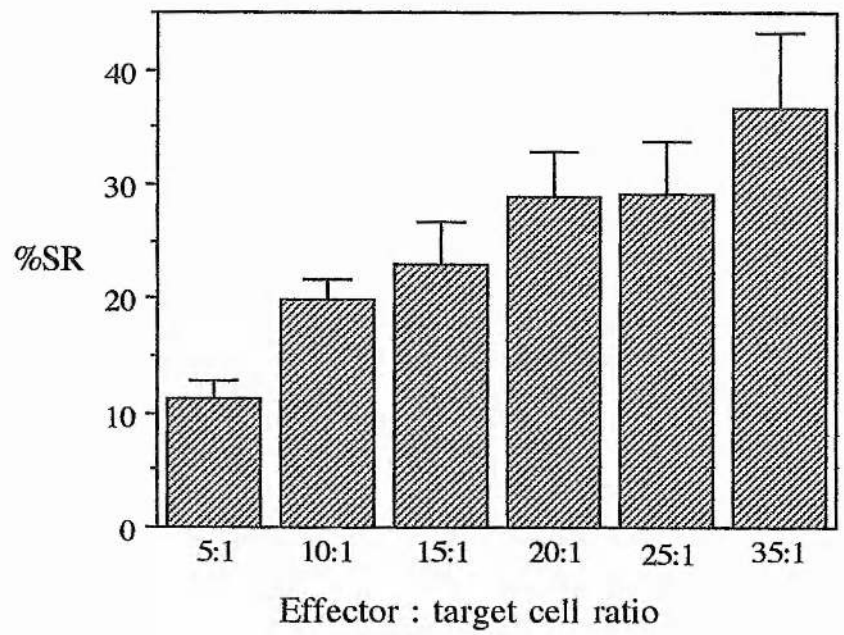
Blood cells, harvested from bands on Percoll continuous density gradient separations (numbered from top to bottom), and target cells were incubated together at a (E:T) ratio 5:1 for 40 min. Eight repetitions with different animals were performed for this experiment and the error bars represent the standard error of the mean. NS - no significant release of CFDA when compared with the controls. *** - $p > 0.001$.

Figure 3.4. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) incubated with enriched effector cells from *Ciona intestinalis* in media of different osmolalities.



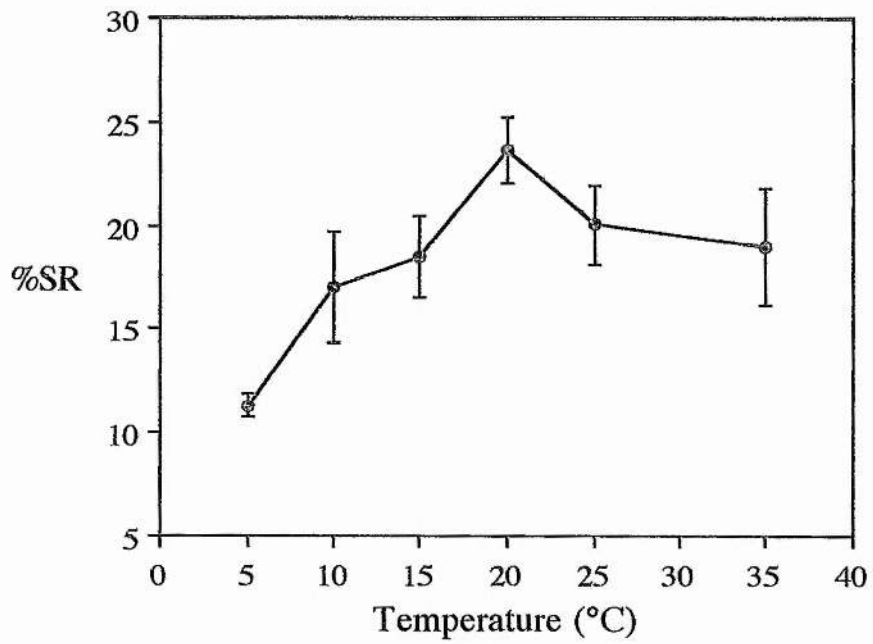
Effector and target cells were incubated together at a (E:T) ratio 10:1 for 40 min at 20°C. The osmolality of each medium was measured using a freezing point osmometer (Roebling). Three repetitions with different animals were performed for this experiment and the error bars represent the standard error of the mean.

Figure 3.5. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) incubated with enriched effector cells from *Ciona intestinalis* at different effector to target cell ratios.



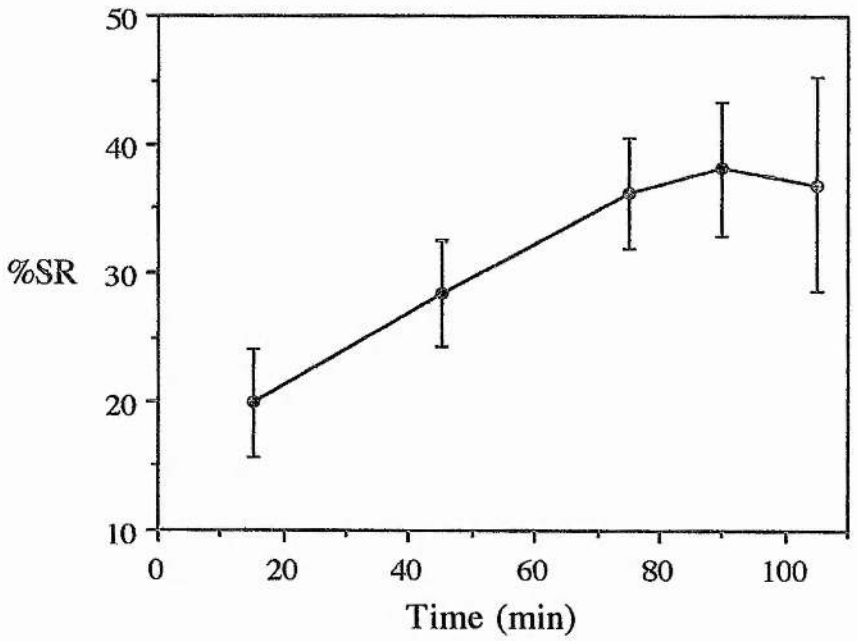
Effector and target cells were incubated together for 40 min at 20°C. Values represent the means of three separate experiments, bars represent standard error of the mean.

Figure. 3.6. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) incubated with enriched effector cells from *Ciona intestinalis* at different temperatures.



Effector and target cells were incubated at an E:T ratio of 10:1, for 40 min at a range of temperatures. Values represent the means of three separate experiments, bars represent standard error of the mean.

Figure 3.7. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) incubated with enriched effector cells from *Ciona intestinalis* for different incubation periods.



Effector and target cells were incubated together at an E:T ratio of 10:1 at 20°C. Values represent the means of three separate experiments, bars represent standard error of the mean.

Table 3.2. The percentage specific release of CFDA (%SR) from different mammalian target cell lines incubated with enriched effector cells from *Ciona intestinalis*

Target cell line	Percentage specific release ± SEM
WEHI 3B	30.71 ± 2.90
K562	23.25 ± 6.84
L929	14.86 ± 4.56
P815	29.65 ± 1.96
YAC-1	42.28 ± 3.13

Effector and target cells were incubated together for 60 min at 20°C at an E:T ratio of 10:1. Each experiment was repeated at least four times, results are means ± standard error of the mean. WEHI - a mouse myelomonocytic leukemic cell (strain 3B); K562 - a human erythromyeloid leukemia cell; L929 - an adherent murine fibroblast; YAC-1 - a murine lymphoma cell induced by Moloney virus in A/Sn mouse; and P815 - a methylcholantrene-induced mastocytoma cell from DBA/2 mouse.

3.4. Discussion

The results presented in this chapter establish that haemocytes from the solitary tunicate, *C. intestinalis*, effect cytotoxic activity against mammalian target cells *in vitro* and that activity is lost when the effector cells are heat treated. The effector cells are enriched in the the upper bands 2 & 3 separated on a continuous density gradient. In addition, cytotoxic activity was found to be rapid, temperature dependent, and effective against a range of human and mouse target cell lines.

In most experiments, cytotoxicity was measured using highly xenogeneic mouse myelomonocytic leukemic target cells. Allogeneic or xenogeneic ascidian haemocytes represent more appropriate targets in terms of non-fusion reactions by colonial ascidians. However, studies by Fuke (1980), Fuke and Numakunai (1982), and Kelly *et al.* (1992) have shown that in such situations both effector and target cells tend to lyse upon contact, thereby confounding investigation of the mechanism(s) of killing (see section 1.2.5). Therefore, whilst the method described in this chapter does not reflect the natural immunological challenges presented to ascidians, it may allow for future detailed analyses of the cellular mechanisms involved in the cytotoxic response.

In the present study, media of different osmolality were tested to achieve the optimal functioning of the effector cells while maintaining low leakage of CFDA from the control target cells over the incubation period. For this purpose, fluorochromasia has many advantages over the ^{51}Cr release assays for cytotoxicity. Namely, it allows for rapid labelling of the target cells (10 minutes as opposed to 1 hour), fast measurement of activity (3 minutes for 96 wells instead of 5 hours for 60 samples), direct visual quantification of labelling and enables the use of small sample volumes (50 μl instead of 200 μl) (Bruning *et al.*, 1980). In the present study, any background leakage was minimised by conditioning the targets in a medium of 740 mOsm kg^{-1} before labelling

and maximal cytolysis by the effector cells was obtained in a medium of 940 mOsm kg^{-1}. As expected for a marine invertebrate, the effector cells functioned best in a medium closest in osmolality to that of tunicate blood (940-1000 mOsm kg^{-1}). This adaptation of the fluorochromasia method (Bruning *et al.*, 1980) provides, for the first time, a method by which the cytotoxic activity of marine invertebrate effector cells against mammalian target cells can be reliably determined.

This is the first report of non-specific *in vitro* cytotoxic activity against mammalian tumour cells by the haemocytes of an ascidian. In this chapter, certain optimal requirements for the cytolytic process are described and it is shown that activity resides only in the upper populations (bands 2 & 3) separated by continuous density gradient centrifugation. Similar to some vertebrate cytotoxic cells, these effector cells from *C. intestinalis* appear to require no *in vitro* activation and effect rapid cytolysis of a wide range of mammalian target cells. Further details of the mechanism of cytotoxic activity and the morphology of the effector cells in *C. intestinalis* are required before further analogies can be drawn with the cytotoxic activity involved in ascidian allogeneic graft responses and non-fusion reactions (see section 1.4.6.), or with, vertebrate cell-mediated cytotoxic activity (see sections 1.2.3 and 1.2.4).

Chapter Four
Mechanism of cytotoxic activity by the blood cells of *Ciona intestinalis*

4.1. Introduction

In section 3.4, it was shown that a blood cell population present in the solitary ascidian *C. intestinalis* mediates cytotoxic activity towards a range of human and mouse target cell lines *in vitro*. This activity was found to increase with the effector to target cell ratio, occur within 15 minutes, and to be temperature-dependent (see section 3.4). However, the mechanisms underlying cytotoxic activity are unknown.

Cytotoxic activity by vertebrate natural killer (NK) cells or cytotoxic lymphocytes has been extensively investigated. Both fish and mammal cytotoxic cells have requirements for electron transport systems, effector cell motility, Mg^{2+} and Ca^{2+} (for binding and cytolysis respectively) and intact secretory apparatus (Carlson *et al.*, 1985; Carpén *et al.*, 1981), whilst superoxide radicals or hydrogen peroxide are not involved (Duwe *et al.*, 1985) (see sections 1.2.3 and 1.2.4).

In invertebrates, the existence of non-specific cytotoxic cells has often been described but few workers have investigated the killing mechanisms involved. The most detailed study by Boiledieu and Valembois (1977a), reports that the cytotoxic activity of sipunculid leucocytes *in vitro* requires effector to target cell contact, divalent calcium ions and intact microtubule assemblages. For molluscs, limited evidence exists to show that haemocyte-mediated cytotoxicity involves reactive oxygen intermediates and/or the action of lysosomal enzymes (see review by Adema *et al.*, 1991).

This chapter aims to investigate the mechanisms of cytotoxic activity by the haemocytes of *C. intestinalis*. In particular, the requirements for effector cell metabolism, and cytoskeletal or secretory processes, are examined. The phagocytic blood cells of *C. intestinalis* have the metabolic capability to generate a respiratory burst (Bell and Smith, 1994). Therefore, the role of oxidative damage by superoxide

anions and/or hydrogen peroxide in cytotoxic activity in *C. intestinalis*.is also investigated.

4.2. Materials and methods

4.2.1. Collection and maintenance of animals

The collection and maintenance of animals was as described in section 2.2.1. The animals were bled as described in section 2.2.2.

4.2.2. Effector cell preparation

The blood cells of *C. intestinalis* were separated on a 60% Percoll continuous density gradient as described in section 3.2.4. The upper bands (2 & 3) containing the enriched cytotoxic effector cells were washed twice in marine saline (MS) at 800 g for 10 min, resuspended in MS at 2×10^7 ml^{-1} and stored on ice until use (see section 3.3.1).

4.2.3. Target cell preparation.

The target cells from a mouse myelomonocytic leukemic line (WEHI, strain 3B) were prepared (section 3.2.2) and labelled with 5-carboxyfluorescein diacetate (CFDA) (Sigma, Poole, Dorset, England) as described in section 3.2.3. Briefly, the target cells were labelled with CFDA in low salt marine saline (MS I) for 15 min at 37°C, washed in MS I and resuspended in MS at a concentration of 2×10^6 ml^{-1}. Staining of the target cells was assessed using the fluorescent attachment of Leitz Diaplan phase contrast microscope. Any cultures with less than 90 % positively staining cells were rejected.

4.2.4 Cytotoxicity assay

As described in section 3.2.5, cytotoxic activity was assayed by incubating the target cells with enriched effector cells from *C. intestinalis*. Labelled target cells (20 μl at a concentration of 2 x 10^6 ml^{-1}) were incubated with effector cell suspensions (20 μl at a concentration of 2 x 10^7 ml^{-1}) and 10 μl of divalent cation chelator, inhibitor, antioxidant enzyme (see below) or MS, in 96 well microtitre plates (U-bottomed for fluorometric use Dynatech, Billinghurst, Sussex). For negative controls, 20 μl of heat inactivated effector cells (15 min, 46°C in water bath) were substituted for the effector cells kept on ice (Bruning *et al.*, 1980). All trays were then incubated in the dark for 45 min at 20°C. After incubation, the trays were centrifuged and the fluorescence was measured as in section 3.2.5. Each assay consisted of at least four well replicates of each treatment and was repeated at least three times.

4.2.5. Ion chelators

The effect of divalent cation chelators on cytotoxic activity was investigated by the inclusion of EDTA or EGTA with Mg^{2+}. These chelators were dissolved in Mg^{2+} and Ca^{2+} depleted-MS (MSD) at a stock concentration of 100 mM. Cytotoxicity was then measured with MSD at EDTA concentrations of 10, 1, or 0.1 mM, or with MSD plus 5 mM Mg^{2+} at EGTA concentrations of 10, 1, or 0.1 mM. Positive controls were performed with MSD and 5 mM Ca^{2+} plus 5 mM Mg^{2+}. Live and heat killed effector cells were incubated in the appropriate saline for 15 minutes prior to the addition of the target cells.

4.2.6. Inhibitors of cellular processes

The effect of inhibitors of cellular processes upon the cytotoxic activity by the haemocytes of *C. intestinalis* was determined by treating both the live and the heat-killed effector cells with various concentrations of the inhibitors for 15 minutes. All

the inhibitors, with the exception of monensin, remained present during the subsequent cytotoxicity assay. Monensin, which acts irreversibly, was removed by washing the treated effector cells, prior to the assay.

Vinblastine sulphate (Sigma) (1 mg ml^{-1}) was dissolved in MS and incubated with the effector cells at concentrations of 50, 100 or 200 μg ml^{-1}. Colchicine (Sigma) (4 mg ml^{-1}) was also dissolved in MS and was incubated with the effector cells at concentrations of 50, 100 or 400 μg ml^{-1}. Cytochalasin B (from *Helmithosporium dematioideum*, Sigma) was dissolved in dimethyl sulfoxide (DMSO) (Sigma) (1 mg ml^{-1}), diluted with MS and used at final concentrations of 0.1, 1 or 10 μg ml^{-1}. Sodium azide (NaN_3) (BDH, Poole, Dorset) was prepared in MS (1 M) and incubated with the effector cells at final concentrations of 10, 1, or 0.1 mM. Monensin (Sigma) was dissolved in absolute ethanol at a concentration of 1 mg ml^{-1}, diluted with MS and incubated with the effectors at concentrations of 2.5, 10 or 50 μg ml^{-1} before washing. Preliminary investigations confirmed that DMSO and ethanol, at the concentrations used, had no effect on target cell viability or effector cell cytotoxicity.

4.2.7 Antioxidant enzymes

To ascertain the role of reactive oxygen metabolites in cytotoxic activity, superoxide dismutase and catalase were used as scavengers of superoxide anions and hydrogen peroxide respectively (Fridovich, 1978; Segal and Abo, 1993). Superoxide dismutase (SOD) (from bovine erythrocytes E.C. 1.15.1.1, Sigma) was used at final concentrations of 50, 100 and 280 u ml^{-1}, while catalase (from bovine liver E.C. 1.11.1.6, Sigma) was used at final concentrations of 100, 200 and 1,150 u ml^{-1}. Assays for cytotoxic activity were carried out in the presence of SOD or catalase either alone or in combination. For positive controls, the antioxidant enzymes were replaced with MS. For every assay, controls for the effect of the enzyme on the

target cells alone, comprised wells containing the enzyme, target cells and heat killed effector cells.

4.2.8. Analysis of results

The percentage specific release of CFDA (%SR) for each assay was calculated as described in section 3.2.8. Data were analysed using a two-way analysis of variance; first, to ascertain whether or not the treatment had a significant effect upon the %SR, and second, to determine whether or not the effect was dose dependent (Sokal and Rohlf, 1981). Differences were considered significant for both analyses when $p \geq 0.05$.

4.3. Results

4.3.1. Effect of divalent cation chelators

Both EDTA and EGTA, at all concentrations, were found to significantly inhibit the cytotoxic activity by the enriched effector cells of *C. intestinalis in vitro* against the target cells ($p=0.006$ and 0.003 respectively) (Figs 4.1 and 4.2).

4.3.2. Energy requirement

Sodium azide was used to investigate the energy requirements of the cytotoxic effector cells during target cell lysis. Sodium azide interferes with electron transfer and affects the active movement of cells, cell surface dynamics and cellular secretion processes (Carlson *et al.*, 1985). In this study, NaN_3, significantly reduced target cell lysis by *C. intestinalis* enriched effector cells ($p<0.001$) in a dose-dependent way ($p=0.001$) (Fig. 4.3).

4.3.3. The effect of cytoskeletal-disrupting agents

Cytochalasin B was used to investigate the role of actin-containing microfilaments and cell motility in target cell lysis. Cytochalasin B has been demonstrated to inhibit amoeboid movement (which is insensitive to colchicine) by competing with cellular proteins for the fast assembly ends of microfilaments (Fulton, 1984). Cytochalasin B significantly inhibited cytotoxic activity at all concentrations ($p=0.014$), although inhibition was not dose-dependent ($p=0.205$) (Fig. 4.4).

Colchicine and vinblastine sulphate were used to disrupt cellular microtubules (Fulton, 1984), thereby assessing the role of microtubule dependent movement in cytotoxic activity. Significant inhibition of cytotoxic activity ($p=0.001$) in a dose-dependent manner ($p=0.004$) was observed following addition of vinblastine sulphate to the effector cells (Fig. 4.5) and, colchicine produced similar inhibition ($p=0.01$) although the reduction was not dose-dependent ($p=0.334$) (Fig. 4.6).

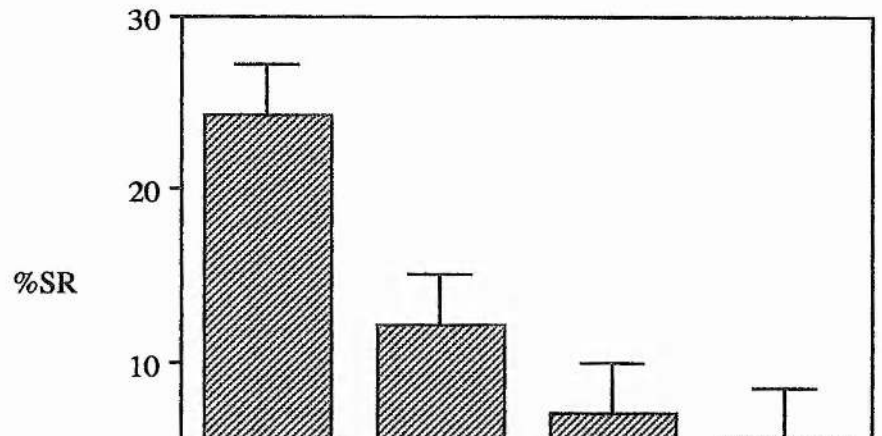
4.3.4. Inhibition of cellular secretion

Experiments to ascertain whether inhibition of cellular secretion effects the cytotoxicity using carboxylic ionophore, monensin, showed that target cell lysis was significantly inhibited by this reagent ($p<0.001$) in a dose dependent manner ($p=0.003$) (Fig. 4.7).

4.3.5. Role of reactive oxygen metabolites

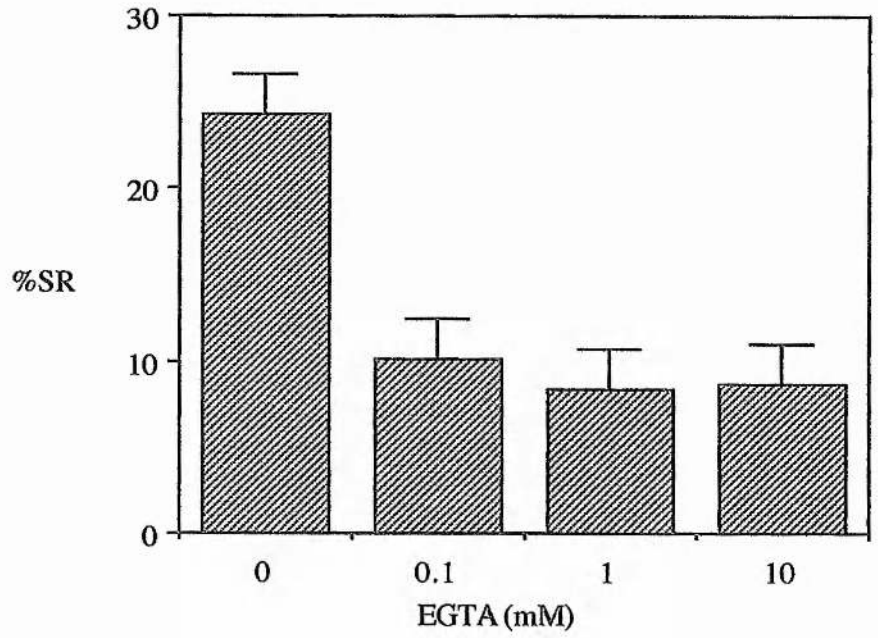
The role of superoxide anions and hydrogen peroxide in cytotoxicity was investigated using the antioxidant enzymes, SOD and catalase. No significant inhibition of cytotoxic activity by the haemocytes from *C. intestinalis* was observed following treatment with SOD ($p=0.148$), catalase ($p=0.846$) or SOD and catalase ($p=0.196$) at any of the concentrations tested (data for SOD at 280 units ml^{-1} and catalase at 1,150 units ml^{-1} shown in Fig. 4.8).

Figure 4.1. The effect of EDTA on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



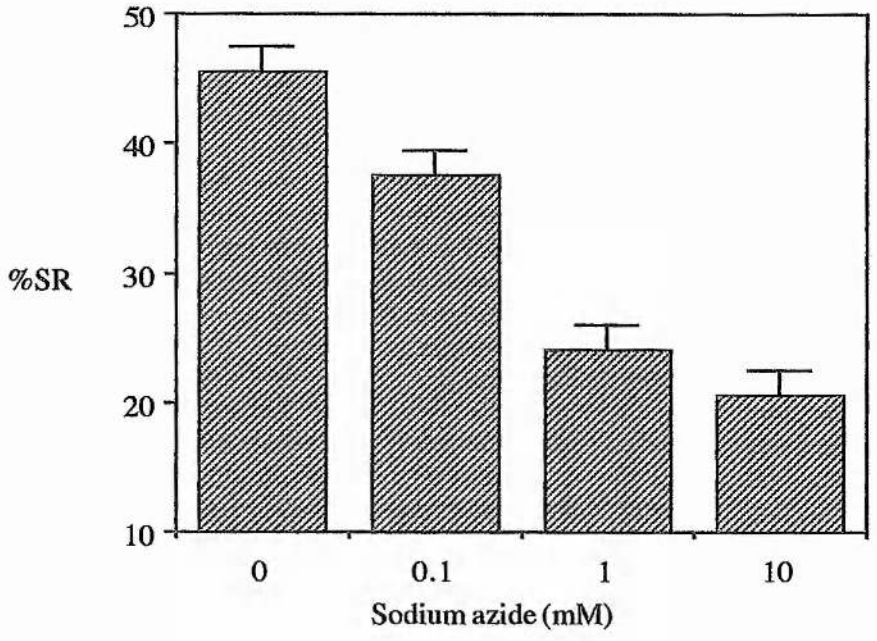
Live and heat killed effector cells were incubated Mg^{2+}- and Ca^{2+}-depleted MS at EDTA concentrations of 10, 1, or 0.1 mM for 15 minutes prior to the addition of the target cells. Positive controls were performed with MS. Values represent the mean of four separate experiments, bars represent the standard error.

Figure 4.2. The effect of EGTA on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



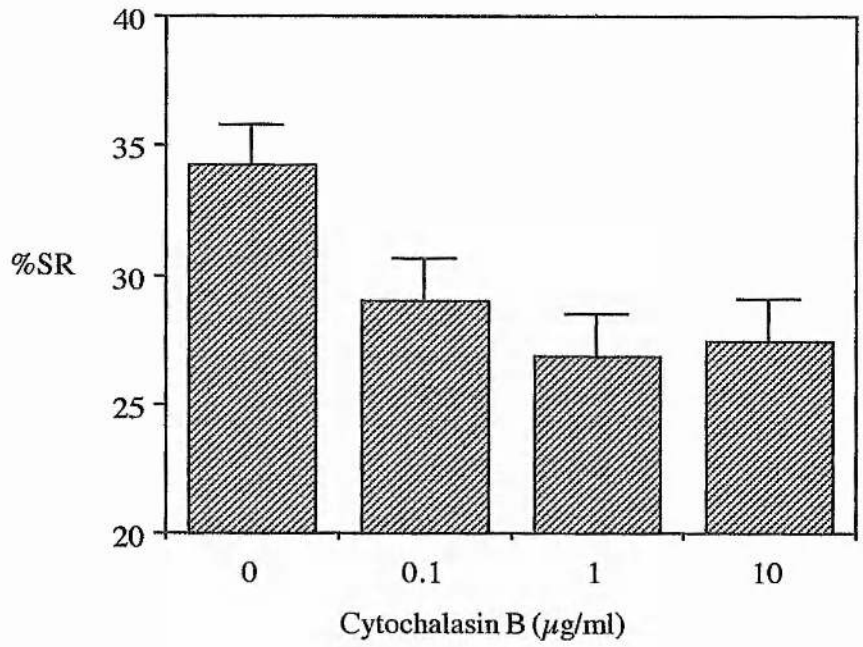
Live and heat killed effector cells were incubated with MS + 5 mM Mg^{2+} but no Ca^{2+} ions at EGTA concentrations of 10, 1, or 0.1 mM for 15 minutes prior to the addition of the target cells. Positive controls were performed with MS. Values represent the mean of four separate experiments, bars represent the standard error.

Figure 4.3. The effect of sodium azide on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



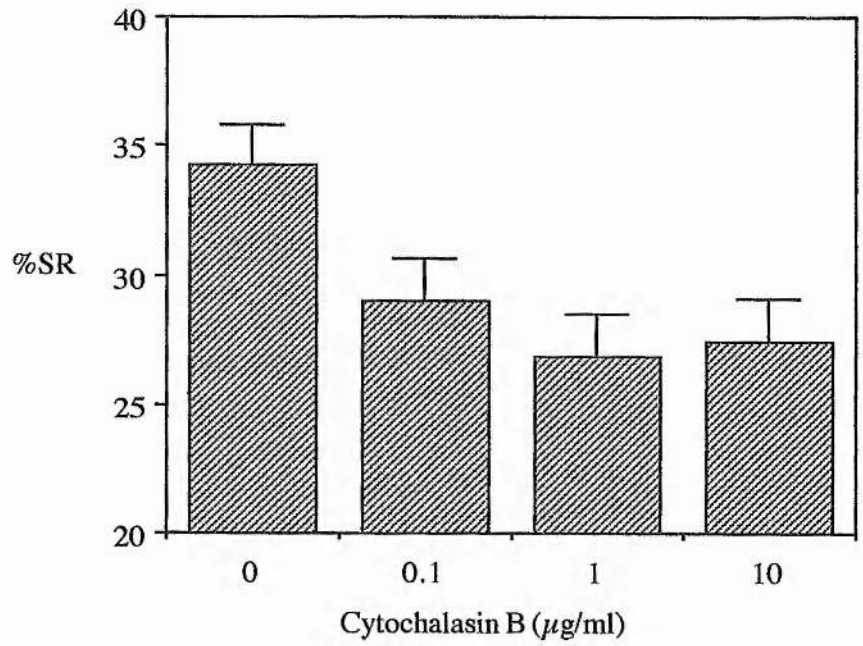
Sodium azide was incubated with the effector cells at final concentrations of 10, 1, or 0.1 mM prior to the addition of the target cells and remained during the assay. Positive controls were performed with MS. Values represent the mean of six separate experiments, bars represent the standard error.

Figure 4.4. The effect of cytochalasin B on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



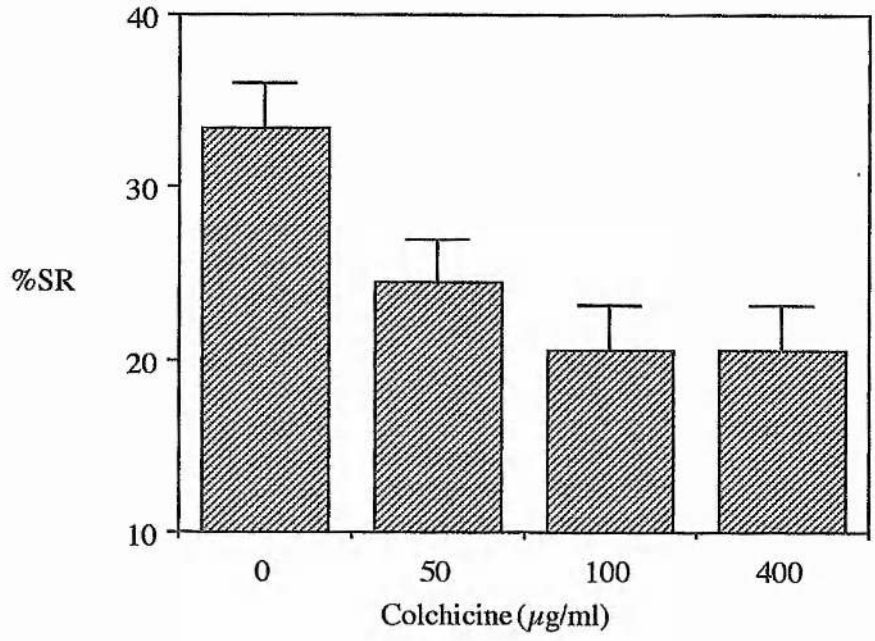
Cytochalasin B at final concentrations of 0.1, 1 or 10 μg ml^{-1}. Live and heat killed effector cells were incubated for 15 minutes prior to the addition of the target cells and remained during the assay. Positive controls were performed with MS. Values represent the mean of six separate experiments, bars represent the standard error.

Figure 4.5. The effect of vinblastine sulphate on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



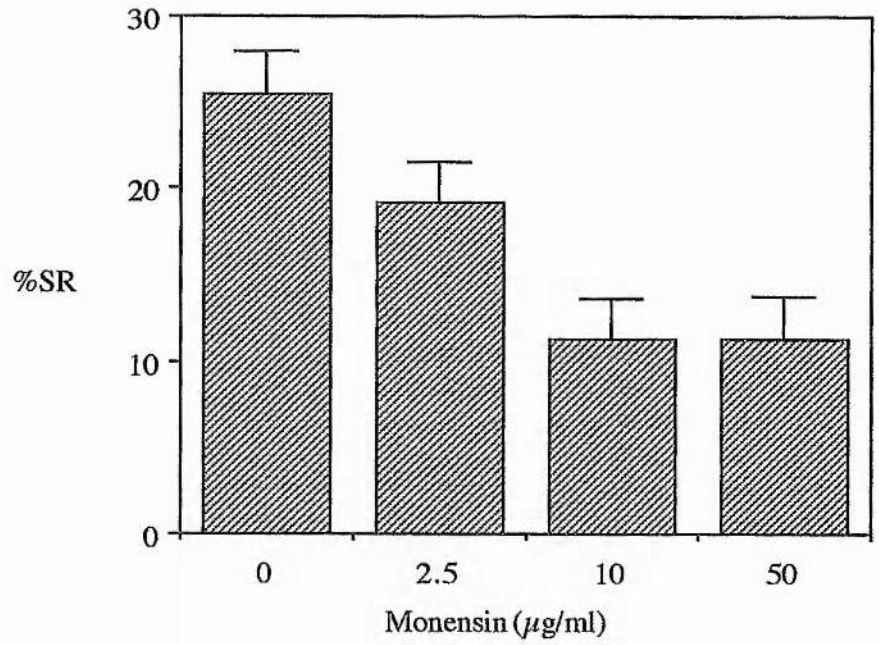
Vinblastine sulphate was incubated with the effector cells at final concentrations of 50, 100 or 200 $\mu g\ ml^{-1}$ prior to the addition of the target cells and remained during the assay. Positive controls were performed with MS. Values represent the mean of three separate experiments, bars represent the standard error.

Figure 4.6. The effect of colchicine on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



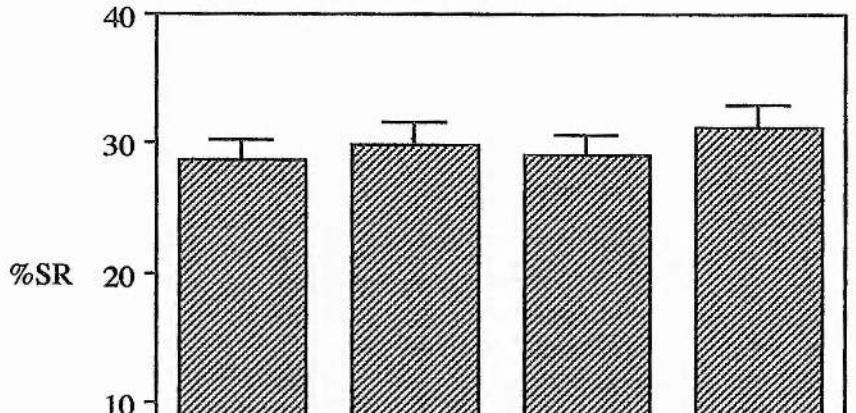
Colchicine was incubated with the effector cells at concentrations of 50, 100 or 400 $\mu g\ ml^{-1}$ prior to the addition of the target cells and remained during the assay. Positive controls were performed with MS. Values represent the mean of three separate experiments, bars represent the standard error.

Figure 4.7. The effect of monensin on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



Monensin was incubated with the effectors at concentrations of 2.5, 10 or 50 $\mu g\ ml^{-1}$ before washing the treated effector cells prior to the addition of the target cells. Values represent the mean of six separate experiments, bars represent the standard error.

Figure 4.8. The effect of antioxidant enzymes on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector cells from *Ciona intestinalis in vitro*.



Superoxide dismutase at 280 u ml^{-1} and catalase at 1,150 u ml^{-1} (final concentrations) were incubated with the effector cells for 15 minutes prior to the addition of the target cells and remained during the assay. For positive controls, the antioxidant enzymes were replaced with MS. Values represent the mean of six separate experiments, bars represent the standard error.

4.4. Discussion

In this chapter, cytotoxic activity by effector cells, enriched from the circulating blood of *C. intestinalis,* towards mammalian tumour cells *in vitro* was found to be reduced by chelation of divalent cations and by inhibition of cellular processes. By contrast, the inclusion of antioxidant enzymes to remove superoxide anions and hydrogen peroxide was found not to affect cytotoxic activity.

The results of experiments using the divalent cation chelators indicate that cytotoxic activity by the effector cells of *C. intestinalis* involves a requirement for both calcium and magnesium ions. Two stage divalent cation dependencies involving first binding and the the target cell lysis have been reported in mammalian (Hiserodt *et al.*, 1982), anuran (Ghoneum and Cooper, 1987), and teleost (Carlson *et al.*, 1985) cytotoxic cells and this aspect of the mechanism requires further investigation for *C. intestinalis*.

The finding that cytotoxic activity is inhibited by sodium azide, a reversible inhibitor of energy metabolism which blocks electron transfer (Carlson *et al.*, 1985), demonstrates that cellular energy metabolism is required for target cell lysis by *C. intestinalis* haemocytes . Agents which affect the cytoskeleton were also found to inhibit cytotoxic activity. Cytochalasin B, disrupts the microfilaments functioning in cell movement (Fulton, 1984), so with *C. intestinalis* may have inhibited cytotoxicity preventing contact formation between the effector and the target cells. Vinblastine sulphate and colchicine are known to disrupt microtubule assemblies and prohibit microtubule dependent movement by blood cells (Fulton, 1984). However, intact microtubule assemblies are also commonly thought to be necessary for cellular secretion (Fulton, 1984). Therefore, vinblastine sulphate and colchicine may inhibit cytotoxic activity by the effector cells from *C. intestinalis* through either mechanism. Monensin is known to specifically block cellular secretion by causing a partial Na/K

equilibrium within cells and to interrupt the vesicular traffic of Golgi-derived vesicles to cell membranes (Carlson *et al.*, 1985). In the present study, monensin was found to inhibit the lysis of the target cells in a dose-dependent manner, indicating that target cell killing is effected through the secretion of one or more factors from the effector cell. Parrinello *et al.* (1993), found that Ca^{2+} dependent factors present within sonicated haemocyte debris from *C. intestinalis* lyse sheep erythrocytes. Whether equivalent lytic factors are actively secreted from intact *C. intestinalis* haemocytes and cause lysis of target cells is unknown.

Finally, in the present study antioxidant enzymes were used to test whether oxidative damage by super oxide radicals and/or hydrogen peroxide mediate cytotoxic activity by the haemocytes of *C. intestinalis*. It was found that inclusion of SOD and catalase in the cytotoxicity assay, even at very high concentrations, produced no inhibition of cytotoxic activity by the haemocytes. Therefore, despite that phagocytes from *C. intestinalis* are capable of a respiratory burst (Bell and Smith, 1994), it seems unlikely that superoxide anions and hydrogen peroxide are involved in the mechanism of cytotoxic activity. Natural killer cell activity, unlike other mammalian antibody-dependent cytotoxic activities (Dongrong *et al.*, 1993), is also unaffected by the antioxidant enzymes catalase and superoxide dismutase (Duwe *et al.*, 1985).

This chapter demonstrates that cytotoxic activity by the haemocytes of *C. intestinalis* involves energy metabolism, a requirement for divalent cations and the active secretion of cytotoxic factor(s) from the effector cells. It is also shown that the phenomenon is probably not mediated by superoxide anion or hydrogen peroxide production. Therefore, in these respects, the mechanism(s) of non specific cytotoxic activity employed by the *C. intestinalis* haemocytes are similar to those by mammalian NK and teleost non-specific cytotoxic blood cells.

Chapter Five
Morphology, ultrastructure and adherence characteristics of the cytotoxic cell population in *Ciona intestinalis*.

5.1. Introduction.

As summarised in section 3.4, a population of cells in the circulating blood of the solitary ascidian, *Ciona intestinalis* effect cytotoxic activity against a range of mammalian target cells *in vitro*. This activity is dependent upon divalent cations, and involves active cellular metabolism and cellular secretion (see section 4.4). Whilst it has been found that the effector cells are enriched in the upper two bands, when separated by density gradient centrifugation (see section 3.4), their morphology has yet to be fully described.

In the past, vertebrate and invertebrate immune cells have been characterised upon their functional, morphological and adherent properties. To date, most circulatory cytotoxic cells identified in invertebrates are macrophage-like (see section 1.2.5). Whether the cytotoxic cells in *C. intestinalis* are also macrophage-like is unknown.

Mammalian cytotoxic cells may be routinely enriched by nature of their differential adherence to glass beads (Shortman *et al.*, 1971) or nylon wool (Julius *et al.*, 1973). Whilst the phagocytic cells in *C. intestinalis* are known to be glass adherent (Smith and Peddie, 1992), it is unclear whether this property is shared by the cytotoxic cells which occur in the same enriched cell bands. Accordingly, this chapter aims to establish the adherence, morphological and ultrastructural nature of the cytotoxic effector cell population in *C. intestinalis*.

5.2. Materials and methods.

5.2.1. Collection and maintenance of animals

The collection and maintenance of animals was as described in section 2.2.1.

5.2.2. Collection of blood cells

The animals were bled according to the method described in section 2.2.2.

5.2.3. Preparation of effector cells

The blood cells were separated by continuous density gradient centrifugation according to the method previously described in section 3.2.4. The enriched effector cells (bands 2 & 3), or mixed blood cells in MAC, were washed twice in marine saline at 800 g for 10 min, resuspended in MS at the required concentration and kept on ice until needed.

5.2.4. Fractionation of cells on glass beads.

Siliconised ('Sigmacote' 5% dimethyldichlorosilane in $CHCl_3$) glass beads (300-600 μm) were placed in a BioRad glass column (internal diameter 9 mm height 8 cm) and incubated with degassed MS for 1 hour to stabilise the column. Six millilitres of mixed blood cells in MS at a concentration of 2.5×10^6 ml^{-1} were added to the column and incubated for 30 min at 20°C. Non-adherent cells were eluted by rinsing the column with 50 ml MS, while adherent cells were removed by incubating the column with 10 ml EDTA buffer for 30 min and rinsing through with a further 50 ml of EDTA buffer (10 mM EDTA; 0.1 M glucose; 15 mM trisodium citrate; 13 mM citric acid; 0.45 M NaCl; pH 7.0). The cell suspensions were washed twice at 800 g for 10 minutes in MS and resuspended in 1 ml of MS. The cell concentration was determined each of the cell preparations, using an new improved Neubauer haemocytometer, and was standardised to 1×10^7 ml^{-1} before measuring the cytotoxic activity.

5.2.5. Fractionation of cells on nylon wool.

Nylon wool (Polysciences Inc., Warrington, USA) was washed in a beaker of boiling distilled water covered with foil for 10 min. The beaker and contents were allowed to cool to 20°C, the water decanted and the nylon wool allowed to drain. This washing procedure was repeated six times, the last two washes being with Milli-Q water. The washed wool was then wrapped in filter paper and squeezed before spreading out on fresh filter paper and drying at 37°C for three days. To prepare the columns, 0.6 g of nylon wool was teased apart with clean forceps and packed into a sterile 10 ml plastic syringe. A short length of silicon tubing was attached to the end of the syringe with a screw valve to control the flow. The columns were finally sealed in foil, autoclaved for 15 minutes and stored until use.

The column was equilibriated by incubation with 50 ml of degassed MS for one hour at 20°C. Six millilitres of mixed cell suspension at a concentration of 2.5×10^6 ml^{-1} were loaded on to the column and incubated for 30 min at 20°C. Non-adherent cells were collected by rinsing the column with 50 ml MS. Adherent cells were eluted by incubating the column with 10 ml EDTA buffer (10 mM EDTA; 0.1 M glucose; 15 mM trisodium citrate; 13 mM citric acid; 0.45 M NaCl; pH 7.0) for 30 min, agitating the nylon wool with the syringe plunger and rinsing through with a further 50 ml of EDTA buffer. The cell suspensions were washed twice at 800 g for 10 minutes in MS and then resuspended in 1 ml of MS. Before determining the cytotoxic activity, the cell concentration in each of the cell preparations was standardised to 1×10^7 cells ml^{-1}.

5.2.6. Cytotoxic activity

Cytotoxic activity was measured as described in section 3.2.5, by incubating CFDA labelled target cells, a mouse myelomonocytic leukemic cell line (WEHI, strain 3B),with glass bead or nylon wool fractionated effector cells from *C. intestinalis*

(see sections 5.2.4 & 5.2.5). Each assay consisted of at least four well replicates and was repeated at least three times.

5.2.7. Cytospin preparation of cells for light microscopy

Cell suspensions of 10^5 cells ml^{-1} were spun onto glass slides using a Cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 min and then fixed in methanol for 5 min and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Cytospin preparations were made, first, from a mixed blood cell suspension, second, from each population (bands 1-6) (see figure 3.2), and third, from the adherent or non-adherent cell populations separated by glass bead and nylon wool fractionation (see sections 5.2.5 & 5.2.6). The cell types were identified according to the classification in Table 1.2.

5.2.8. Preparation of cells for transmission electron microscopy

Continuous density gradient enriched effector cell preparations (bands 2 & 3) (see section 3.2.4.) were centrifuged for 5 min at 800 g and the pellet was then pre-fixed for 2 h in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde, 20 % sucrose (pH 7.4) for two hours (Sabatini *et al.*, 1963). The pellet was then gently washed three times in 0.2 M cacodylate buffer and post-fixed in the same buffer containing 1% osmium tetroxide for 15 minutes (Palade, 1952; Hirsch and Fedorko, 1968). Following fixation, the samples were washed twice in cacodylate buffer and dehydrated through a series of ethanols and cleared with epoxypropane. The samples were then embedded in Araldite and polymerized for 36-48 hours at 60°C. Gold sections were cut with glass knives, placed on 200μm grids and stained with uranyl acetate and lead citrate (7 min each) with intermediate washes in distilled water (Reynolds, 1963). Samples were then examined and photographed using a Philips transmission electron microscope at 60 kv. The cell types were identified according to Table 1.2.

5.3. Results

5.3.1. Fractionation of cells on glass beads

Only 83 % of the mixed cell population was recovered following fractionation of the cells on glass beads, with 10 % were found to be non-adherent (Table 5.1). Cytotoxic activity was detectable only in the glass bead non-adherent fraction and was slightly higher (although not significantly) ($p>0.05$) than in the mixed cell population (Table 5.1).

5.3.2. Fractionation of cells on nylon wool.

Difficulty was experienced in removing the adherent cells from the nylon wool column, with ca. 40 % of the mixed cell population recovered following fractionation. Of the total mixed blood cell population, 5 % were found to be non-adherent (Table 5.2). Cytotoxic activity significantly increased from 7.6 ± 1.95 to 13.73 ± 1.31 (means $\pm$ SE) ($p=0.011$) following passage of the effector cell preparation through nylon wool (Table 5.2).

5.3.3. Examination of the Cytospin preparations.

The Cytospin preparations enabled the identification and clear morphological description of several different cell types in *C. intestinalis* following enrichment by density gradient centrifugation.

Cytospins of band 2 contained three different cell types (Fig. 5.1). The majority of the cells were rounded with a large nuclear : cytoplasmic ratio and deeply basophilic cytoplasm (Fig. 5.1). Another type were slightly larger, with a weakly staining cytoplasm and small central nucleus; with the third, less common cell type

characterized by cytoplasm containing large spindle-shaped eosinophilic granules and a small and often eccentric nucleus (Fig. 5.1). Following the classification presented in Table 1.4, the terminology ascribed to these three cell types is undifferentiated cell, hyaline amoebocytes and granular amoebocytes, respectively.

The cytospin of cells in band 3 contained a similar cell profile to band 2, except that there was a greater proportion of undifferentiated cells, and that, cells with lightly staining cytoplasm containing one or more vacuoles were present (Fig. 5.2). Following to the classification given in Table 1.4, this additional cell type was designated a vacuolar amoebocyte.

Cytospin preparations of band 4 contained very few undifferentiated cells, and the granular amoebocytes were absent (Fig. 5.3). Instead, hyaline and vacuolar amoebocytes predominated (Fig. 5.3). Other cells present included those in which the nucleus is displaced to one side of the cell, and, the cytoplasm contained either several (designated morula cells, see table 1.4) or occasionally, a single large vacuole (designated signet ring cells, see table 1.4) containing a grey flocculent material (Fig. 5.3). Interestingly, cells with deep violet-blue granules were present only in this band and constituted less than 1% of the cells (Fig. 5.3). These cells often lysed and released the darkly stained granules which then appeared to form the focus for aggregating hyaline cells (Fig. 5.4 & 5). This cell type has not been described in the literature for *C. intestinalis*, and is not directly comparable with cell types described for other species, but resembles the basophilic or granular cells described by Fuke and Fukomoto (1993) for *Halocynthia roretzi* .

Cytospin preparations of band 5 showed four different cell types (Fig. 5.6). The undifferentiated cells and granular amoebocytes were absent, although some vacuolar amoebocytes, morula cells and signet ring cells were present (Fig. 5.6). Very noticeable, were the cells with highly orange/yellow pigmented inclusions occupying all of the cytoplasm and a small and condensed nucleus which, in

Cytospin preparations, is not obscured by the pigment granules (Fig. 5.6). These coloured cells were the pigment cells (see Table 1.4). All the cells in band 6, had a highly eccentric nucleus and a single large vacuole occupying the remaining cellular space (Fig. 5.7). The vacuole was either empty or, occasionally, as in the previous band, contained a grey flocculent material (Fig. 5.7). According to the classification presented in Table 1.4., these are signet ring cells.

Subsequent examination of Cytospin preparations of the glass bead fractionated cells established that 85% of the non-adherent cells had a deeply basophilic cytoplasm and a high nuclear to cytoplasmic ratio (Fig. 5.8). Far fewer (15%) of these cells were present in the glass adherent population (Fig.5.9). Examination of Cytospin preparations of the nylon wool fractionated cells revealed that 90% of the non-adherent cells had a deeply basophilic cytoplasm and a high nuclear to cytoplasmic ratio (Fig. 5.10). Few (10%) of these cells were present in the cell population adherent to nylon wool (Fig. 5.11).

5.3.4. Transmission electron microscopy of effector cell population.

The effector cell population enriched by density gradient centrifugation (bands 2 & 3) and examined by transmission electron microscopy consisted of vacuolar amoebocytes and undifferentiated cells. The phagocytes had a small nuclear : cytoplasmic ratio, with a cytoplasm containing large vacuoles, small amounts of rough endoplasmic reticulum and protoplasmic extensions (Fig. 5.12) (Rowley, 1982b). In the present study, the undifferentiated cells had a large nuclear : cytoplasmic ratio, the nucleus often showing peripheral condensed chromatin and a prominent nucleolus (Fig. 5.13). The cytoplasm was notable for the lack of differentiation, many free ribosomes, few large mitochondria and some profiles of rough endoplasmic reticulum (Fig. 5.13). These undifferentiated cells were similar in intracellular morphology to those described in ascidians as stem cells (Rowley, 1982b), haemoblast (Milanesi and Burighel, 1978), or lymphocyte (Overton, 1966).

Table 5.1. Cytotoxic activity of cells after fractionation on glass beads.

Cell population	Percentage of total blood cell population	Cytotoxic activity (%SR)
Glass bead adherent	73%	ND
Glass bead non-adherent	10%	9.05 ± 3.25
Mixed cell population	100%	7.09 ± 2.65

Mixed cell suspensions were added to a column containing siliconised glass beads (300-600 μ) and incubated for 30 min at 20°C. Non-adherent cells were eluted with MS, while adherent cells were removed by incubating the column and rinsing through with EDTA buffer. The cell suspensions were washed twice at 800 g before measuring their cytotoxic activity. Values are means ± SE; n=3.

Table 5.2. Cytotoxic activity of cells after fractionation on nylon wool.

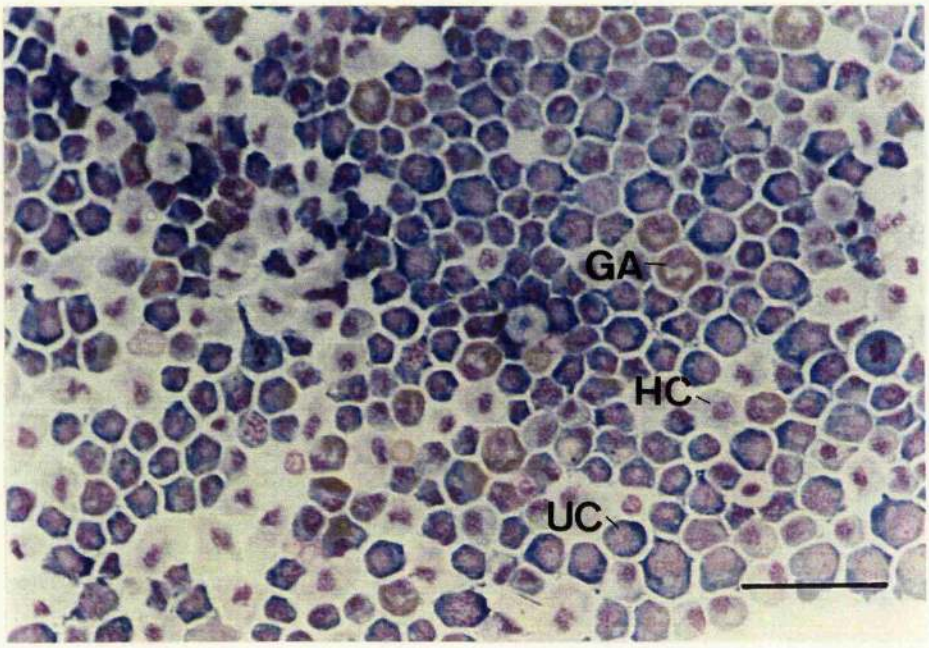
Cell population	Percentage of total blood cell population	Cytotoxic activity (%SR)
Nylon wool adherent	35%	2.4 ± 1.38
Nylon wool non-adherent	5%	13.73 ± 1.31
Mixed cell population	100%	7.60 ± 1.95

Mixed cell suspensions were added to a column containing nylon wool and incubated for 30 min at 20°C. Non-adherent cells were eluted with MS, while adherent cells were removed by incubating the column and rinsing through with EDTA buffer. The cell suspensions were washed twice at 800 g before measuring the cytotoxic activity. Values are means ± SE; n=3.

Chapter Five. Abbreviations on figures.

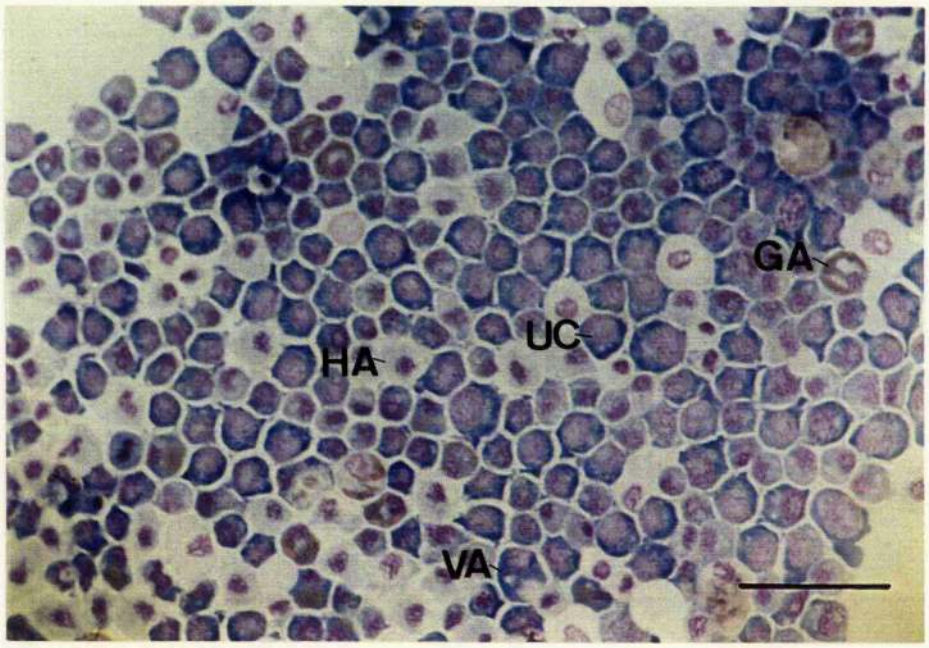
UC	Undifferentiated cell
GA	Granular amoebocyte
HA	Hyaline amoebocyte
VA	Vacuolar amoebocyte
MC	Morula cell
SC	Signet ring cell
VGC	Violet granular cell
VG	Violet granule
AHA	Aggregating hyaline amoebocytes
PC	Pigment cell

Figure 5.1. Cytospin of continuous density gradient enriched cells. Band 2.



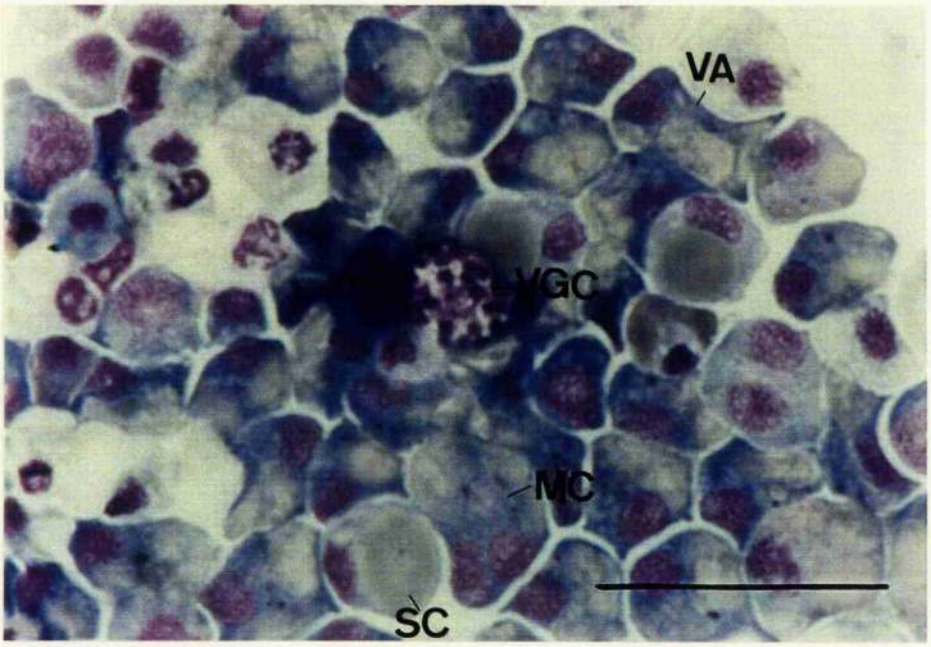
The band of cells was removed from the gradient and washed once in MS before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm.

Figure 5.2 Cytospin of continuous density gradient enriched cells. Band 3.



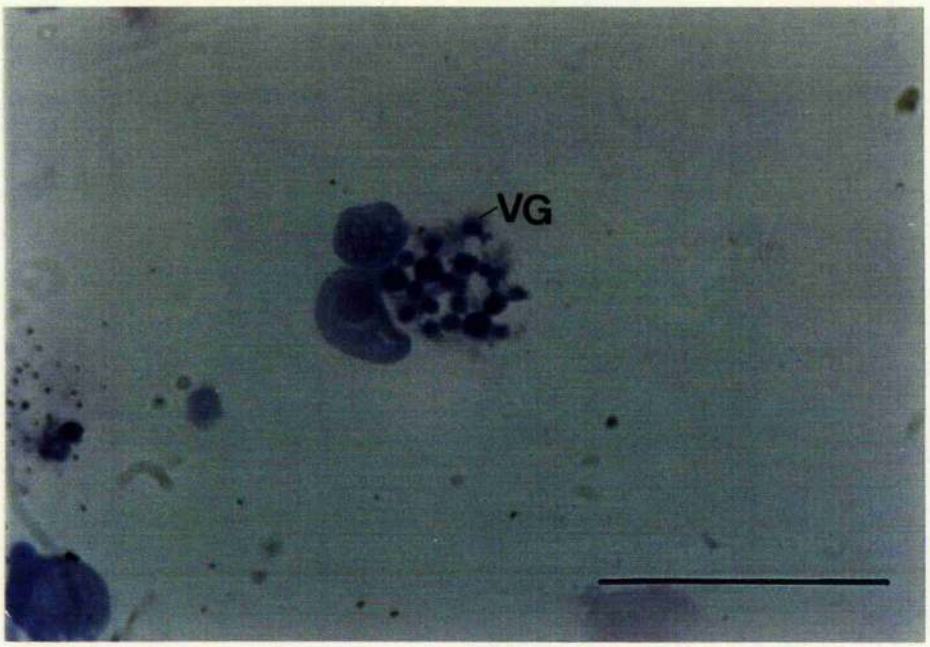
The band of cells was removed from the gradient and washed once in MS before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm

Figure 5.3. Cytospin of continuous density gradient enriched cells. Band 4.



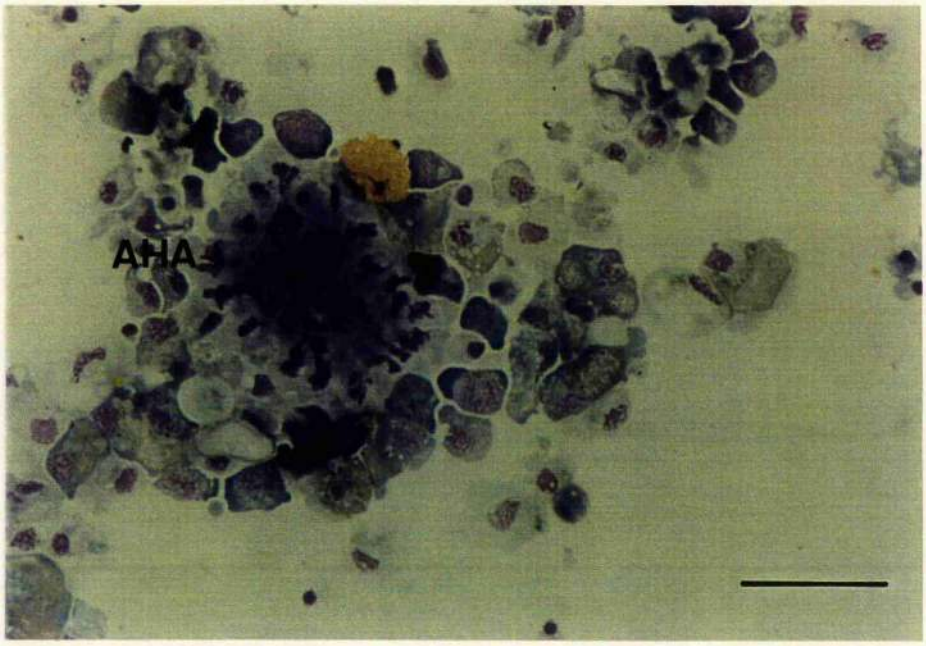
The band of cells was removed from the gradient and washed once in MS before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm

Figure 5.4. Cytospin of continuous density gradient enriched cells. Band 4.



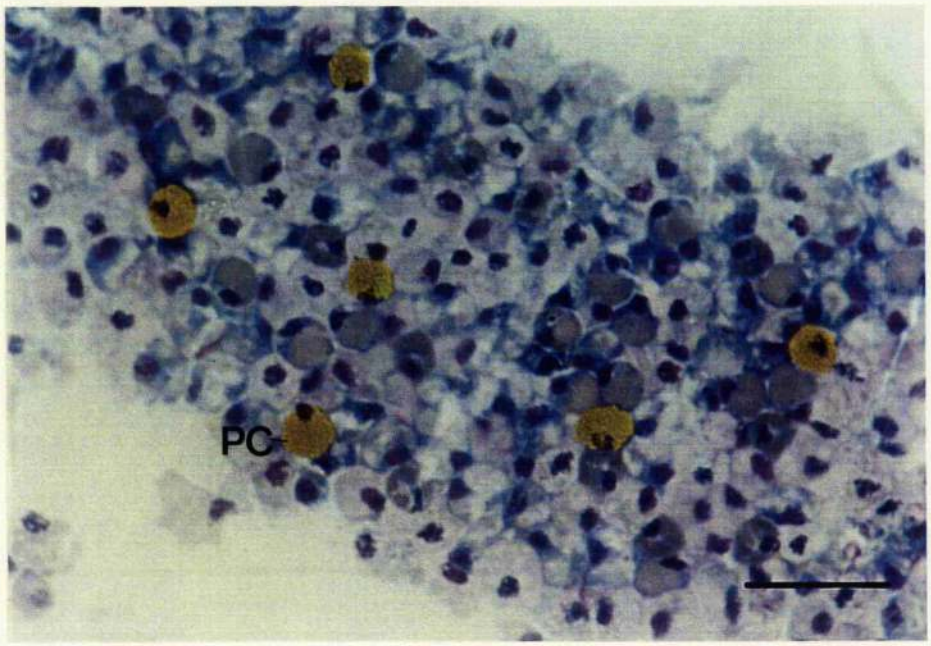
The band of cells was removed from the gradient and washed once in MS before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm

Figure 5.5. Cytospin of mixed blood cells.



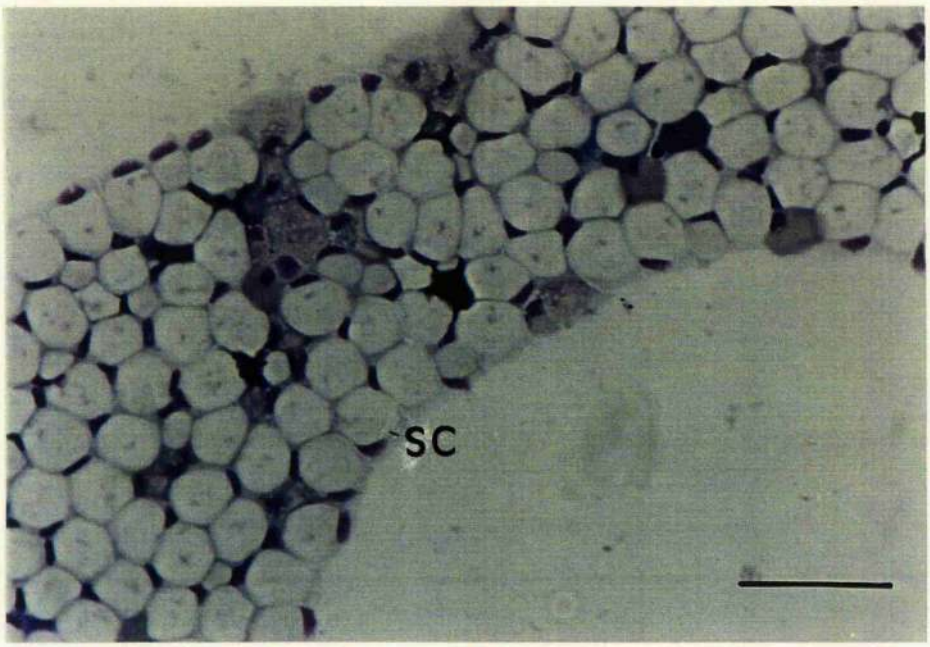
The mixed blood cells were washed once in MS before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm

Figure 5.6. Cytospin of continuous density gradient enriched cells. Band 5.



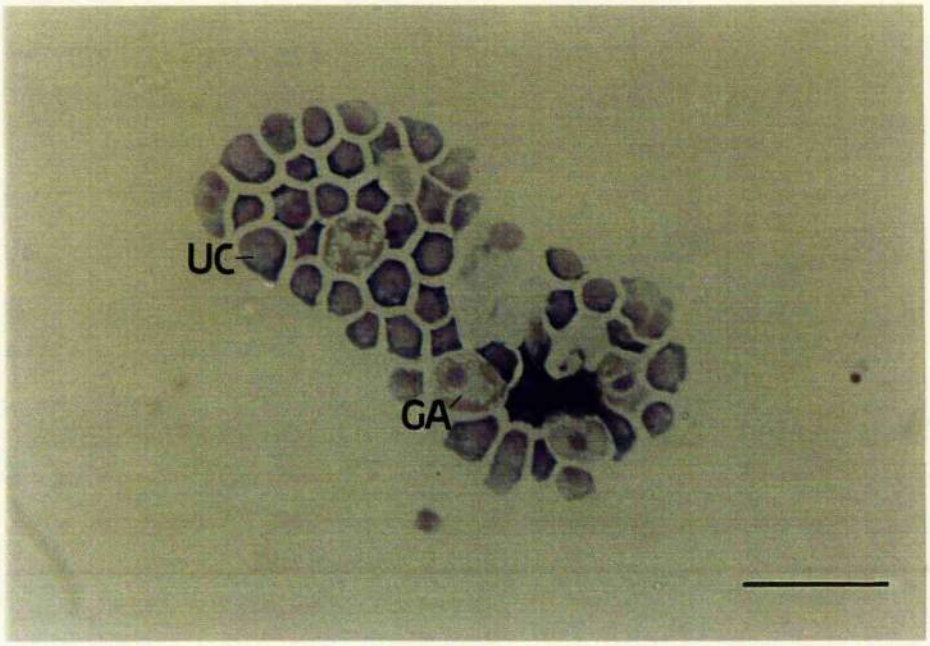
The band of cells was removed from the gradient and washed once in MS before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm

Figure 5.7. Cytospin of continuous density gradient enriched cells. Band 6.



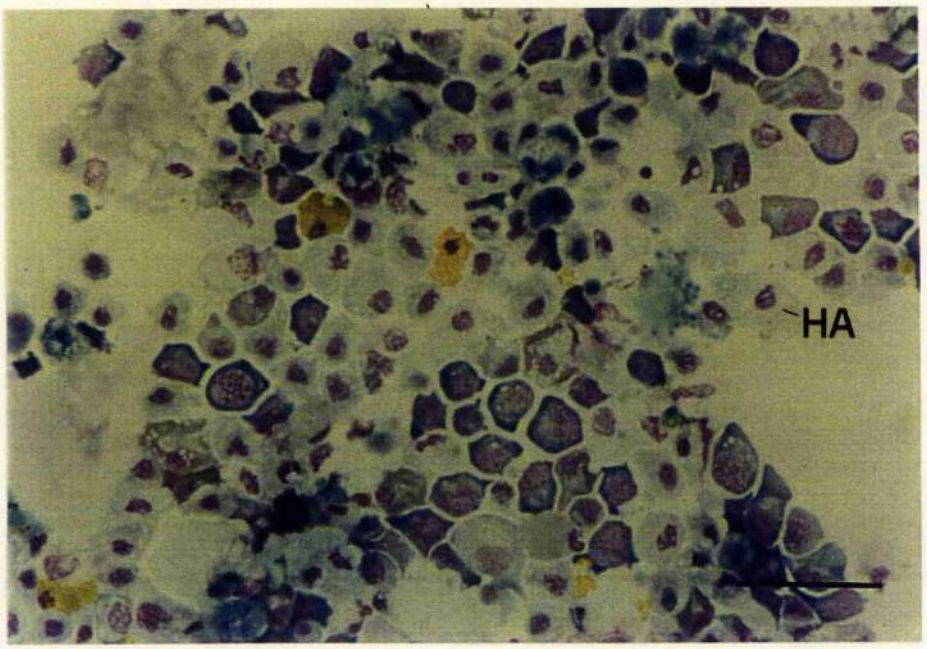
The band of cells was removed from the gradient and washed once in MS before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm

Figure 5.8. Cytospin of blood cells from *Ciona intestinalis* non-adherent to glass beads.



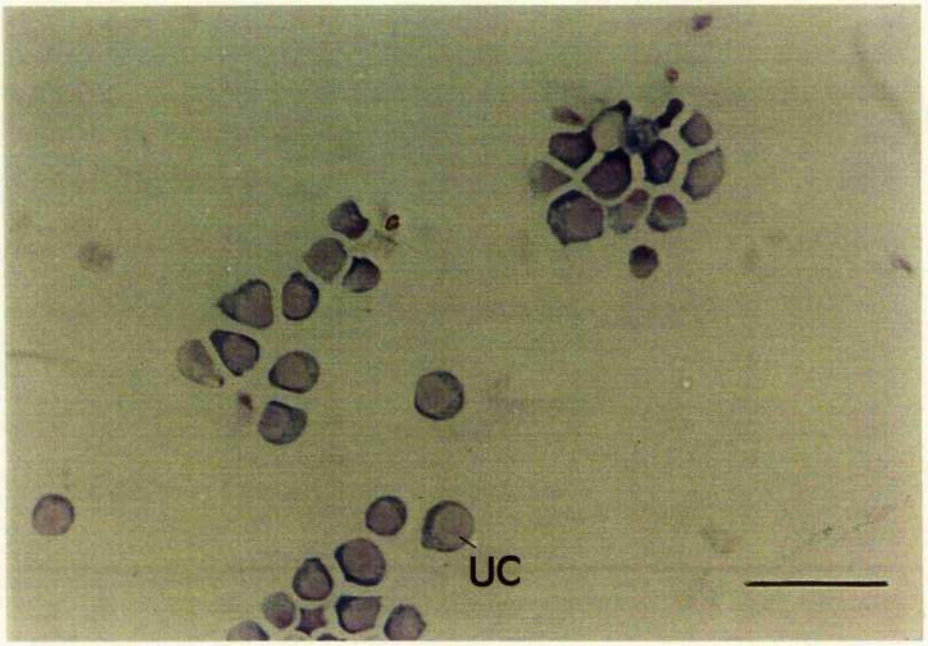
Mixed cell suspensions were added to a column containing siliconised glass beads (300-600 μ) and incubated for 30 min at 20°C. Non-adherent cells were eluted with MS and washed twice at 800 g before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar= 20 μm.

Figure 5.9. Cytospin of blood cells from *Ciona intestinalis* adherent to glass beads.



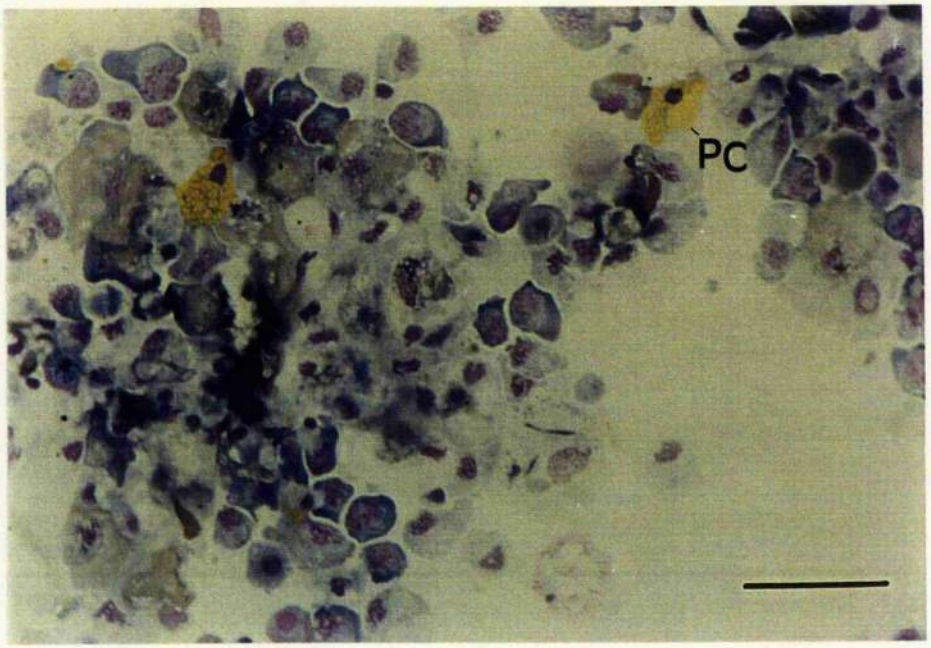
Mixed cell suspensions were added to a column containing siliconised glass beads (300-600 μ) and incubated for 30 min at 20°C. Adherent cells were removed by incubating the column and rinsing through with EDTA buffer and washed twice at 800 g before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm.

Figure 5.10. Cytospin of cells non-adherent to nylon wool.



Mixed cell suspensions were added to a column containing nylon wool and incubated for 30 min at 20°C. Non-adherent cells were eluted with MS and washed twice at 800 g before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm

Figure 5.11. Cytospin of cells adherent to nylon wool.

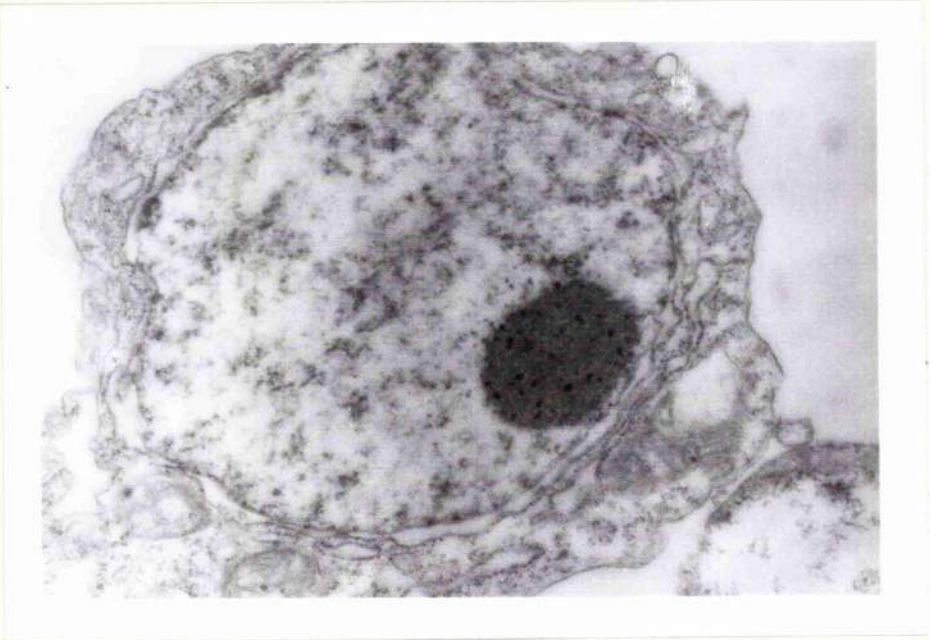


Mixed cell suspensions were added to a column containing nylon wool and incubated for 30 min at 20°C. Adherent cells were removed by incubating the column and rinsing through with EDTA buffer and washed twice at 800 g before spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland) Scale bar = 20 μm

Figure 5.12. Transmission electron micrograph of a phagocyte.

Cells in bands 1 and 2 were pre-fixed for 2 h in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde, 20 % sucrose (pH 7.4) for two hours. The pellet was washed three times and post-fixed in 1% osmium in 0.2 M cacodylate buffer (pH 7.4) for 15 minutes. The samples were washed twice in cacodylate buffer, dehydrated through a series of ethanols and cleared with epoxypropane. Then embedded in araldite and baked for 36-48 hours in a 60°C oven. Gold sections were stained with uranyl acetate and lead citrate, then examined and photographed using a Philips Transmission Electron Microscope at 60 Kv. Magnification x 18 300.

Figure 5.13 Transmission electron micrograph of an undifferentiated cell.



Cells in bands 1 and 2 were pre-fixed for 2 h in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde, 20 % sucrose (pH 7.4) for two hours. The pellet was washed three times and post-fixed in 1% osmium in 0.2 M cacodylate buffer (pH 7.4) for 15 minutes. The samples were washed twice in cacodylate buffer, dehydrated through a series of ethanols and cleared with epoxypropane. Then embedded in araldite and baked for 36-48 hours in a 60°C oven. Gold sections were stained with uranyl acetate and lead citrate, then examined and photographed using a Philips Transmission Electron Microscope at 60 Kv. Magnification x 23 180.

5.4. Discussion.

There is considerable variation between authors as to the classification of ascidian blood cells (see section 1.4.4). Difficulties in obtaining a consensory classification have arisen because of interspecific variation in blood cells, differences in the methods of fixation and observation, and the failure to link functional properties with morphological characteristics (see section 1.4.4). The identification of cell types in this chapter was carried out in accordance with the scheme presented in Table 1.2.

Superficially, the unidentified cell type found in band 4, resembles the basophils of mammals, which are also characterised by deep violet-blue granules and found in very small numbers in the circulation (see Roitt *et al.*, 1989). Mammalian basophils degranulate in response to antigen to release a suite of pharmological mediators (Roitt *et al.*, 1989). In *C. intestinalis* this rare cell type, present in band 4, may be responsible, through a similar degranulation mechanism, for the initiation of haemostasis (see section 5.3.3), the release of antibacterial factors (Findlay and Smith, 1995), and the release of opsonins in response to foreign challenge (Smith and Peddie, 1992).

Transmission electron microscopy shows that the cytotoxic cell population enriched by density gradient centrifugation consists principally of phagocytes and undifferentiated cells. Separation of this population, by differential adherence to nylon wool or glass, shows that the cytotoxic cells reside in the non-adherent fraction. The glass or nylon wool non-adherent fractions consist of mostly undifferentiated cells. It is reasonable to hypothesize therefore that the non-adherent undifferentiated cells are responsible for cytotoxic activity in *C. intestinalis*.

Nylon wool non-adherent cells are the mediators of cytotoxic activity in salmonids (Hayden and Laux, 1985, Moody *et al.*, 1985) and catfish (Evans *et al.*, 1984). Whilst shark cytotoxic cells are glass-adherent macrophages (McKinney *et al.*,

1986). Evans and Cooper (1990) have suggested that cytotoxicity is mediated by macrophage-like cells in all animals at a phylogenetically lower level than teleosts. The present study, is the first to link cytotoxic activity in an invertebrate to an undifferentiated cell, however, further investigations are necessary to ascertain the interaction between the effector and target cells.

Chapter Six
The cationic requirements, morphology and ultrastructure of the interaction between effector and target cells

6.1 Introduction.

In vertebrates, cytotoxic cells cannot be defined simply in terms of morphology; their ultrastructural characteristics tend to differ from group to group, however, there are a few features that appear to be common to most cytotoxic cells. For example, teleost non-specific cytotoxic cells (NCC) have been described as small, agranular, lymphocyte-like cells (Evans and McKinney, 1990). They resemble mammalian NK cells in possessing a kidney shaped nucleus with margination of nuclear chromatin, a high nuclear : cytoplasmic ratio and a prominent Golgi apparatus (Evans and McKinney, 1990). In frogs, a peripheral blood cell, morphologically similar to NCC, constitutes almost all of the cytotoxic target-binding cells (Smith *et al.*, 1988). However, by contrast, the spontaneous cytotoxic cell population in elasmobranchs comprises phagocytic macrophages, not lymphoid cells (Evans and McKinney, 1990).

In mammals, the mechanisms underlying cell mediated cytotoxic activity typically involve the close interdigitation of the effector and target cell membranes and the active secretion of either calcium-dependent pore-forming factors or perforin independent factors, which generate apoptosis and membrane lysis in the target cell (Doherty, 1993). There is often a change in shape of the effector cells accompanied by reorientation of vesicles towards the target-effector interface (Zychlinsky *et al.*, 1988). In particular, the cytotoxic cells form an intimate contact area with their targets, leading to the formation of the so-called 'closed chamber' between the effector and target cell (Zychlinsky *et al.*, 1988). This provides ideal conditions for enhancing the lethal potential of various lytic factors (Zychlinsky *et al.*, 1988).

In chapters three and four, cytotoxic activity has been identified in the non-adherent blood cell population of the solitary ascidian, *C. intestinalis*. As yet, the morphology of the effector cells is unclear, and whether the activity is dependent upon the

formation of a similar 'closed chamber' with the target cells or involves toxic factors released by the cells in the vicinity of the targets independent of effector : target cell bonding. To date, over 68 metabolites that are cytotoxic to a variety of tumour cells lines have been isolated from the homogenates of a variety of ascidian species (see review by Davidson, 1993).

The present chapter is aimed at describing the morphology and ultrastructure of the cytotoxic target-binding cells in *C. intestinalis* and nature of their association with the target cells. The inhibition of conjugate formation should clarify whether or not formation of E:T conjugates are prerequisites for cytotoxic activity, whilst, ultrastructural examination of effector and target cell preparations by TEM may indicate if 'closed chambers' are formed.

6.2 Materials and Methods.

6.2.1. Collection and maintenance of animals

The collection and maintenance of animals was as described in section 2.2.1. The animals were bled according to the method previously described in section 2.2.2.

6.2.2. Preparation of effector cells.

The blood cells of *C. intestinalis* were separated on a 60% Percoll continuous density gradient as described in section 4.2.2. The enriched effector cells (bands 2 & 3) were washed twice in marine saline (MS) at 800 g for 10 min, resuspended in MS at 2 x 10^7 ml^{-1} and stored on ice until use (see section 3.2.4).

6.2.3. Preparation of target cells.

The target cells from a mouse myelomonocytic leukemic line (WEHI, strain 3B) were washed in MS I and resuspended in MS at a concentration of 2 x 10^6 ml^{-1}.

6.2.4. Preparation of effector and target cells.

Effector and washed target cells were incubated together in MS at a ratio of 10:1 for 10 minutes prior to preparation for light, scanning or transmission microscopy. Preliminary trials showed that a 10 minute incubation period allowed for the formation of conjugates without target cell disintegration. Agarose was then prepared by dissolving a 1:5 blend of agarose (Sigma types I and IV) in boiling water to make a 2% solution which was stored in 2 ml aliquots at 4°C. As required, each aliquot was melted, cooled to 45°C, supplemented with 1 ml of triple strength marine saline to give the correct salt concentration and then held at 39°C in a water bath. Meanwhile, target (150 μl of 10^6 ml^{-1}) and effector cells (150 μl of 10^7 ml^{-1}) were incubated together for 10 minutes at 20°C (see above), then gently centrifuged (200 g) for 5 minutes and the supernatant discarded. The target and effector cell pellet was then gently resuspended in 50 μl MS and subsequently dispersed in 50 μl of the prepared molten agarose. This cell suspension was quickly spread over agarose pre-coated glass slides and after air drying for two minutes, stored in dishes filled with MS. The proportion of 100 target cells which had conjugated effector cells were recorded under light microscopy (Lietz Diaplan), from each slide. Three replicate slides were prepared for each treatment.

6.2.5. Cytotoxic activity

Cytotoxic activity was measured as described in section 3.2.5, by incubating CFDA labelled target cells, a mouse myelomonocytic leukemic line (WEHI, strain 3B) (see section 3.2.3),with prepared cytotoxic cells from *C. intestinalis*. Each assay consisted of at least four well replicates and was repeated at least three times.

6.2.6. Divalent cations

To assess the importance of divalent cations for effector to target cell binding and cytotoxic activity. Effector and target cells were incubated together in the presence of EDTA (10 mM), EGTA (10 mM) plus Mg^{2+} (5 mM), or MS. The cytotoxic activity and the coincident number of effector to target cell conjugates formed were measured. Data were analysed with the students *t*-test (Sokal and Rolf, 1981). Results were considered significant when $p \leq 0.05$.

6.2.7. Preparation of cells for light microscopy

Cytospin preparations of incubated effector and target cells (section 6.2.4) were prepared as described in section 5.2.7.

6.2.8 Low temperature scanning electron microscopy

Low temperature scanning electron microscopy (LTSEM) was employed to preserve the structure of the effector-target cell conjugates. This technique facilitates the preservation of conjugates in an undisturbed manner, because the cells retain most or all of their water and are rapidly immobilized and stabilised by the cryofixation (Sargent, 1988). Moreover, the cells are not subjected to chemical or dessicatory modification, thereby reducing the possibility of artifacts (Sargent, 1988).

Effector cells alone, target cells alone or effector to target cell conjugates were prepared for examination. Freshly prepared cell samples (50 μl of ca. 10^6 cells ml^{-1}) were gently pipetted onto the surface of 5 mm^2 0.22 μm Millipore filters. After allowing the MS to drain through the filter, the cells were washed with 1 ml of fresh MS. Care was taken never to allow the sample to dry out completely. The samples were cut to size, placed on a brass stub, immediately cryofixed by plunging into nitrogen "slush", and transferred to the LTSEM stage at -180°C. In the cryochamber (Oxford, CT1500) excess water was allowed to sublimate from the preparation at

120°C for 15 min or until the Millipore filter was exposed. The sample was then sputtered with gold palladium and examined on a Jeol (JSM-35CF) scanning electron microscope at 7 or 10 Kv.

6.2.9. Transmission electron microscopy

Effector-target cell conjugates were centrifuged for 5 min at 800 g and the pellet was then pre-fixed for 2 h in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde, 20 % sucrose (pH 7.4) for two hours (Sabatini *et al.*, 1963). The pellet was gently washed three times and post-fixed in 1% osmium in 0.2 M cacodylate buffer (pH 7.4) for 15 minutes (Palade, 1952; Hirsch and Fedorko, 1968). Following fixation, the samples were washed twice in cacodylate buffer, dehydrated through a series of ethanols and cleared with epoxypropane. The samples were then embedded in Araldite and polymerized for 36-48 hours at 60°C. Gold sections, cut with glass knives, were placed on 200 μm grids and stained with uranyl acetate and lead citrate (7 min each) with intermediate washes in distilled water (Reynolds, 1963) Samples were finally examined and photographed using a Philips transmission electron microscope at 60 kv.

6.3 Results

6.3.1. Divalent cations

In the experiments to measure effector to target cell conjugate formation and related cytotoxic activity, EDTA, a chelator of both magnesium and calcium ions, reduced both effector to target cell binding ($p=0.002$) and cytotoxic activity ($p=0.010$) (Table 6.1). By contrast, EGTA (a chelator of calcium ions only) plus Mg^{2+} had no significant effect on effector to target cell binding ($p=0.882$) but inhibited cytotoxic

activity (p=0.016) (Table 6.1). The ability of Ca^{2+} to facilitate binding was not investigated because a chelator of Mg^{2+} ions alone was not available.

6.3.2 Light microscopy

Examination of the effector and target cells prepared in agarose showed that the target cells often had effector cells bound to them (Fig. 6.1). Light microscopical examination of Cytospin preparations of these effector-target cell conjugates showed cells with a high nuclear to cytoplasmic ratio and a densely basophilic cytoplasm in direct contact with the target cells (Fig. 6.2). Often the effector cells were seen to form 'rosettes around' the target cell (Fig. 6.2). The target cells appeared to be in different stages of cell degradation ranging from healthy to completely lysed (Fig. 6.2). Target cells often had more than one effector cell in contact with them (Fig. 6.2).

6.3.3. Low temperature scanning electron microscopy

Low temperature scanning electron microscopy of effector cells alone or target cells alone showed that the effector cells were 3-5 μm diameter (Fig. 6.3) whilst the target cells were distinguishably larger at 8-10 μm diameter (Fig. 6.4). Examination of effector-target cell conjugates showed that the effector cells were often in close contact with the target cell, the boundary between the effector and target often being indeterminable (Fig. 6.5).

6.3.4. Transmission electron microscopy

In contrast to the effector cell population, which consists of two main cell types; the undifferentiated cells and phagocytes (see section 5.3.4), the target cell population was homogeneous, with a large, irregular kidney shaped or multi-lobed nucleus and in general the surface of the cells was very villous (Fig. 6.6). Examination of the effector-target preparations showed that the target binding cells were readily

distinguishable from the target cells by size (3-5 μm versus 8-10 μm in diameter respectively) (Fig. 6.7). At the ultrastructural level, the target binding cells had a high nuclear to cytoplasmic ratio, a relatively undifferentiated cytoplasm, large mitochondria, many free ribosomes and some small profiles of endoplasmic reticulum (Fig. 6.8). In detail, figure 6.9, shows that the point of contact between the effector and target cells was characterised by close interdigitating processes from the effector cell forming enclosed spaces between the effector and target cell membranes.

Table 6.1. The effect of divalent cations on the percentage conjugate formation between effector and target cells and the related percentage specific release of carboxyfluorescein diacetate (CFDA) from the target cells.

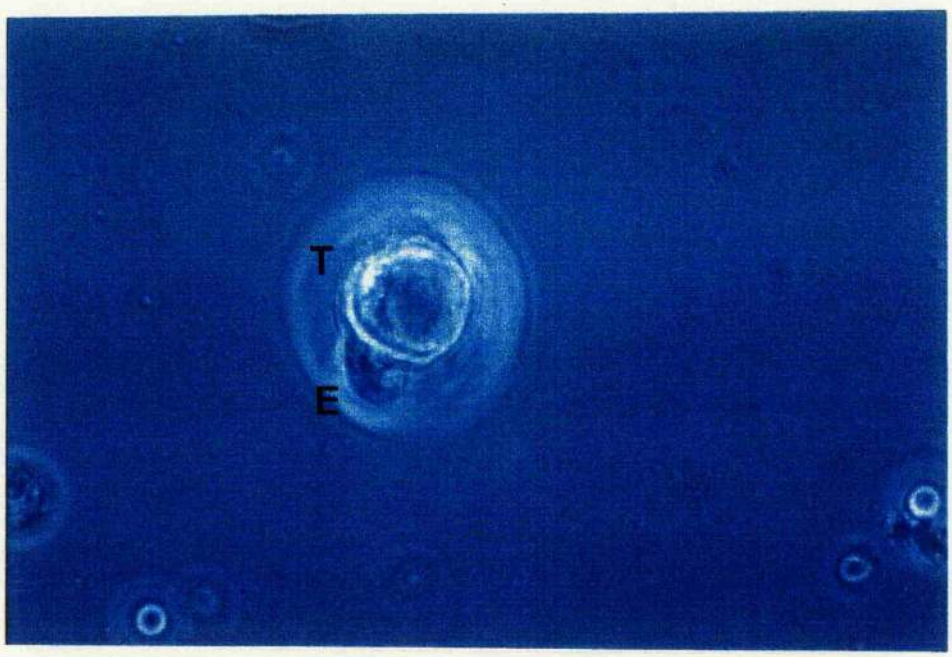
Treatment	Percentage of conjugates (%C)	Percentage specific release (%SR)
10 mM EDTA	4.63 ± 1.13	5.56 ± 2.15
10 mM EGTA + 5 mM Mg^{2+}	19.82 ± 4.81	8.57 ± 1.29
5 mM Ca^{2+} + 5 mM Mg^{2+}	20.68 ± 7.71	24.28 ± 2.15

Target (150 μl of 10^6 ml^{-1}) and effector cells (150 μl of 10^7 ml^{-1}) were incubated together for 5 minutes at 20°C with EDTA, EGTA + Mg^{2+} or for controls, MS, centrifuged and the pellet was resuspended in 50 μl MS and then with 50 μl of prepared molten agarose. The pellet was then spread over agarose pre-coated glass slides, air dried and then stored in saline. The slides were examined by light microscopy and the proportion of 100 target cells bound to effector cells (%C) recorded. Three replicate slides were prepared for each treatment. To measure cytotoxic activity (%SR), effector and labelled target cells were incubated together for 45 min at 20°C at an E:T ratio of 10:1 (see section 3.2.5). Each experiment was repeated at least four times. All values represent the mean ± SE. n=4.

Chapter Six. Abbreviations on figures.

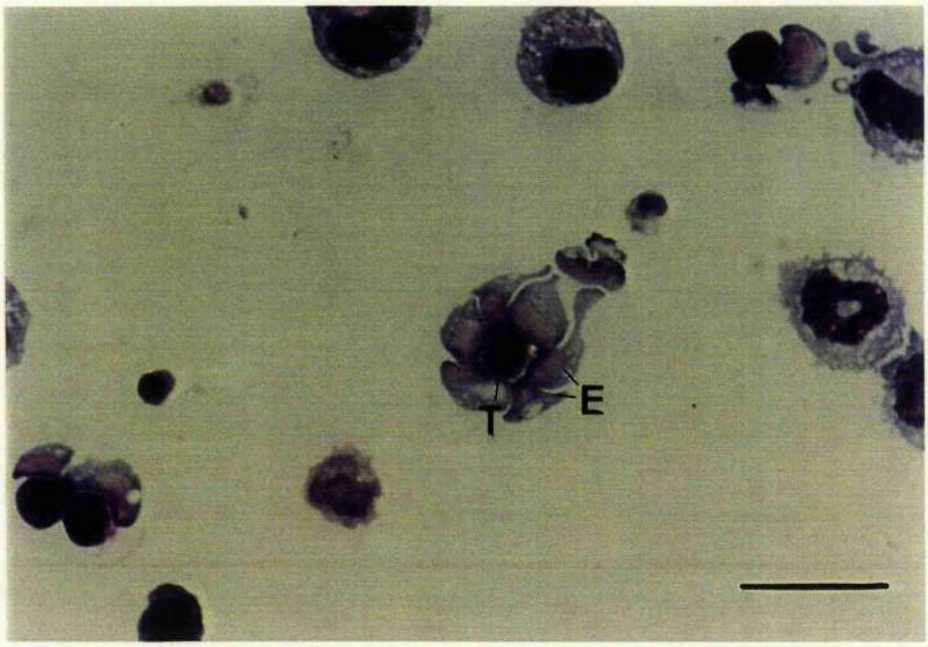
E	Cytotoxic effector cell
T	Target cell (WEHI 3B)

Figure 6.1 Effector to target cell conjugates in agarose



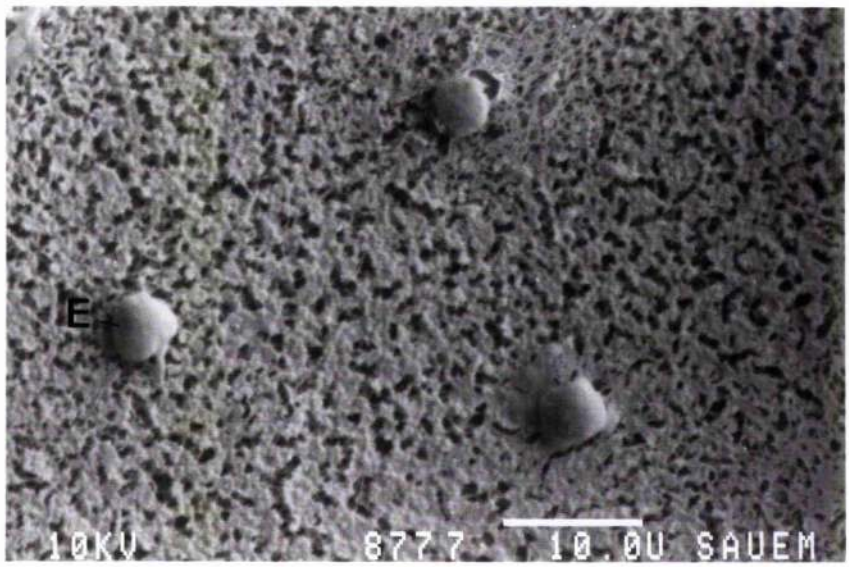
Target (150 μl of 10^6 ml^{-1}) and effector cells (150 μl of 10^7 ml^{-1}) were incubated together for 10 minutes at 20°C, centrifuged, then gently resuspended in 50 μl MS and 50 μl of molten agarose (1:5 blend of Sigma types I and IV). The cell suspension was quickly spread over agarose pre-coated glass slides and examined under light microscopy (Lietz Diaplan). Scale bar = 20 μm.

Figure 6.2. Effector to target cell conjugates on cytospins.



Target to effector cell conjugates were spun onto glass slides using a cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes and then fixed in methanol for 5 minutes and stained using a Romanovsky stain (Diff-Quik staining system, Merz und Dade AG, Switzerland). Scale bar = 20 μm.

Figure 6.3. Low temperature scanning electron micrograph of effector cell preparation.

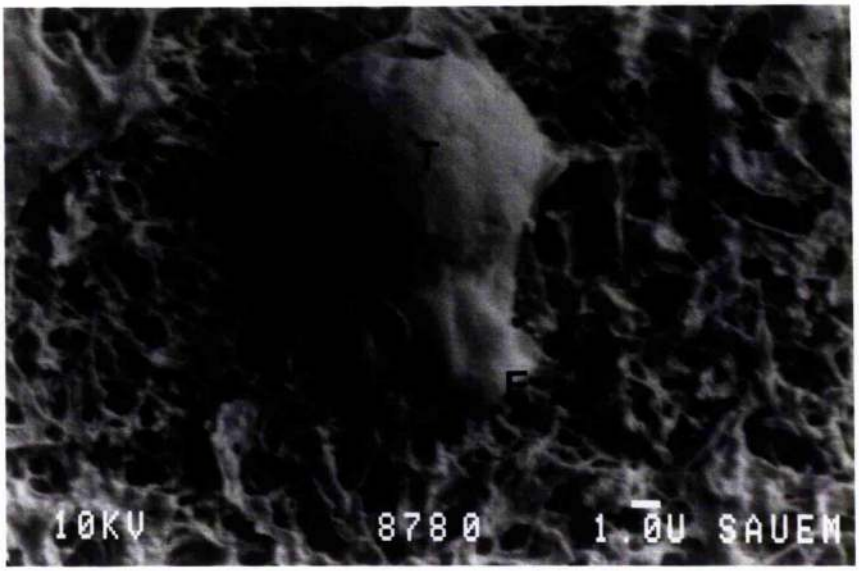


Freshly prepared effector cells (50 μl of ca. 10^6 cells ml^{-1}) were gently pipetted onto the surface of a 5 mm^2 0.22 μ Millipore filter, cut to size, placed on a brass stub, immediately cryofixed by plunging into nitrogen "slush". The sample was transferred to the LTSEM stage at -180°C, where excess water was allowed to sublimate from the preparation at -120°C for 15 min or until the Millipore filter was exposed. The sample was then sputtered with gold palladium and examined under a Jeol scanning electron microscope at 10 Kv. Scale bar = 10 μm.

Figure 6.4. Low temperature scanning electron micrograph of target cell preparation.

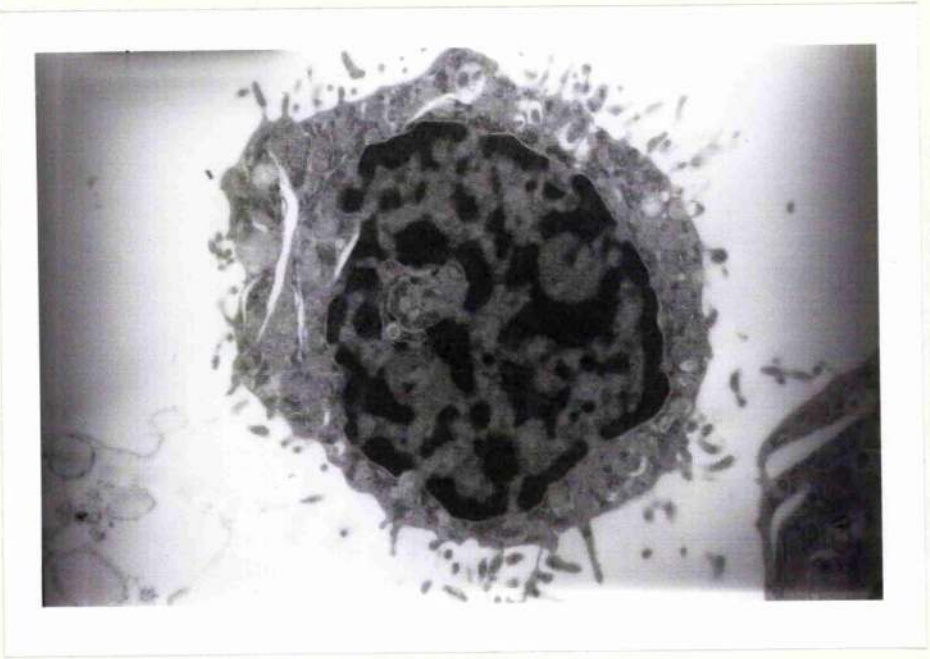
Freshly target cells (WEHI 3B) (50 μl of ca. 10^6 cells ml^{-1}) were gently pipetted onto the surface of a 5 mm^2 0.22 μ Millipore filter, cut to size, placed on a brass stub, immediately cryofixed by plunging into nitrogen "slush". The sample was transferred to the LTSEM stage at -180°C, where excess water was allowed to sublimate from the preparation at -120°C for 15 min or until the Millipore filter was exposed. The sample was then sputtered with gold palladium and examined under a Jeol scanning electron microscope at 7 Kv. Scale bar = 10 μm.

Figure 6.5. Low temperature scanning electron micrograph of effector and target cell preparation.



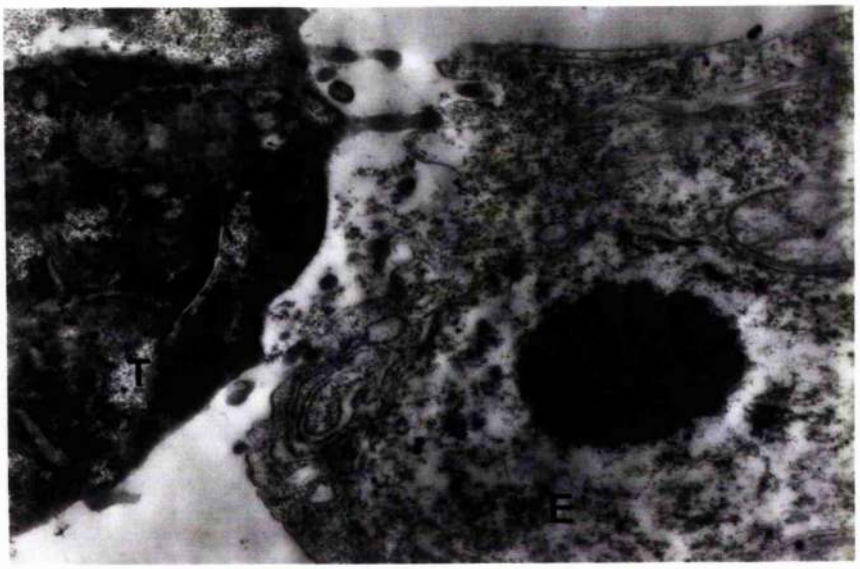
Freshly prepared effector and target cell conjugates were gently pipetted onto the surface of a 5 mm^2 0.22 μ Millipore filter, cut to size, placed on a brass stub, immediately cryofixed by plunging into nitrogen "slush". The sample was transferred to the LTSEM stage at -180°C, where excess water was allowed to sublimate from the preparation at -120°C for 15 min or until the Millipore filter was exposed. The sample was then sputtered with gold palladium and examined under a Jeol scanning electron microscope at 10 Kv. Scale bar = 1 μm.

Figure 6.6. Transmission electron micrograph of a target cell, WEHI 3B.



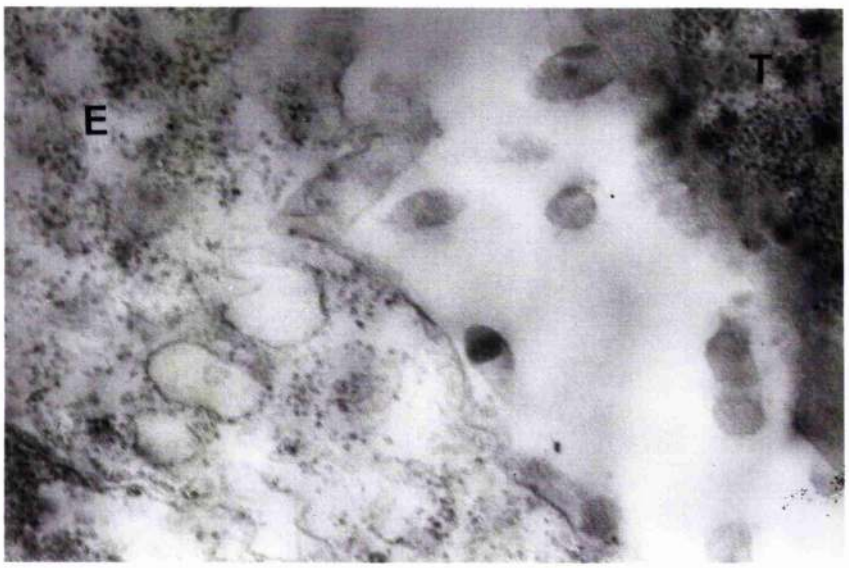
Target cells (WEHI 3B) were pre-fixed for 2 h in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde, 20 % sucrose (pH 7.4) for two hours. The pellet was washed three times and post-fixed in 1% osmium in 0.2 M cacodylate buffer (pH 7.4) for 15 minutes. The samples were washed twice in cacodylate buffer, dehydrated through a series of ethanols and cleared with epoxypropane. Then embedded in araldite and baked for 36-48 hours in a 60°C oven. Gold sections were stained with uranyl acetate and lead citrate, then examined and photographed using a Philips Transmission Electron Microscope at 60 Kv. Magnification x 11,102.

Figure 6.7. Transmission electron micrograph of effector to target cell conjugate.



Effector to target cell conjugates were pre-fixed for 2 h in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde, 20 % sucrose (pH 7.4) for two hours. The pellet was washed three times and post-fixed in 1% osmium in 0.2 M cacodylate buffer (pH 7.4) for 15 minutes. The samples were washed twice in cacodylate buffer, dehydrated through a series of ethanols and cleared with epoxypropane. Then embedded in araldite and baked for 36-48 hours in a 60°C oven. Gold sections were stained with uranyl acetate and lead citrate, then examined and photographed using a Philips Transmission Electron Microscope at 60 Kv. Magnification x 23,400.

Figure 6.8. Transmission electron micrograph of effector to target cell conjugate, detail.



Effector to target cell conjugates were pre-fixed for 2 h in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde, 20 % sucrose (pH 7.4) for two hours. The pellet was washed three times and post-fixed in 1% osmium in 0.2 M cacodylate buffer (pH 7.4) for 15 minutes. The samples were washed twice in cacodylate buffer, dehydrated through a series of ethanols and cleared with epoxypropane. Then embedded in araldite and baked for 36-48 hours in a 60°C oven. Gold sections were stained with uranyl acetate and lead citrate, then examined and photographed using a Philips Transmission Electron Microscope at 60 Kv. Magnification x 41,000.

6.4. Discussion

The results of the series of experiments described in this chapter show that cytotoxic activity by the target-binding cells of *C. intestinalis* is dependent upon effector to target cell contact and involves at least two stages, distinguishable by their divalent cation requirements. The first stage entails recognition and binding of the effector to the target cell, while the second entails lysis of the target cells. The binding of the effector to the target cells is dependent only upon the presence of magnesium ions. Target cell lysis, by contrast, is calcium dependent. Similar two stage divalent cation dependencies have been reported in mammalian (Hiserodt *et al.*,1982), anuran (Ghoneum and Cooper, 1987), and teleost (Carlson *et al.*, 1985) cytotoxic cells.

In the present study, there was some indication that vesicles within the cytoplasm of *C. intestinalis* target-binding cells, but not free effector cells, form near the site of contact during binding. This observation offers some evidence that a factor or suite of factors are secreted by the effector cell into the 'closed chamber' formed between the effector and target cell, and may be responsible for the subsequent death of the target cell. A hypothesis supported by the observation that cytotoxic activity is suppressed by the cellular secretory inhibitor monensin (section 4.3.4).

This study also provides clear evidence that the cells responsible for cytotoxic activity by *C. intestinalis* are undifferentiated cells and not macrophage-like. The ultrastructural morphology of the target binding cells in *C. intestinalis* is similar to the 'lymphocyte-like' cells previously described by Overton (1966) or the 'stem cells' described by Rowley (1982b). Interestingly, there are also striking ultrastructural similarities between the target binding cells of *C. intestinalis* and the cytotoxic (NK) cells of mice (Roder *et al.*, 1978). Both types of cytotoxic cells have a high nuclear : cytoplasmic ratio, scanty cytoplasm, relatively large mitochondria, little endoplasmic reticulum and many free ribosomes. Moreover, the contact structure between the NK

cells of mice and their target cells is similar to that between the target binding cells in *C. intestinalis* and their targets. In mice, the contact area between NKs and target cells usually involves broad irregular or villous surface areas of the effector cells (Roder *et al.*, 1978). This physical interaction is established by point contact of interdigitating villi or processes extending between the cells (Roder *et al.*, 1978). In *C. intestinalis*, similar interdigitation of target and effector cell membranes was observed at the site of contact.

As shown in previous chapters, the cytotoxic effector cells of *C. intestinalis* are active against a range of mammalian tumour cells, dependent upon effector to target cell contact and mediated through a mechanisms similar to those of vertebrate cytotoxic cells (see chapters 3 and 4). They also have similar morphological and ultrastructural features to vertebrate cytotoxic lymphocytes (see chapters 5 and 6). Thus, the cytotoxic target-binding cells of *C. intestinalis* fulfil some criteria required of an evolutionary precursor of the vertebrate lymphocyte (see section 1.5).

Chapter Seven
Proliferation of the blood cells of *Ciona intestinalis in vitro*

7.1. Introduction

Numerous studies concerning the phylogeny of immunity have been aimed at showing a morphological and functional homology between the blood cells of ascidians and vertebrate lymphocytes (see review by De Leo, 1992). Vertebrate lymphocytes are a heterogeneous group of cells characterised by their structural and functional properties. Functionally, they may be cytotoxic (cytotoxic T lymphocytes, NK cells), produce antibodies (B cells), or proliferate in response to allogeneic or mitogenic stimulus (T- & B-cells) (Roitt *et al.*, 1989). Morphologically, they are relatively undifferentiated cells with a large nuclear to cytoplasm ratio, many free ribosomes and a nucleus with dense peripheral chromatin (Roitt *et al.*, 1989).

In preceding chapters, certain functional and morphological similarities between the vertebrate lymphocyte and the undifferentiated cells of *C. intestinalis* have been identified. In particular, it is shown that a population of circulating blood cells from *C. intestinalis* are capable of spontaneous cytotoxic activity against mammalian foreign target cells *in vitro* (see section 3.4). The mechanism of the response appears to be similar to that of vertebrates, in that it requires effector to target cell binding and involves active effector cell metabolism and secretion (see section 4.4). Ultrastructural analysis of effector to target cell conjugates have also revealed, that the cytotoxic cells of *C. intestinalis* have morphological features resembling those of the ascidian cell type described as 'lymphocyte-like' (see section 6.4), and that the nature of the interaction between the effector and target cells is similar to that described for vertebrate effector to target cell bonds.

Equivalent 'lymphocyte-like cells' have been reported to proliferate within haemopoietic crypts of solitary ascidians challenged with allogeneic tissue (Raftos and Cooper, 1991), with mitogens, or human recombinant interleukin-2 (Raftos *et al.*, 1991a,b). In addition, autoradiographic preparations of haemopoietic tissue have

revealed labelled 'lymphocyte-like cells' in the circulating blood, suggesting that cell proliferation outside the nodule can also occur (Ermak, 1976). However, attempts to confirm lymphocyte-like activity by stimulating circulating ascidian blood cells to proliferate *in vitro* with plant lectins have produced conflicting results (Warr *et al.*, 1977; Tam *et al.*, 1976). The lack of information about the functional capabilities of the 'lymphocyte-like cells' in ascidians has prompted several workers to classify the undifferentiated cells as stem cells, haemocytoblasts, haemoblasts or progenitor cells (De Leo, 1992). The aim of this chapter is to investigate proliferation from *C. intestinalis* by developing a culture method for enriched populations of circulating cytotoxic cells and to characterise the proliferating cells on the basis of their response, if any, to mitogens or allogeneic cells.

7.2. Materials and methods

7.2.1. Collection and maintenance of animals

The collection and maintenance of animals was as described in section 2.2.1.

7.2.2. Preparation of plasma

The animals were bled according to the method described in section 2.2.2. and plasma was prepared from the blood as described in section 2.2.6.

7.2.3. Preparation of cell culture medium

The cell culture medium (CCM) was modified a previously developed tissue culture medium for the culture of pharyngeal extracts from *Styela plicata* (Raftos *et al.*, 1990). The modified medium contained RPMI 1640 powder (4.5 g l^{-1}), streptomycin (500 mg l^{-1}), penicillin (1000 units/l), amphotericin B (2.5 mg l^{-1}), sea salts (34 g l^{-1}) and 20% homologous plasma.

7.2.4. Collection of blood cells

The animals were bled according to the method described in section 2.2.2. The effector cells were enriched, in bands 2 & 3, by one stage continuous density gradient centrifugation (see sections 3.2.4), collected aseptically, pooled and then washed and resuspended in CCM usually at a concentration of 1.5×10^6 cells ml^{-1}.

7.2.5. Cytospin and autoradiograph preparations

Cytospin preparations of density gradient enriched cells (bands 2 & 3) (see 3.2.4) of 10^5 cells ml^{-1} were processed for examination by light microscopy as described in section 5.2.7.

Autoradiographs were prepared to confirm the presence of active mitotic cells in the circulating blood. Replication of DNA differs from that of RNA in that thymidine is incorporated into the chromatin. Therefore DNA replication can be detected by the uptake of radioactive thymidine into the nucleus. This uptake can be visualised by overlaying the cells with photographic emulsion which blackens in response to the emitted beta waves from the incorporated thymidine. The cells can also be stained in the conventional manner resulting in stained cells in which any nuclei in DNA synthesis are superimposed with blackened silver grains. Enriched cell suspensions (see section 7.2.4) were flash labelled with tritiated thymidine (^{3}H-TdR 185 GBq $mmol^{-1}$) (65 KBq ml^{-1}) in CCM for 1 h and then washed thoroughly. Cell suspensions at 10^5 cells ml^{-1} were placed in Cytospin sample chambers and spun at 250 g onto glass slides in a Cytospin centrifuge (Shandon, Berks, UK). The preparations were air dried for 30 minutes, then overlaid with K2 gel photographic emulsion (Ilford) and incubated in the dark for 4 days. The slides were then developed and washed before staining as for cytospins (see section 5.2.7). Nuclei superimposed with more than five black spots were considered positive for DNA synthesis. Background levels were checked in every case.

7.2.6. Measurement of cell proliferation

Cultures of enriched cell populations (bands 2 & 3) (see seciton 3.2.4) were prepared in wells of a sterile flat bottomed 96-well tissue culture grade plates and incubated in an atmosphere of 5% CO_2 in air at 15°C. The level of cell proliferation in each microculture was quantified by the measurement of incorporation of radio-labelled thymidine into newly synthesized DNA. Briefly, 25 μl of CCM containing 7.5 kBq ^{3}H-TdR was added to each well and the cells were incubated for a further 16 hours. The cells were then harvested onto glass fibre filter discs using an automated cell harvester (Titertek Multiple Cell Harvester) (Flow Laboratories Ltd., Ayrshire, Scotland). After drying for 2 hours at 60°C the individual filter discs were placed in scintillation vials containing 5 ml of Optiphase Safe scintillation fluid (FFA Laboratory Supplies, Loughborough, Leics, UK). Radioactivity in each vial was counted in a Beckman liquid scintillation counter.

7.2.7. Determination of culture conditions

The optimal cell dose per well and duration of culture were determined using density gradient enriched cell populations (bands 2 & 3) (see section 3.2.4), pooled from four animals. Cell doses ranging from 0.4 to 4.0 x 10^5 cells per well were incubated over 1-4 days (see section 7.2.6). Contamination controls were included in each run.

7.2.8. Measurement of mixed leucocyte-type responses

Mixed leucocyte-type responses were measured using both bidirectional and unidirectional methods.

For the bidirectional assay, density gradient enriched cell populations (bands 2 & 3) (see section 3.2.4) collected from individual animals were cultured alone or with similarly enriched cell populations collected from one other animal. For this, 100 μl of enriched cell population (bands 2 & 3) (see section 3.2.4) (3 x 10^5 cells per well)

from each of 12 animals were cultured (as in section 7.2.6) with 100 μl of CCM. In addition, 50 μl of enriched cell populations (see section 7.2.5) from each of a pair of individuals within the twelve (to give a total of 3×10^5 cells per well) was cultured with 100 μl of CCM. The experiment gave six mixed culture assays prepared from twelve animals, and twelve individual cultures from the same twelve animals.

The unidirectional assay was used to avoid the possibility that stimulator cells from one individual might not provide stimulation for cells collected from another. Accordingly, the cells from eight animals were use as a stimulation cocktail. To prepare these stimulator cells, enriched cell populations (bands 2 & 3) (see section 3.2.4) pooled from eight animals were treated with mitomycin C to arrest cell division. For each millilitre of cell suspension, 50 μl of stock mitomycin C (0.5 mg ml^{-1} in sterile CCM, stored in the dark) was added and then incubated for 20 minutes at 15°C, protected from light. The cells were then washed three times in excess CCM and resuspended in CCM at 1.5×10^6 cells ml^{-1} before use as stimulators.

To prepare the responder cells, enriched cell populations (bands 2 & 3) (see section 3.2.4) from eleven animals were prepared separately in sterile CCM at concentrations of 1.5×10^6 cells ml^{-1}. To control for background proliferation in the responder cells, 100 μl of responder cells were cultured with 100 μl of sterile CCM. In a separate control, to confirm that the mitomycin C had inhibited proliferation in the stimulator cells, 100 μl treated stimulator cells were cultured with 100 μl of sterile CCM. For the experimental cultures, 100 μl responder cells were mixed with 100 μl of treated stimulator cells. All cultures were run in triplicate.

7.2.9. Measurement of mitogen-induced proliferation.

Lipopolysaccharide (LPS) from *Salmonella enteritidis* (Sigma), concanavalin A (Con A) from *Canavalia ensiformis*, type IV-S (Sigma), or phytohaemagglutinin (PHA -B) from *Phaseolus vulgaris* (Sigma) were tested for their ability to stimulate proliferation

by blood cells from *C. intestinalis*. All mitogens were dissolved in sterile CCM at 1 mg ml^{-1} and diluted to give the required concentration per well. Sterile suspensions of cells from individual animals in CCM were prepared as above and incubated in the wells with LPS at final concentrations of 5, 10, 20, 25, 50 or 100 μg ml^{-1}. Other cell suspensions were incubated with Con A at final concentrations of 5, 10, 20, 25, 50, or 100 μg ml^{-1}, or PHA-B at final concentrations of 5, 10, 20, 100 or 200 μg ml^{-1}.

The results are expressed as the ratio of the mean counts per minute (CPM) of experimental cultures to the mean CPM of the control cultures. This ratio is defined as the Stimulation Index (SI). The results were analysed using the students *t*-test and stimulation was considered to be significant if $p \leq 0.05$ (Sokal and Rolf, 1981).

7.3. Results

7.3.1. Examination of Cytospin and autoradiographic preparations

Cytospin preparations of the density gradient enriched cell population (bands 2 &3) (see section 7.2.5) revealed mitotic figures (cells in M-phase) in <1% of the undifferentiated cells (Fig. 7.1). In addition the autoradiographs showed that ca 17 % of undifferentiated cells had 5 or more silver grains superimposed over the nuclear region (cells in S-phase) (Fig. 7.2). The cells in S-phase had a deeply basophilic cytoplasm with a high nuclear to cytoplasmic ratio (Fig. 7.2). No other cell type was noted to be undergoing DNA synthesis. Negligible levels of background developed emulsion were present on all slides.

7.3.2. Culture conditions

The experiments designed to determine the optimal time and cell dose to detect proliferative responses were carried out with enriched cell populations (see section 7.2.5) pooled from eight animals. It was found that a dose of 3×10^5 cells per well

(Fig. 7.3) and an incubation period of 3 days gave optimal responses (Fig. 7.4). These parameters were used in all subsequent experiments.

7.3.3. Mitogen induced proliferation.

Con A , PHA and LPS all significantly stimulated cell proliferation of the density gradient enriched cell population (bands 2 & 3) from *C. intestinalis in vitro* (Figs. 7.5, 6 & 7). With Con A, the stimulation index was 1.68 ± 0.20 at a concentration of 5 μg /ml (p=0.001), and increased to 1.99 ± 0.31 at a concentration of 20 μg/ml (p=0.009) (Fig. 7.5) although concentrations of Con A greater than 50 μg/ml did not significantly stimulate proliferation (p>0.05) (Fig. 7.4). Stimulation by PHA was significant only at 200 μg/ml, a stimulation index of 2.06 ± 0.38 (p=0.01) (Fig. 7.6). Greatest stimulation of cell proliferation was induced by LPS with concentrations of LPS at 10 μg/ml found to give a mean stimulation index of 3.67 ± 0.58 (p=0.005) (Fig. 7.7). Significant stimulation was also recorded with LPS concentrations of 5, 20, 25, 50 and 100 μg/ml (p=0.026, 0.001, 0.042, 0.039, 0.021, respectively) (Fig. 7.7).

7.3.4. Mixed leucocyte-type responses

The baseline levels of uptake of ^{3}H-TdR varied considerably between animals (Table 7.1). However, four of the six pairs of co-cultured cell populations, showed significant increases in the uptake of ^{3}H-TdR above the control values (p<0.05) (Table 7.1). For these pairs of animals, stimulation indices of between 1.67 and 3.57 were recorded (Table 7.1).

With the unidirectional assay, mitomycin C inhibited the uptake of ^{3}H-TdR within the stimulator cell population thereby facilitating accurate recording of proliferation by the responder cells (Fig 7.8). The mean uptake of ^{3}H-TdR by the responder cells significantly increased from 858 ± 159 to 1557 ± 191 CPM following incubation

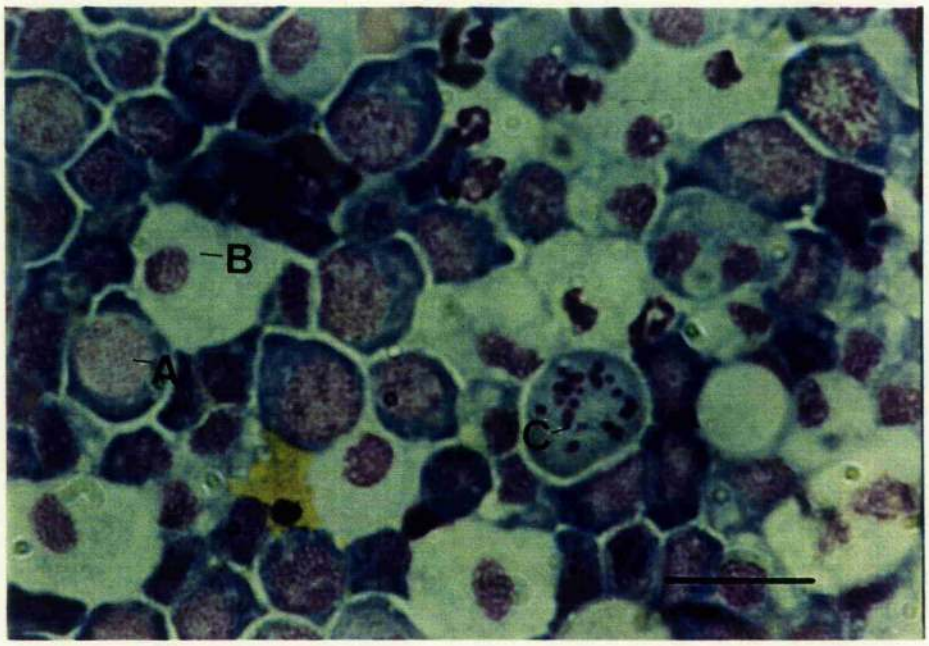
with the treated stimulator cells ($p<0.001$, $n=11$) (Fig 7.8). The responder cell population showed a mean stimulation index of 2.82 ± 0.42 (Fig 7.8).

Table 7.1. The incorporation of ^{3}H-TdR into the undifferentiated blood cells of *Ciona intestinalis* at a concentration of 3 x 10^5 cells per well during a 16 hour pulse in response to allogeneic cells.

Expt.	Mixed Values (CPM)	Control Values (CPM)	Stimulation Index (SI)	Student *t* - test p value
1	1214 ± 123	728 ± 137	1.67	0.020
2	1467 ± 33	707 ± 234	2.08	0.021
3	321 ± 83	90 ± 30	3.57	0.009
4	1241 ± 33	709 ± 227	1.75	0.039
5	280 ± 24	207 ± 42	ND	>0.05
6	130 ± 17	163 ± 52	ND	> 0.05

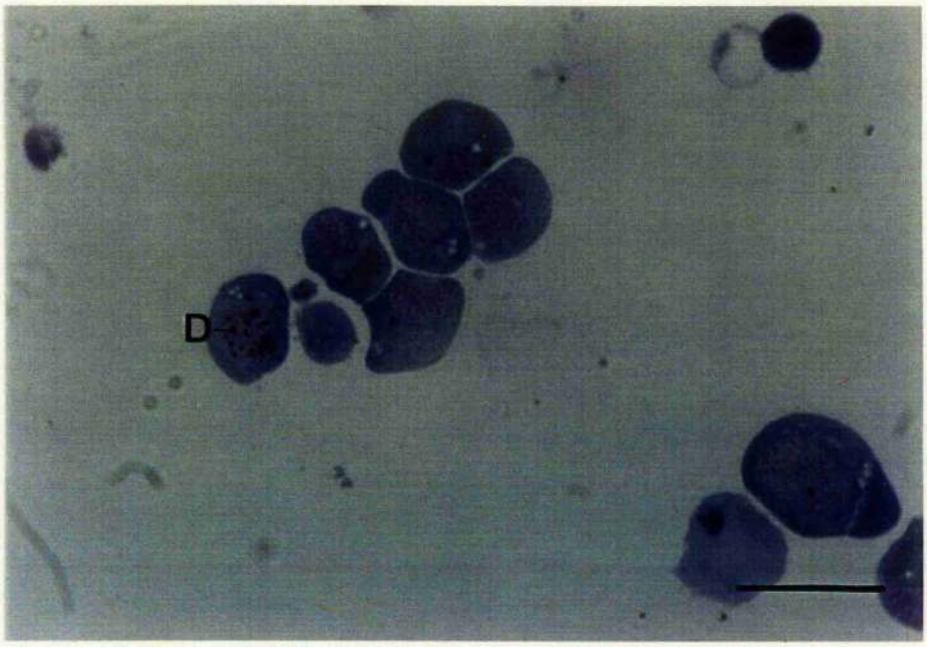
Each culture consisted of 100 μl of density gradient enriched blood cells (bands 2 & 3) (3 x 10^5 cells per well) from each of 12 animals with 100 μl of CCM (see section 7.2.3). In addition, 50 μl of the density gradient enriched blood cells (bands 2 & 3) from both of two different individuals (to give a total of 3 x 10^5 cells per well) was cultured with 100 μl of CCM. The experiment was repeated to give six mixed culture assays prepared from twelve animals. The cultures were incubated, pulsed, then harvested to determine counts per minute as described in section 7.2.9. Results are expressed as the average counts per minute (CPM) with bars as standard error. Results are expressed as the CPM ± SEM. ND = no significant stimulation.

Figure 7.1. Light micrograph of a cytospin preparation of density gradient enriched blood cells (bands 2 & 3) from *Ciona intestinalis*.



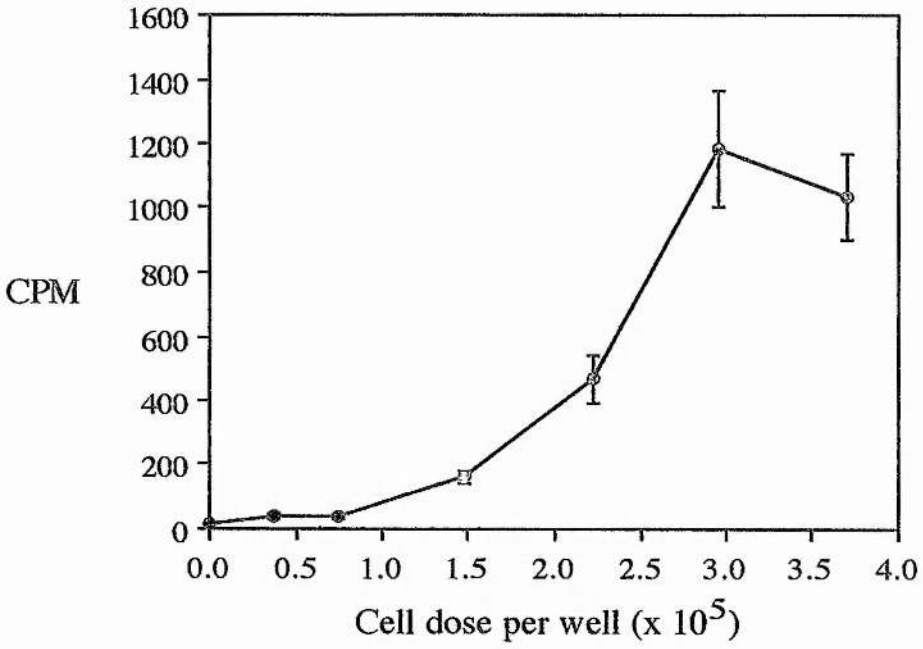
The micrograph shows undifferentiated blood (A) cells and amoebocytes (B) stained with a Romanovsky stain. Mitotic figures are present in <1% of the undifferentiated blood cells (C). Scale bar = 10 μm.

Figure 7.2. Autoradiograph of undifferentiated blood cells from *Ciona intestinalis*.



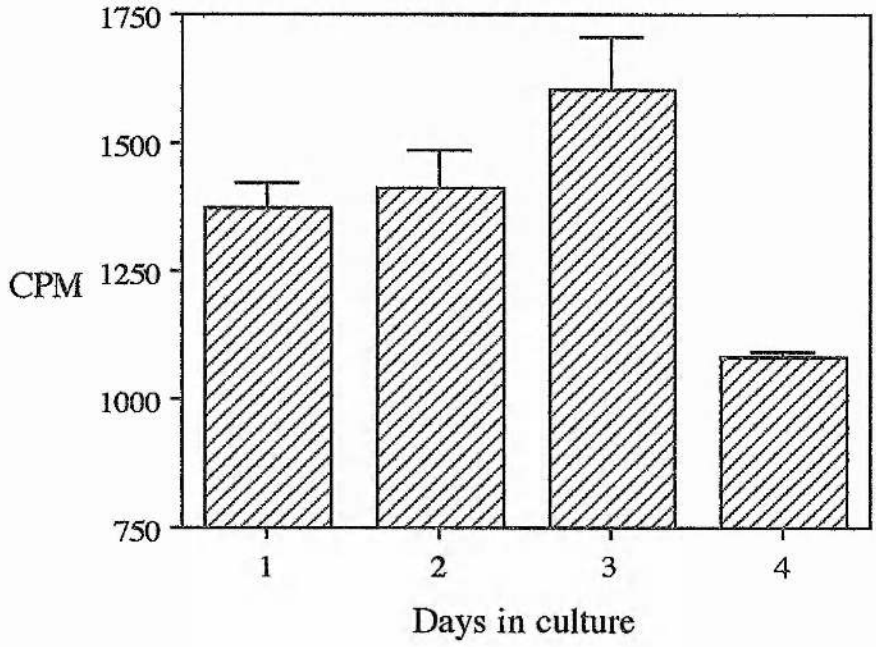
Of the undifferentiated cells ca 17% had nuclei with more than five superimposed silver grains denoting cells in active DNA synthesis (D). Scale bar = 10μm.

Figure 7.3. The incorporation of ^{3}H-TdR into pooled enriched effector cell populations, cultured at different cell doses, during a 16 hour pulse after three days.



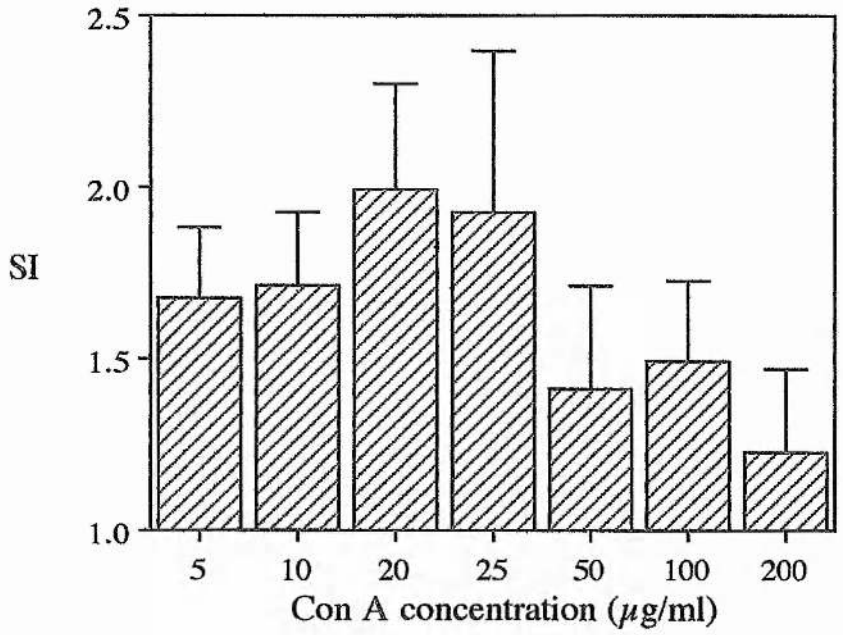
Each well consisted of 200μl of culture medium (see section 7.2.3) containing enriched effector cells (bands 2 & 3) from four animals. The cultures, at a range of cell doses, were incubated for three days at 15°C in a humidified incubator with 5% CO2 and then pulsed with 7.5 KBq ^{3}H-TdR in 25 μl culture medium for 16 hours. The wells were then harvested onto filter discs to determine counts per minute. Results are expressed as the average counts per minute (CPM) ± standard error (n=4).

Figure 7.4. The incorporation of ^{3}H-TdR into pooled enriched effector cell populations cultured at a concentration of 3 x 10^{5} cells per well during a 16 hour pulse after 1, 2, 3 and 4 days.



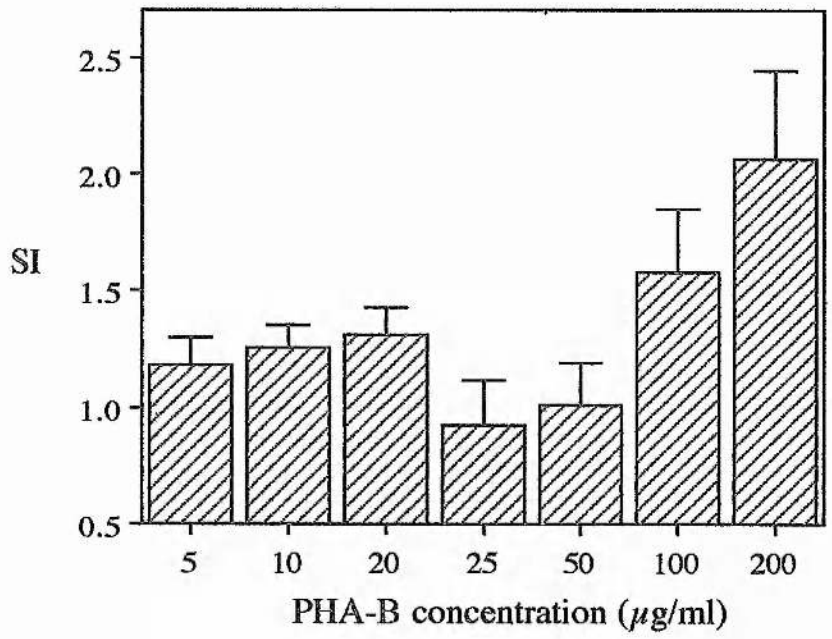
Each well consisted of 200 μl of culture medium (see section 7.2.9) containing bands 2 and 3 pooled from four animals at 3 x 10^{5} per well. The cultures were incubated for each time period at 15°C in a humidified incubator with 5% CO_2 and then pulsed with 7.5 KBq ^{3}H-TdR in 25μl culture medium for 16 hours. The wells were then harvested onto filter discs to determine counts per minute. Results are expressed as the average counts per minute (CPM) ± standard error (n=4).

Figure 7.5. The incorporation of ^{3}H-TdR into enriched effector cell populations cultured at a concentration of 3 x 10^{5} cells per well during a 16 h pulse in response to concanavalin A (Con A).



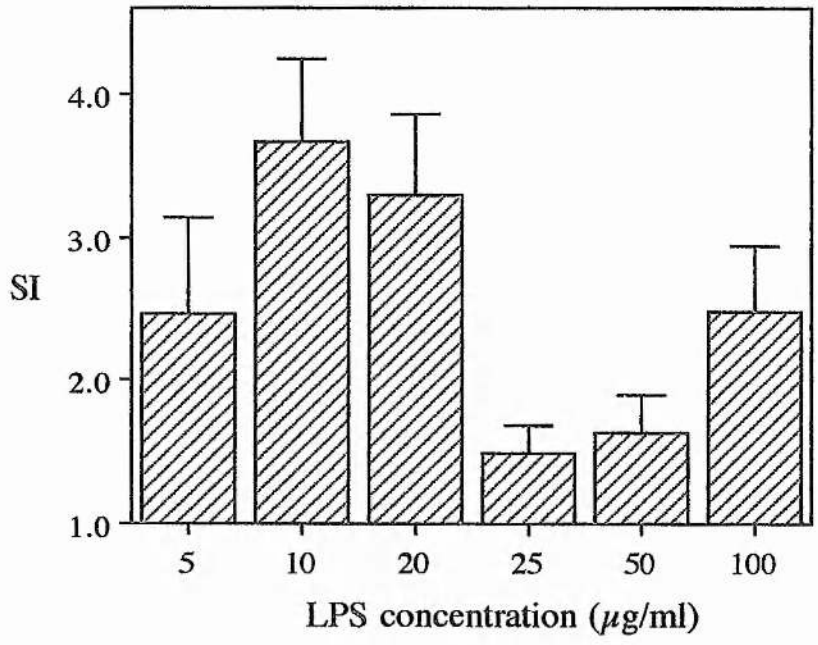
Each culture consisted of 200 μl of culture medium (see section 7.2.3) containing enriched effector cells (bands 2 & 3) (see section 3.2.4) prepared from one animal at 3 x 10^{5} per well and Con A (from *Canavalia ensiformis*, type IV-S) at the required concentration. The cultures were incubated for three days and then pulsed with 7.5 KBq ^{3}H-TdR in 25 μl culture medium for 16 hours. The wells were then harvested to determine counts per minute. Results are expressed as the stimulation index (SI) ± standard error (n=12).

Figure 7.6. The incorporation of ^{3}H-TdR into enriched effector cell populations at a concentration of 3 x 10^{5} cells per well during a 16 h pulse in response to phytohaemagglutinin-B (PHA-B).



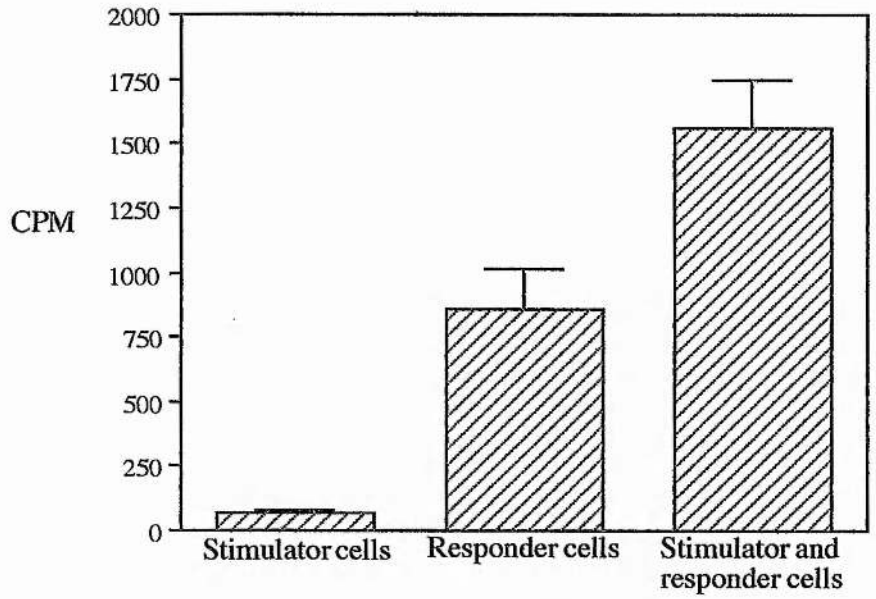
Each culture consisted of 200 μl of culture medium (see section 7.2.3) containing enriched effector cells (bands 2 & 3) (see section 3.2.4) prepared from one animal at 3 x 10^{5} per well and PHA-B (from *Phaseolus vulgaris*) at the required concentration. The cultures were incubated for three days and then pulsed with 7.5 KBq ^{3}H-TdR for 16 h. The wells were then harvested to determine counts per minute. Results are expressed as the stimulation index (SI) ± standard error (n=12).

Figure 7.7. The incorporation of ^{3}H-TdR into enriched effector cell populations at a concentration of 3 x 10^{5} cells per well during a 16 hour pulse in response to lipopolysaccharide (LPS).



Each culture consisted of 200 μl of culture medium (see section 7.2.3) with enriched effector cells (bands 2 & 3) (see section 3.2.4) prepared from one animal at 3 x 10^{5} per well and LPS (from *Salmonella enteritidis*) at the required concentration. The cultures were incubated for three days and then pulsed with 7.5 KBq ^{3}H-TdR for 16 h. The wells were then harvested to determine counts per minute. Results are expressed as the stimulation index (SI) ± standard error (n=12).

Figure 7.8. The incorporation of ^{3}H-TdR into cytotoxic blood cell populations at a concentration of 3 x 10^{5} cells per well during a 16 h pulse in response to allogeneic cells.

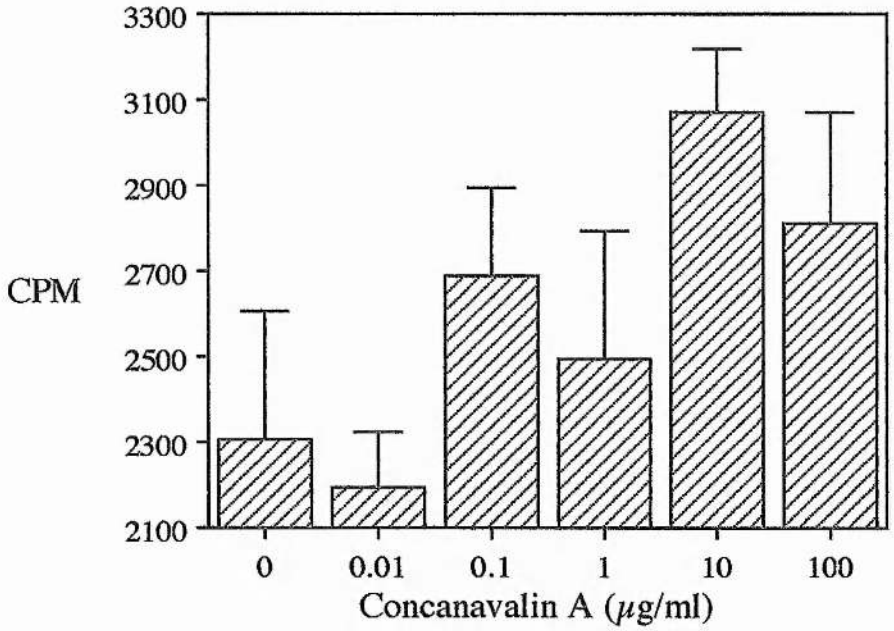


Each culture consisted of 200 μl of culture medium (see section 7.2.3) containing enriched effector cells (bands 2 & 3) (see section 3.2.4) cells prepared from one animal at 3 x 10^{5} per well and pooled blood cells from eight animals treated with mitomycin C to arrest cell division. The cultures were incubated for three days and then pulsed with 7.5 KBq ^{3}H-TdR for 16 h. The wells were then harvested to determine counts per minute. Results are expressed as the average counts per minute (CPM) with bars as standard error (n=11).

7.4. Discussion.

The results presented in this chapter show that enriched populations of undifferentiated circulating blood cells from *C. intestinalis* undergo low levels of spontaneous cell division within the circulation and display enhanced proliferation following treatment with mitogens or allogeneic cells *in vitro*. Importantly, the present study confirms earlier indications by Ermak (1976) that proliferation of the undifferentiated (lymphocyte-like) blood cell population occurs outside the haemopoietic crypts and is the first to demonstrate mitogen-induced enhanced proliferation in response to both B- and T-cell mitogens in an ascidian. Previous attempts to demonstrate proliferative responses by ascidian blood cells have met with limited success. For example, Tam *et al.* (1976) showed that blood cells from *C. intestinalis* synthesize DNA but found increased proliferation only in response to very high concentrations of phytohaemagglutinin over long culture periods. By contrast, Warr *et al.* (1977) apparently failed to demonstrate increased cell division of 'lymphocyte-like' cells from *Pyura stolonifera* by treatment with Con A, wheat germ agglutinin or soy bean lectin at any concentration over periods of 4-13 days. These authors suggested that the failure of their cultures may be due to the commitment of circulatory cells to advanced differentiation states and/or inappropriate culture conditions (Warr *et al.*, 1977). However, it should be noted that some of the experiments in this study were conducted with no repetition (Warr *et al.*, 1977). For example, closer examination of the results obtained by Warr *et al.*, (1977) for concanavalin A (see fig 7.9), indicate that with enlarged sample sizes significant proliferation may have become evident, especially at concentrations in the range 10-100 μg ml^{-1}.

Figure 7.9. The uptake of ^{125}IdU into blood cells from *Pyura stolonifera* in response to concanavalin A on day 5 (Warr *et al.*, 1977).



The values for this graph are extracted from Table 3 of Warr *et al.*, 1977. Uptake of ^{125}IdU is used as a measure of cell proliferation. Values are counts per minute ± S.E. for one animal on day 5 in response to Con A.

Furthermore, the present study differs from the earlier investigations of Warr and others in using cell populations enriched by density gradient centrifugation rather than whole blood. In my experience, the cells need to be carefully extracted and prepared for assay from individual animals and under sterile conditions to avoid pre-stimulation. Thus, key aspects for successful measurement of the proliferative

response by the blood cells of *C. intestinalis* are, the need for suitable sample sizes, the condition of the cells, and the type and concentration of the stimulus used.

The stimulation indices obtained with *C. intestinalis* , are generally lower than equivalent values obtained for mammalian cells by other workers. For example, stimulation indices of ca 30-40 and ca 15 have been reported for mouse spleen cells exposed to con A or LPS respectively (Byrd *et al.*, 1977), while values of ca 2-5 and ca 5-12 have been determined for human peripheral blood lymphocytes following treatment with PHA or Con A (Hebebrand *et al.*, 1979). In *C. intestinalis* stimulation indices of ca 2 for Con A and PHA and ca 3.5 for LPS were recorded. Similarly, the MLR SI in human peripheral blood nucleated cells is ca 10, at the optimal incubation time and cell concentration (Lavignac *et al.*, 1992), although, for mouse spleen cells, Frindel *et al.* (1992) have derived values of ca 2.2 - 4.6, depending on the number of days in culture. In the present study, *C. intestinalis* stimulation indices in response to allogeneic cells achieved values of ca. 1.5 - 3.5. Differences in the magnitude of the responses between ascidians and mammals may be related, at least in part, to the lower incubation temperature used with the ascidian cells and to the greater degree of purity of mouse and human cells in culture.

In addition, as the cell fractions assayed comprised of both undifferentiated cells and phagocytic amoebocytes, it is unclear whether the mitogens act directly on the undifferentiated cells, or whether the responses are mediated through the phagocytic amoebocytes in a manner resembling macrophage-activation of T-lymphocytes in vertebrates(see section 6.4). Such interaction between different blood cell types is not improbable as co-operation between the phagocytes and morula cells has already been demonstrated for *C. intestinalis in vitro* (Smith and Peddie, 1992).

In vertebrates, the MLR is associated with the major histocompatibility complex (MHC) and is used as a functional marker of genetic disparity between individuals (Roitt *et al.*, 1989). Within the Invertebrata, ascidians are known to reject foreign

tissue grafts and exhibit non-fusion reactions *in vivo* under the control of a single, highly polymorphic (MHC-like) gene locus (see section 1.4.6). In the present study, the demonstration of an MLR-type response by *C. intestinalis* blood cells, provides information about the cellular events involved in histoincompatibility reactions in ascidians and offers a useful laboratory method to quantify allorecognition in solitary species. Indeed, it is possible that the failure of two animals out of the six tested to exhibit MLR-type reactions may be explained by the degree of relatedness between the individuals from which the cell samples were taken. In *C. intestinalis*, the majority of eggs are spawned in mucus strings which are retained epibenthically (Svane and Havenhand, 1993). Offspring generally settle in the proximity of their parents (Svane and Havenhand, 1993), so closely related individuals are likely to occur within semi-enclosed populations. The collection site used for the present study is such a semi-enclosed area (see section 2.2.1). Variations between animals of baseline values of ^{3}H-TdR uptake, on the other hand, may be due to either differences in the immunological status of the hosts or differences in the proportion of bioactive cells.

In previous chapters, evidence is presented for, morphological similarities between vertebrate lymphocytes and undifferentiated cells (see review by De Leo, 1992 and section 1.4.7), and the cytotoxic activity by these undifferentiated cells (see section 6.4). This chapter, demonstrating MLR and mitogen-induced responses *in vitro*, provides further evidence that undifferentiated cells of ascidians represent, morphologically and functionally, a primordial form of lymphocyte.

Chapter Eight
The culture of haemopoietic tissue from *Ciona intestinalis*

8.1. Introduction

In ascidians, blood cell renewal occurs both in the circulation (see Chapter Seven) and in haemopoietic tissues located in three main parts of the body: the pharynx, around the digestive tract and, in advanced species, the body wall (Wright and Ermak, 1982). The haemopoietic tissue is arranged as diffuse clusters of cells or as discrete nodules (Wright and Ermak, 1982). In *C. intestinalis*, these nodules are located in the transverse and longitudinal bars and along the endostyle (Wright and Ermak, 1982).

A haemopoietic nodule consists of a group of undifferentiated cells surrounded by maturing blood cells undergoing various stages of differentiation. These undifferentiated cells have a high nuclear : cytoplasm ratio, a prominent nucleolus, sparse chromatin, numerous polyribosomes, and a few cytoplasmic granules (Wright and Ermak, 1982). By measurement of the incorporation of tritiated thymidine and their sensitivity to X-rays, these cells have been demonstrated to proliferate *in situ* and to differentiate into other blood cell types (Wright and Ermak, 1982). Differentiating cells lose their prominent nucleolus and gain chromatin within the nucleus (Ermak, 1976). They also lose their polyribosomes and develop long cisternae of endoplasmic reticulum, elongate mitochondria and a larger Golgi apparatus (Ermak, 1976). Blood cell proliferation rates within these nodules have been determined by measuring the incorporation of tritiated thymidine (^{3}H-TdR) and autoradiography (Wright and Ermak, 1982). Twenty days after ^{3}H-TdR administration most of the labelled cells are found either in the circulation or in the peripheral parts of the nodules and after 60 days most blood cells in the nodules are unlabelled with only a few present in blood channels (Wright and Ermak, 1982). These findings led the authors to conclude that, in *C. intestinalis*, blood cell renewal is of the order of several weeks (Wright and Ermak, 1982).

In 1990, Raftos and co-workers developed a technique for the *in vitro* culture of pharyngeal explants from the solitary ascidian, *Styela clava* (Raftos *et al.*, 1990). They were able to maintain viable and proliferative tissue for up to 72 days, and, by measuring the incorporation of the thymidine analog, bromodeoxyuridine (BrDu), into proliferative hemocytes, were able to assess proliferation rates within the nodules in response to various stimuli (Raftos *et al.*, 1990). These authors recorded stimulated cell proliferation: firstly in the body wall in response to allogeneic stimuli (Raftos and Cooper, 1991), secondly, in pharyngeal explants in response to phytohaemagglutinin and recombinant human interleukin-2 (IL-2) (Raftos *et al.*, 1991b), and thirdly, in pharyngeal explants in response to a factor present in tunicate plasma called tunicate interleukin-1beta (IL-1ß) (Raftos *et al.*, 1991a). In all cases, the cells that showed increased proliferation from the haemopoietic tissue in response to the stimuli were the 'lymphocyte-like cells' (Raftos *et al.*, 1991a,b; Raftos and Cooper, 1991).

The hypothesis upon which this chapter is based is that immunocompetent 'lymphocyte-like' cells capable of cytotoxic activity against mammalian target cells originate in the haemopoietic tissue of the pharynx. It may be expected, therefore, that increased proliferation of cytotoxic 'lymphocyte-like' cells from the pharyngeal explants of *C. intestinalis in vitro* will occur in response to factor(s) contained within the plasma, as seen by Raftos *et al.*, (1991a) for *S. clava.* In order to test this hypothesis, it was necessary to modify the *in vitro* culture method developed for *S.clava* (Raftos *et al.*, 1990) to suit pharyngeal explants from *C. intestinalis* and then, to examine the cells, migrating from the explants, for morphological and functional similarities to the cytotoxic cells described in section 6.3.2.

8.2. Materials and methods

8.2.1. Collection and maintenance of animals

The collection and maintenance of animals was as described in section 2.2.1.

8.2.2. Preparation of plasma

Sterile plasma was prepared as described in section 7.2.2.

8.2.3. Dissection of pharyngeal explants

To prepare the explants, individuals of *C. intestinalis* were first rinsed in sterile seawater and then surface sterilised with 70% ethanol. The outer test of each animal were then removed to expose the mantle. The pharyngeal basket was then isolated by cutting horizontally through the animal just below the basket. This upper portion was rinsed once more in sterile seawater and the pharyngeal basket exposed by making vertical cuts through both siphons. The pharyngeal tissue is characterised by vertical rows of pharyngeal grooves with horizontal striations. This tissue was removed and dissected into 1 mm squares. All surgical procedures were performed under sterile conditions.

8.2.4. Preparation of tissue culture medium

The CCM, as described in section 7.2.3., was used for tissue culture.

8.2.5. *In vitro* culture of pharyngeal explants

Sixteen explants were removed from each of seven animals, and, to reduce contamination of by protozoa and bacteria, the explants were washed three times for 15 minutes in 5 ml of sterile CCM medium containing no plasma but antibiotics were replaced with 5 mg ml^{-1} streptomycin and 10000 units ml^{-1} penicillin in sterile

bijoux mounted on a shaker. For each animal, eight explants were incubated in CCM with plasma and eight were incubated in CCM without plasma. The explants were incubated in tissue culture 24-well plates in groups of four explants per well. Each well contained 1 ml of medium and the medium was changed every four days. Each day the cultures were checked through an inverted light microscope for muscle contraction, ciliary action and blood cell streaming within the explant.

8.2.6. Harvesting of cells

After every four days, the explants were removed from the wells using sterile forceps and placed in fresh wells containing fresh media. The supernatant, containing cells which had migrated from the explants, was aspirated into 10 ml sterile polycarbonate tubes and the wells were rinsed vigorously with ice-cold media to remove adherent cells. The contents of each well, together with all washings, was harvested into one individual tube. The cells were then spun at 800 g for 20 min, the supernatant decanted and the pellet resuspended in 500 μl MS. For cell counting (see section 2.2.4), viability (see section 2.2.4) and cytospin preparations (see section 5.2.7) 100 μl was removed, whilst the remaining 400 μl was kept for measurement of cytotoxic activity, without adjustment of cell concentration, at an E:T of ca 20:1.

8.2.7. Measurement of cytotoxic activity

Cytotoxic activity of the cells which had migrated from the explants was measured as described previously (section 3.2.5) using WEHI (strain 3B) as targets. Each assay consisted of at least four well replicates and was repeated for each of the seven animals.

8.2.8. Preparation of cells and explants for light microscopy

Cytospins were prepared (see section 5.2.7) from cell suspensions which had migrated from explants incubated in CCM with and without plasma. The cell types

were identified according to the classification in Table 1.2. For LM, pharyngeal explants were fixed in Bouins fixative, dehydrated through a series of alcohols, cleared with chloroform and embedded in paraffin wax. Sections, 7 μm thick, cut with a rotary microtome, were stained with haematoxylin and eosin.

8.2.9. Transmission electron microscopy of pharyngeal explants.

Pharyngeal explants cultured for four days with or without plasma were pre-fixed in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde and 20 % sucrose (pH 7.4) for two hours (Sabatini *et al.*, 1963). The explants were then gently washed three times in 0.2 M cacodylate buffer and post-fixed in the same buffer containing 1% osmium for 15 minutes (Palade, 1952; Hirsch and Fedorko, 1968). The explants were washed twice in cacodylate buffer and dehydrated through a series of ethanols and cleared with epoxypropane. After embedding in araldite and baking for 36-48 hours at 60°C, the block was then trimmed and sections were cut using glass knives. Thick sections were stained with toludine blue and examined under light microscopy to find nodules of undifferentiated cells. For E.M., gold sections were cut and placed on 200μm grids, stained with uranyl acetate and then, lead citrate (7 min each) with intermediate washes in distilled water (Reynolds, 1963). The haemopoietic nodules and the migrating blood cells were then examined and photographed using a Philips transmission electron microscope at 60 kv. The cell types were identified according to the classification in table 1.2.

8.3. Results

8.3.1. *In vitro* culture of pharyngeal explants

Cultures of pharyngeal explants remained viable for up to 21 days. A culture was considered viable if ciliary beating on the surface of the explants, or muscle

contraction, or blood cell streaming was visible. The most common reason for abandoning a culture was contamination by protozoa or fungal growths which often became noticeable after 2-3 weeks of culture. Due of contamination problems the cytotoxicity assays were carried out only after 4 days culture. Wax sections of 4 day old explants showed the transverse bars within the pharynx (Fig. 8.1). Clusters of pigment cells were seen between each bar and diffuse nodules of undifferentiated cells were more clearly noted in the toluidine blue section (Fig. 8.2).

8.3.2. Cell proliferation, viability and cytotoxic activity.

Using light microscopy of living cultures, cells were seen to continuously migrate from the explants into the culture medium continuously. After the first four days, significantly more cells had migrated from the cultures incubated in plasma ($1.23 \pm 0.26 \times 10^{-7}$ ml^{-1}) than from the explants incubated without plasma ($1.09 \pm 0.27 \times 10^{-7}$ ml^{-1}) ($p=0.004$) (Table 8.1). Moreover, viability was significantly higher in the cells which had migrated from the explants incubated in plasma (98.14 ± 0.76 % viable) than the cells incubated without plasma (94.00 ± 1.04 %) ($p=0.006$) (Table 8.1). However, despite these differences in cell viability and proliferation, greater cytotoxic activity was found for the cells which had migrated from the explants incubated without plasma (7.47 ± 1.01 %SR) than for the cells which had migrated from the cultures incubated in plasma (2.38 ± 1.38 %SR) ($p=0.03$). It was noted, that despite the high effector to target cell ratio (20 : 1) the percentage specific release from the target cells (7.47 ± 1.01) was much lower than with an equivalent E : T ratio of enriched cytotoxic blood cells (28.94 ± 4.03) (see section 3.3.1).

8.3.3. Examination of cytospin preparations.

Cytospin preparations showed that only ca 20% of the cells which had migrated from the explants during the first four days were morphologically similar to the cytotoxic target-binding cells described in Chapter Six (section 6.3.2) (see Fig. 8.3). This was

the case for the explants cultured both with plasma or without plasma Instead, the majority of cells were atypical haemocytes, either migrating with amoeboid movement or adhered to the culture vessel. In cytospin preparations, these cells were seen to have a darkly stained nucleus with a small nuclear : cytoplasmic ratio and lightly basophilic cytoplasm (Fig 8.3).

8.3.4. Examination of pharyngeal explants under T.E.M.

The transmission electron micrographs of the pharyngeal explants from *C. intestinalis* show haemopoietic nodules slightly more diffuse than those of *S. clava* described by Ermak (1976) (Fig.8.4). The undifferentiated cells in the centre of the nodule have a high nuclear : cytoplasmic ratio, a distinct nucleolus, sparse chromatin many polyribosomes and very few or no cytoplasmic granules (Fig.8.4). Noticeably, some cells, appeared to have lost the distinct nucleolus and gained cytoplasmic granules, in manner similar to the differentiating haemoblasts described for *S. clava* by Ermak (1976) (Fig.8.4). Cells resembling both the target-binding cells described in section 6.3.4, and the undifferentiated cells in the haemopoietic nodules (see above), were seen in blood channels outside the haemopoietic nodules, apparently migrating into the circulation (Fig.8.5). No noticeable differences were evident between pharyngeal explants cultured with or without plasma.

Table 8.1. The viability, number, and cytotoxic activity of cells, migrating from pharyngeal explants, from *Ciona intestinalis,* after four days of culture.

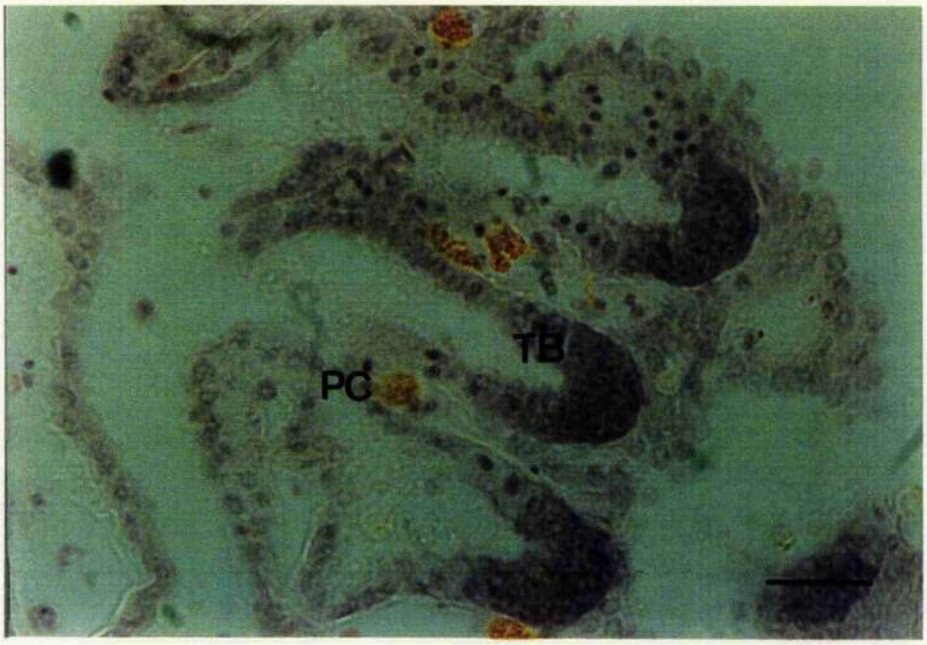
	With plasma	Without plasma	p value
Cell counts ($\times 10^7$ ml^{-1})	1.23 ± 0.26	1.09 ± 0.27	0.004
% Viability	98.14 ± 0.76	94.00 ± 1.04	0.006
% SR	2.38 ± 1.38	7.47 ± 1.01	0.027

Viability and cell counts were measured as described in section 2.2.4. using eosin-Y exclusion and New Improved Neubauer counting chambers. Cytotoxic activity (percentage specific release - %SR) was measured as described in section 3.2.5 using WEHI (strain 3B) as targets. Values expressed are means ± SE, n = 7. Each assay consisted of four well replicates of both treatments, with and without plasma, and was repeated for each of the seven animals.

Chapter Eight. Abbreviations on figures.

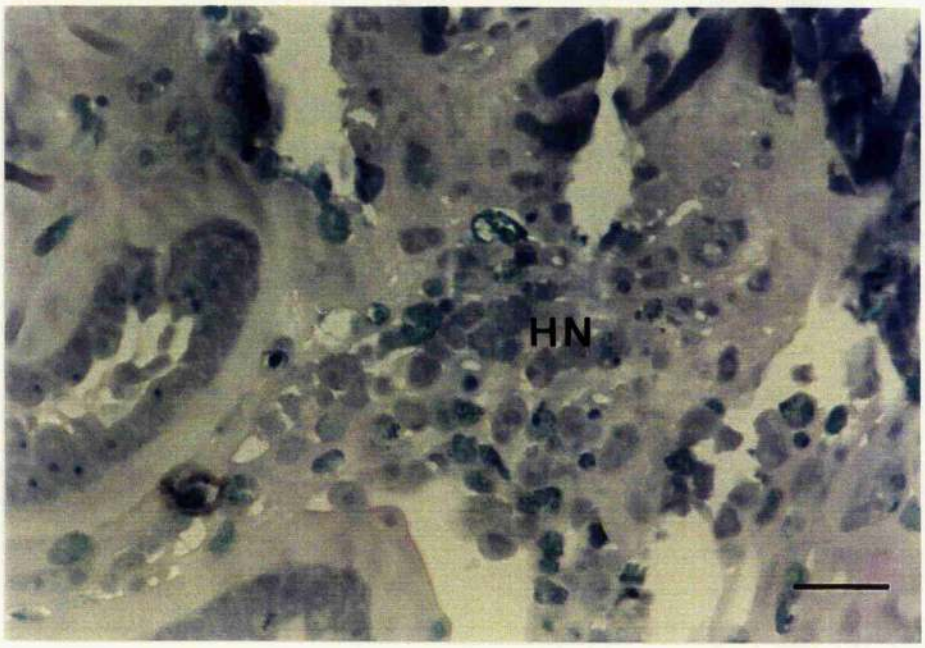
TB	Transverse bar
PC	Pigment cell
HN	Haemopoietic nodule
A	Atypical haemocytes
B	Undifferentiated cell
C	Signet ring cell
UC	Undifferentiated cell

Figure 8.1. Light micrograph of a pharyngeal explant from *Ciona intestinalis* after 4 days culture stained with haematoxylin and eosin-Y.



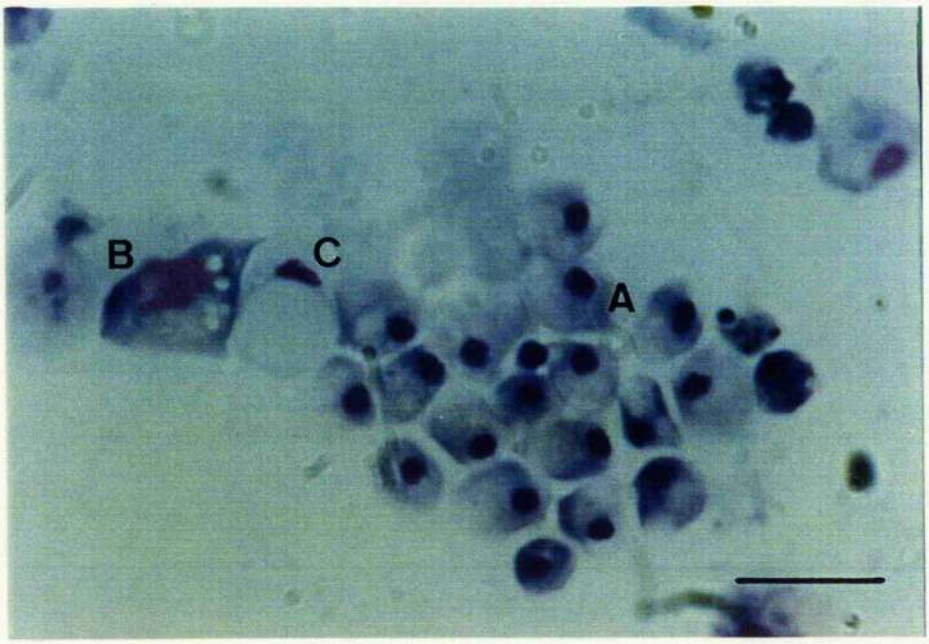
The explant was cultured for four days in CCM (see section 7.2.2.), fixed, dehydrated and embedded in paraffin wax. Sections were stained with haematoxylin and eosin-Y. Scale bar = 50 μm.

Figure 8.2. Light micrograph of a pharyngeal explant from *Ciona intestinalis* after 4 days culture stained with toludine blue.



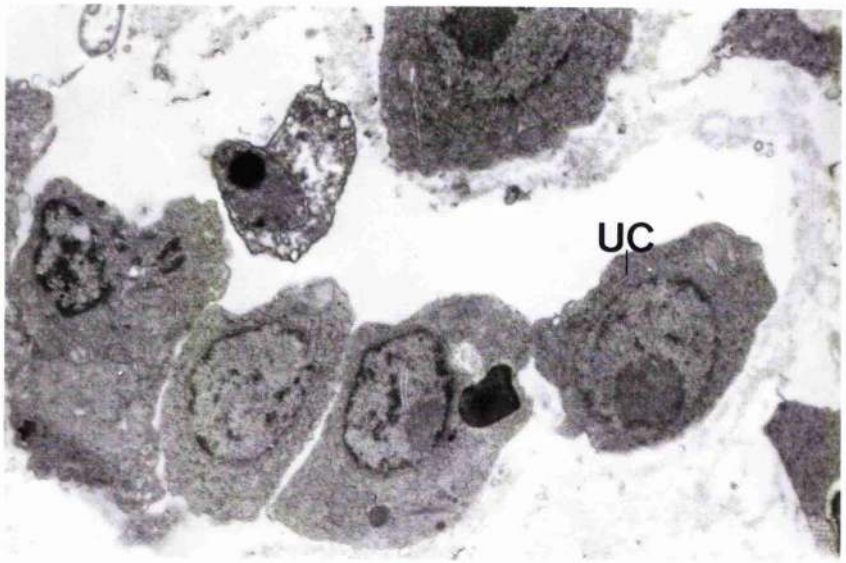
The explant was cultured for four days in CCM (see section 7.2.2.), fixed, dehydrated and embedded as for TEM, see section 8.2.9. Sections were stained with haematoxylin and eosin-Y. Scale bar = 20 μm.

Figure 8.3. Light micrograph of cells migrated from pharyngeal explants after 4 days.



The cells were prepared on cytospins and stained with Diff-quik see section 5.2.7. The migrating cells were mostly atypical haemocytes (A) with some circulatory haemocytes, such as the undifferentiated cell (B) and signet ring cell (C). Scale bar = 10 μm.

Figure 8.4. Transmission electron micrograph of a haemopoietic nodule in a pharyngeal explant from *Ciona intestinalis* cultured for 4 days.



Pharyngeal explants, cultured for four days, were pre-fixed in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde and 20 % sucrose (pH 7.4) for two hours, then washed in 0.2 M cacodylate buffer, 1% osmium for 15 minutes. The explants were washed, dehydrated through a series of ethanols, cleared with epoxypropane, and embedded in araldite. Gold sections stained with uranyl acetate and lead citrate and then photographed using a Philips transmission electron microscope at 60 Kv. Magnification x 6,954.

Figure 8.5. Transmission electron micrograph of a blood cells migrating from a pharyngeal explant from *Ciona intestinalis* cultured for 4 days.

Pharyngeal explants, cultured for four days, were pre-fixed in 0.2 M cacodylate buffer, 0.5 % paraformaldehyde, 0.5 % glutaraldehyde and 20 % sucrose (pH 7.4) for two hours, then washed in 0.2 M cacodylate buffer, 1% osmium for 15 minutes. The explants were washed, dehydrated through a series of ethanols, cleared with epoxypropane, and embedded in araldite. Gold sections stained with uranyl acetate and lead citrate and then photographed using a Philips transmission electron microscope at 60 Kv. Magnification x 5,000.

8.4. Discussion

This chapter describes the *in vitro* culture of pharyngeal explants from *C. intestinalis* over three weeks and examines the morphological and functional properties of the cells migrating from the explants during this period. In the present study it was found that migrating cells showed greater viability and that the rate of cell production increased in the presence of homologous plasma, corroborating the findings of Raftos and co-workers (1990) working on pharyngeal explants from *S. clava*. In contrast to the findings of Raftos *et al.*, (1990), who found that the migrating cells comprised in the majority normal blood haemocytes, in the present study it was noted that the majority of cells produced were atypical haemocytes rather than undifferentiated 'lymphocyte-like' cells. Sawada *et al.* (1994) examined the living cells released from cultured pharyngeal explants of *Styela clava* and also found that after 7 days ca. 30% were atypical. Raftos *et al.*, (1990) also found that the percentage of 'lymphocyte-like' cells in the migrating population increased upon the addition of homologous plasma, however, in the present study, no differences in the proportion of undifferentiated cells was detected.

That cytotoxic activity is shown only by the cells migrating from *C. intestinalis* explant cultures incubated in plasma-free media indicates that whilst the plasma stimulates greater proliferation of cells from the explants, and enhances the viability of the cells, it does not promote cytotoxic activity *in vitro*. Conversely, plasma seems to inhibit such immunologically active cells, possibly by inhibiting the differentiation process necessary to produce active cytotoxic cells or, alternatively, by reducing their rate of production from the explant.

At the ultrastructural level, some of the cells emerging from the explants appear to resemble, morphologically, the cytotoxic cells within the circulation. However, no differences were seen in the proportion of cytotoxic cell types migrating from the

explants cultured, with or without plasma. The presence of plasma may reduce the production of functional cells from the explant, or, alternatively inhibit a possible differentiation process necessary to produce cytotoxic cells in culture. Additionally, transmission electron micrographs of explants of pharyngeal tissue cultured with or without plasma, show that some of the emerging cells resemble, morphologically, the cytotoxic cells within the circulation. Possibly, the cytotoxic cells may form in other haemopoietic tissues and migrate to the pharynx, or after migration from the pharynx the cells need to migrate through another tissue, to become fully functionally competent. Further research is necessary to ascertain whether or not growth factors, mitogens or cytokine-like factors are required to produce a full complement of immunologically active blood cells from the haemopoietic tissue.

The *in vitro* culture of pharyngeal explants from *C. intestinalis* in the absence of plasma was successful in producing some functionally active cytotoxic cells, indicating that this tissue may be responsible for the production of cytotoxic cells *in vivo*. The presence of primitive, haemopoietic tissue in the pharyngeal region of *C. intestinalis* from which cytotoxic and proliferative 'lymphocyte-like' cells migrate, may conceivably indicate that the phylogenetic origins of a functional vertebrate thymus can be visualised in extant ascidians.

Chapter Nine
General Discussion

9.1 General Discussion

This thesis has presented new evidence for immunological functions by the blood cells of the solitary ascidian, *C. intestinalis*. In particular, it was found that blood cells present in the circulating blood of *C. intestinalis*, mediate haemolysis of sheep red blood cells (section 2.4) and, more significantly, cytolysis of a range of mammalian tumour cell lines *in vitro* (section 3.4). The cell-mediated cytotoxic effector cells are enriched in two bands by continuous density gradient centrifugation, and activity is ameliorated by heat-treatment of the effector cells (section 3.4). Parameters of cytotoxic activity against the target cell, WEHI, a mouse myelomonocytic leukemic cell (strain 3B) were ascertained and the phenomenon was found to be rapid, temperature dependent and sensitive to osmotic conditions (section 3.4). Aspects of the underlying cytolytic mechanism were determined using divalent cation chelators or inhibitors of cellular processes, and the cytotoxic activity was found to be dependent upon the presence of calcium and magnesium ions, effector to target cell binding, and active cellular cytoskeletal and secretory processes (section 4.4). The cytotoxic effector cells were found to non-adherent to glass and nylon wool (section 5.4). Transmission electron microscope studies of the target-binding cells showed that they were undifferentiated with a high nucleus to cytoplasmic ratio (section 6.4), a morphology similar to those previously been described in ascidians as 'lymphocytes' or 'lymphocyte-like cells' (De Leo, 1992). In addition, TEM studies revealed the presence of close inter-digitation between the effector and target cell membranes and the formation of vesicles, in the area of target cell binding, within the effector cell (section 6.4). The effector cell population was cultured *in vitro* and proliferation in response to concanavalin A (Con A), phytohaemagglutinin-B (PHA-B), lipopolysaccharide (LPS), or allogeneic leucocytes showed that these cells respond to T and B cell mitogens and exhibit a mixed leucocyte reaction (section 7.4). Pharyngeal

explants from *C. intestinalis* were cultured *in vitro* and the morphological and functional properties of the cells which migrate from the explants were determined (section 8.4).

The original aim of this thesis was to examine lymphocyte-like functions in the solitary ascidian, *C. intestinalis* (see section 1.5), with a view to clarifying the phylogenetic origin of lymphocytes in invertebrates. To achieve this, the blood cells of the solitary ascidian *C. intestinalis* were examined for lymphocyte-like characteristics or properties. Members of the Ascidiacea are thought to be close to the origin of the vertebrate line (Bone, 1979) and therefore represent a useful extant species for comparative analysis. *C. intestinalis* is a large, common and widely distributed species which is thought to represent the most 'primitive' of the ascidiacea (Berrill, 1936). To ascertain whether *C. intestinalis* possesses blood cells with properties akin to vertebrate lymphocytes, the blood cells were examined for haemolytic, cytotoxic, proliferative and morphological characteristics *in vitro*.

Cytotoxic activity by the blood cells of *C. intestinalis* was measured, firstly as the haemolysis of SRBC, and secondly, as cytolytic activity against mammalian tumour cell lines. In vertebrates, haemolysis may not only be a measure of cell mediated cytotoxic activity, but also of humoral complement-like lytic factors (Roitt *et al.*, 1993). In the present study, measurement of haemolysis was additionally complicated by phagocytosis of the erythrocytes (section 2.4). While the blood cells of *C. intestinalis* were found to lyse SRBC, related work by Parrinello and co-workers (1994), found that the cells responsible for mediating haemolytic activity in *C. intestinalis* are enriched on the lower bands of a density gradient separation, and that the cellular repository of the haemolytic factors in *C. intestinalis* are the granulocytes or morula cells (Parrinello *et al.*, 1994; personal communication). In contrast, in the present study, the mediators of cytolytic activity against mammalian tumour cells are enriched on the upper bands of a continuous

density gradient (section 5.4), and are undifferentiated cells (section 6.4). Therefore, it is concluded that, in *C. intestinalis*, haemolysis and cell-mediated cytotoxic activity are two independent functional processes mediated by different cell types.

Importantly, in *C. intestinalis*, cytotoxic activity against mammalian tumour cells has many similarities with vertebrate cell-mediated cytotoxicity. In vertebrates, lymphocyte-mediated cytotoxicity also increases with E:T ratio, and is active against a range of target cell lines (Roitt *et al.*, 1993). In mammals, cytotoxic T-cells recognise antigen associated with class 1 MHC molecules, and NK cells recognise determinants expressed on neoplastic cells (Roitt *et al.*, 1993). Cytotoxic activity by the blood cells of *C. intestinalis* also increases with the effector to target cell ratio, and similarly, is effective against both mouse (L929, YAC-1, P815, and WEHI (3B)) and human (K562) target cell lines (section 3.4). Further investigations into the means by which the target cells are recognised as foreign would clarify the recognition mechanisms active in *C. intestinalis*. Future experiments, either with cell lines which are NK-resistant, or with cell lines which differ only by the presence or absence of class 1 MHC molecules may indicate further homology with mammalian recognition mechanisms.

In general, vertebrate cell-mediated cytotoxic activity is dependent upon active metabolic, cytoskeletal, and secretory processes (Taylor and Cohen, 1992). Cytolysis also involves at least two stages; first, effector to target cell conjugate formation is dependent upon magnesium, whereas, the final lytic stage is calcium dependent (Carpén *et al.*, 1981). In addition, natural killer cell activity , is not mediated by superoxide anions or hydrogen peroxide, because the antioxidant enzymes, superoxide dismutase and catalase, do not affect target cell lysis (Duwe *et al.*, 1985). Crucially, this study establishes that cytotoxicity by the blood cells of *C. intestinalis* is also influenced by temperature, is rapid, involves energy metabolism, requires an intact cytoskeleton and entails active

secretory processes (sections 3.4 and 4.4). Likewise cell-mediated cytotoxic activity in *C. intestinalis*, has two stages distinguishable by their requirements for divalent cations (see section 6.4). In particular, effector to target cell conjugate formation is dependent upon magnesium, whereas, the final lysis of the target cell is calcium dependent.. Similarly, cytotoxic activity by the undifferentiated cells of *C. intestinalis* is not mediated by the products of a respiratory burst (see section 4.4). Therefore, it appears that blood cells in *C. intestinalis* mediate cytotoxic activity through mechanisms similar to those of vertebrate lymphocyte-mediated cytotoxic activity.

There are also ultrastructural similarities between the target binding cells of *C. intestinalis* and cytotoxic cells present in vertebrates. Morphologically, vertebrate lymphocytes are difficult to define, although they do have some unifying characteristics, such as a large nuclear : cytoplasm ration and an undifferentiated cytoplasm (Roitt *et al.*, 1993). The present study shows that the target-binding cells in *C. intestinalis* also have a high nuclear to cytoplasmic ratio, a relatively undifferentiated cytoplasm with many free ribosomes and some profiles of endoplasmic reticulum (see section 6.4). These ultrastructural similarities between the target binding cells in *C. intestinalis* and, for example, the cytotoxic (NK) cells of mice are discussed in section 6.4. The cellular mediators of cytotoxic activity in salmonids (Hayden and Laux, 1985, Moody *et al.*, 1985) and catfish (Evans *et al.*, 1984) and mammals (Roitt *et al.*, 1993) are non-adherent to nylon wool and glass. Likewise, the cytotoxic cell population in *C. intestinalis* was found to be non-adherent to nylon wool and glass beads. These non-adherent undifferentiated cells of ascidians have frequently been referred to, in previous studies, as 'lymphocytes' or 'lymphocyte-like' cells, terms which have been adopted mostly upon the basis of morphological similarities (De Leo, 1992).

Vertebrate cytotoxic activity is mediated by the secretion of lytic factors into a 'closed chamber', this chamber forms upon the interdigitation of effector and target cell membranes (Roder *et al.*, 1978). The interdigitation of the membranes of the target-binding cells in *C. intestinalis* and their targets to form a 'closed chamber', is similar to that which forms between NK cell membranes of mice and their target cells (Roder *et al.*, 1978). The presence of vesicles within the cytoplasm of the target-binding cells of *C. intestinalis* near the site of contact, also offers evidence that a factor or suite of factors are secreted by the effector cell into the 'closed chamber', and may be responsible for the subsequent death of the target cell (see section 6.4). This hypothesis is supported by the observation that cytotoxic activity is dependent upon intact cellular secretory mechanisms (section 4.4). In vertebrates, the factors secreted by the effector cells into the 'closed chamber' have been characterised as calcium dependent-perforins and/or granzymes (Roitt *et al.*, 1993). In the present study, results indicated that the lytic factor may also be calcium dependent (see section 6.4), but, time precluded the further characterisation of putative cytolytic factors secreted from the effector cells. Future isolation, purification and characterisation of the lytic factors in *C. intestinalis,* is needed to ascertain the degree of homology between vertebrates and *C. intestinalis.*

The identification of a cell in the circulation of *C. intestinalis* ,which mediates cytotoxic activity and has morphological and adherence properties similar to vertebrate lymphocytes, fulfils the first aim of this thesis. The cytotoxic and adherence properties of the undifferentiated blood cells, coupled with their accepted morphological resemblance to vertebrate lymphocytes, offers some justification for the use of the term 'lymphocyte-like cell' to describe this particular cell type and indicates that in terms of cytotoxic capability these cells may represent pre-immunoglobulin precursors to vertebrate lymphocytes.

The second aim of this thesis was to investigate proliferation by the blood cells of *C. intestinalis in vitro* in response to mitogens (con A, PHA or LPS) and/or allogeneic cells, in order to ascertain whether or not these cells show proliferative capabilities in common with vertebrate lymphocytes (see section 1.3). Confirming the ideas of Ermak (1976), the undifferentiated cells in *C. intestinalis* were shown to undergo spontaneous cell division and DNA synthesis within the circulation (see section 7.4). Additionally, in contrast to previous studies (Tam *et al.*, 1976; Warr *et al.*, 1977), the present study demonstrated that, under suitable conditions, these low levels of background proliferation are enhanced by culture *in vitro* in the presence of T or B-cell mitogens or allogeneic cells (see section 7.4). The levels of stimulation were found to be slightly reduced over those expected from mammalian cultures of lymphocytes (see section 7.4), however development of improved cell purification techniques and culture media for the undifferentiated cells of *C. intestinalis* may increase stimulation indices. In addition further work, using amoebocyte-free cell populations, is necessary to determine whether the mitogens act directly on undifferentiated cells or whether the response is mediated through the phagocytic amoebocytes in a manner resembling macrophage-activation of T-lymphocytes in vertebrates (Roitt *et al.*, 1993).

In most vertebrates, lymphocytes comprise a heterogeneous population, distinguishable by mitogen sensitivity in *vitro*, cell surface receptors, immunological function and membrane bound immunoglobulin (Roitt *et al.*, 1993). In the present study, the heterogeneity of the undifferentiated cell population in *C. intestinalis* with respect to mitogen sensitivity was not investigated. It also remains plausible that the proliferative cells and the cytotoxic cells of *C. intestinalis* are two, morphologically similar, distinct sub-populations of the undifferentiated cells; or that undifferentiated cells are capable of proliferation or cytoxicity at different stages in development. In vertebrates, lymphocyte

heterogeneity is also determined by differential functional or adherence properties within the cell population. For example, proliferation of nylon-wool adherent B-cells only in response to LPS and nylon wool non-adherent T-cells in response to Con A (Roitt *et al.*, 1993). Presently, lymphocytes can be characterised by a variety of cell surface markers (the CD system) (Roitt *et al.*, 1993). The cell surface markers, for functional lymphocytes have only been determined for the higher vertebrates and have been found to vary even between human and murine lymphocyte populations (Roitt *et al.*, 1993). However, the definitive T-cell marker in humans is the T-cell antigen receptor (TCR) and in mice, Thy-1 (Roitt *et al.*, 1993). The surface phenotype of the NK cell in humans has also been delineated (Roitt *et al.*, 1993). Given the variation in cell surface markers between mice and humans, it would seem futile to look for homologous markers between the mammalian lymphocytes and the undifferentiated cells of *C. intestinalis*. Mansour and Cooper (1984) succeeded in detecting Thy-1 cross reacting membrane bound and soluble determinants in the tunicate, *S. clava*, but failed to link this phenomena with immunological function. The finding of these cross reactive homologs in ascidians, such as Thy-1, would have greater significance if linked to functional attributes such as cytotoxic activity and proliferation in response to mitogens. The generation of such definitive cell surface markers for the cytotoxic and proliferative undifferentiated cells in *C. intestinalis* would enable the levels of heterogeneity within the cell population to be understood.

In mice, cytotoxic T-cell lines can be prepared by the use of primed cells cultured with allogeneic cells and induced to proliferate with IL-2, and the cytotoxic activity *in vitro* of NK cells can be increased after incubation for several hours in interferon or IL-2 (Roitt *et al.*, 1993). In the present study, time precluded the investigation of the functional attributes of cells produced from the proliferative cultures (see section 8.4) but future

studies should consider the effects of human recombinant IL-2, interferon or other growth and stimulatory factors upon the functional capabilities of these cells.

As mixed leucocyte reactions by vertebrate lymphocytes is a functional marker of disparity at the major histocompatibility complex (Roitt *et al.*, 1993); it is necessary o establish whether the MLR in *C. intestinalis* conforms to the genetic rules which guide the MLR in vertebrates to completely fulfil this second aim of this thesis. Close examination of the genetic recognition mechanisms which regulate the control of MLR in *C. intestinalis*, possibly by linking MLR reactions to the mechanisms that control the non-fusion reactions in compound ascidians, or the graft rejections in solitary ascidians, would give further indication of the level of homology with vertebrate MHC-controlled responses. In the event that MLR between individuals of *C. intestinalis* is linked to genetic differences, the simple *in vitro* assay for MLR could be utilised as a measure of population genetics. An analysis of the incidences of MLR between and within spatially separated populations of *C. intestinalis* could generate values of gene flow, and contribute to studies on the dispersal mechanisms of the species.

Finally, the third aim of the thesis, was to investigate haemopoiesis in *C. intestinalis,* to ascertain the site of production of the cytotoxic cells with a view to drawing comparisons with vertebrate lymphopoiesis. In the present study, the *in vitro* culture of pharyngeal explants from *C. intestinalis* was achieved and cytotoxically active undifferentiated cells were recorded migrating from the explants. This does not conclusively identify the pharyngeal region as the anatomical origin of the undifferentiated (cytotoxic) cells in *C. intestinalis,* as it is possible that the cells originated elsewhere and migrated through the body. Further studies, using definitive cell surface markers, would enable the

development of cytotoxic or proliferative undifferentiated cells to be tracked, and the haemopoietic origins to be, indisputably, determined. However, the presence of, 'primitive', haemopoietic tissue in the pharyngeal region of *C. intestinalis* from which cytotoxic 'lymphocyte-like' cells migrate, may indicate that the phylogenetic origins of a functional vertebrate thymus can be visualised in extant ascidians. To date, therefore, the evidence presented in this thesis indicates a homologous anatomical origin for the vertebrate T-lymphocytes and the undifferentiated cytotoxic cells in *C. intestinalis*.

In vertebrates, there are many cytokines, such as the interleukins and interferons, which mediate cellular immunological responses (Roitt *et al.*, 1989). Previous studies by Raftos and co-workers (1990) with *S. clava*, had indicated that factors present in the plasma, stimulate the production of 'lymphocyte-like' cells. The results presented in this study for *C. intestinalis*, using cytotoxic activity as a measure of 'lymphocyte-like activity' shows that plasma inhibits the production of cytotoxically active cells and may be contraindicatory to Raftos's findings. An important goal for future work would be to determine the functional properties of cells produced from pharyngeal explants in ascidians, especially after treatment with interleukins, interferons, or isolated fractions of plasma.

The findings presented in this thesis, also open questions upon the importance of the cell co-operation molecules to the immunological capacity of *C. intestinalis* . The morula cells have been shown to store opsonic factor(s), proteases, and antibacterial factors which are released *in vitro* and *in vivo* in response to foreign challenge (Smith and Peddie, 1992; Azumi *et al.*, 1991; Findlay and Smith, 1995; Azumi *et al.*, 1990). In the present study, because separated cell bands were used, the morula cells were absent from cultures in which mitogen induced proliferation or MLR responses were detected (section 7.4). In previous studies, unsuccessful attempts to measure proliferation in response to

mitogens had used cells prepared from whole blood (Tam *et al.*, 1976 ; Warr *et al.*, 1977. It is possible, that the morula cells, when present in such cultures, exocytose in response to the *in vitro* conditions, factors which stimulate proliferation of the undifferentiated cells. Consequently, any subsequent attempt to stimulate such cultures would be unsuccessful and no stimulatory effect measurable. Morula cells are also present in non-fusion reactions, allograft and autograft rejections (Taneda & Watanabe, 1982c; Reddy *et al.*, 1975; Raftos *et al.*, 1987a,b) and appear to release a battery of non-specific defense molecules e.g. antibacterial factors (Findlay and Smith, 1995) in response to immunological challenge *in vitro*. Therefore, future investigations, should explore the possibility that the morula cell releases cytokine-like factors, in response to immunological challenge, which simulate the proliferation of functionally active cytotoxic cells both *in vivo* and *in vitro*.

Vertebrate lymphocytes are characterised by their function, anatomical origin and morphology. They are cytotoxic (cytotoxic T-cells, NK cells), proliferate in response to certain stimuli (T- and B- cells), produce immunoglobulins (B-cells only), originate from primary lymphoid organs (thymus and bone marrow), interact with macrophages in the regulation of immunity and are relatively undifferentiated cells (Roitt *et al.*, 1989). To conclude, this thesis has fulfilled the original aims by demonstrating that the undifferentiated cells of *C. intestinalis*, are cytotoxic to mammalian tumour cells, have the capacity to proliferate in response to mitogenic or allogeneic stimulation, and are produced in thymus-like haemopoietic nodules. Whilst the absence of membrane-bound immunoglobulins has been established and the extent of interaction with macrophage-like cells remains unknown, the undifferentiated cells of the solitary ascidian *C, intestinalis*, have many functional, as well as morphological, similarities with vertebrate lymphocytes. This thesis, therefore, offers compelling evidence that the undifferentiated

'lymphocyte-like' cells of ascidians represent a primordial form of vertebrate lymphocyte.

It has been suggested that T-cell mediated immune functions may have preceded the emergence of the vertebrates (Hildemann and Reddy, 1973; Cooper, 1980), and that building blocks for adaptive immunity, such as allorecognition, cell mediated cytotoxicity, and proliferative mixed leucocyte reactions, can be examined in lower deuterostomes (Raftos and Raison, 1992). In 1989, Anderson *et al.* postulated that the capacity to generate immunoglobulins in response to foreign antigen is an evolutionary recent addition to pre-existing, non-clonal systems of host defence. Contrary evidence showed that the echinoderms produce membrane-bound and secreted molecules resembling vertebrate immunoglobulins (Delmotte *et al.*, 1986). However, recent studies by Larson and Bayne (1994), confirmed that immunoglobulin did not evolve until after the emergence of the echinoderms, thus, confirming the idea that rearranging immunoglobulin systems probably arose at the time of the evolution of the chordates from the protochordates (Schulter *et al.*, 1994). That lower deuterostomes, such as the ascidians and echinoderms, are the ideal experimental system in which functional T-cell/MHC recognition can be studied in the absence of B-cells and immunoglobulin (Marchalonis, 1977), has not been without criticism (Klein, 1989; Smith and Davidson, 1992), as Klein (1989) considers that true vertebrate lymphocytes have no evolutionary origins in extant invertebrate cells. The findings presented in this thesis, for non-specific cell mediated cytotoxic activity, and mitogen-induced or MLR proliferation, by undifferentiated cells which originate in primitive thymus-like tissue of *C. intestinalis*, support the hypothesis that rearranging immunoglobulin is an evolutionary recent addition to pre-existing defence systems and that functional lymphocytes with T-cell/MHC-like recognition systems are present in the ascidians .

Bibliography

ADEMA, C.M., VAN DER KNAAP, W.P.W.AND SMINIA, T. (1991). Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. *Reviews in Aquatic Sciences* **4**:201-223.

ANDERSON, D.H. AND SWINEHART, J.H. (1991). The distribution of vanadium and sulfur in the blood cells, and the nature of vanadium in the blood cells and plasma of the ascidian, *Ascidia ceratodes*. *Comparative Biochemistry and Physiology* **99A**:585-592

ANDERSON, P., CALIGIURI, M., RITZ, M.J. AND SCHLOSSMAN, S. F. (1989). FcϒR receptor type III CD16 is included in the ∂NK receptor complex expressed by human natural killer cells. *Nature* **341**:159-162

ANDERSON, R.S. (1971). Cellular responses to foreign bodies in the tunicate *Molgula manhattenis* (DeKay). *Biological Bulletin* **141**:91-98

ARIZZA, V., PARRINELLO, N., CAMMARTA, M. AND PICCIURRO, A. (1993). Immunocytochemical localization of cellular lectins in *Phallusia mamillata* hemocytes. *Animal Biology* **2**:83-90

AZUMI, K., SATOH, N. AND YOKOSAWA, H. (1993). Functional and structural characterization of hemocytes of the solitary ascidian, *Halocynthia roretzi*. *Journal of Experimental Zoology* **265**:309-316.

AZUMI, K., YOKOSAWA, H. AND SHIN-ICHI, I. (1990). Halocyamines : Novel antimicrobial tetrapeptide-like substances isolated from the hemocytes of the solitary ascidian *Halocynthia roretzi*. *Biochemistry* **29**:159-165.

AZUMI, K., YOKOSAWA, H. AND SHIN-ICHI, I. (1991). Lipopolysaccharide induces release of a metallo-protease from hemocytes of the ascidian, *Halocynthia roretzi*. *Developmental and Comparative Immunology* **15**:1-7

BALLARIN, L., CIMA, F. AND SABBADIN, A. (1993). Histoenzymatic staining and characterization of the colonial ascidian *Botryllus schlosseri* hemocytes. *Bollettino di Zoologia.* **60**:19-24.

BALLARIN, L., CIMA, F. AND SABBADIN, A. (1994). Phenoloxidase in the colonial ascidian, *Botryllus schlosseri* (Urochordata : Ascidiacea). *Animal Biology* **3**:41-48

BAYNE, C.J., BUCKLEY, P.M. AND DEWAN, P.C. (1980). *Schistosoma mansoni :* Cytotoxicity of hemocytes from susceptible snail hosts for sporocyts in plasma from resistant *Biomphalaria glabrata. Experimental Parasitology* **50**:409-416.

BELL, K.L. AND SMITH, V.J. (1994). A comparative study of the respiratory burst produced by the phagocytic cells of marine invertebrates. *Annals of the New York Academy of Sciences* **712**:330-331.

BERRILL, N. J. (1936). Studies in tunicate development V. The evolution and classification of ascidians. *Philosophical Transactions. Royal Society of London. Series B. Biological Sciences. B* **226**:43-70

BERRILL, N. J. (1955). In : The origin of the vertebrates. Clarendon Press, Oxford

BERTHEUSSEN, K. (1979). The cytotoxic reaction in allogeneic mixtures of echinoid phagocytes. *Experimental Cell Research* **120**:373-381.

BOILEDEAU, D. AND VALEMBOIS, P. (1977a). Natural cytotoxic activity of sipunculid leukocytes on allogeneic and xenogeneic erythrocytes. *Developmental and Comparative Immunology* **1**:207-216.

BOILEDEAU, D. AND VALEMBOIS, P. (1977b). The mechanism of leukocyte cytotoxicity studied by time-lapse microcinematography and its inhibition. An example of *in vitro* specific recognition in invertebrates. In : Developmental Immunobiology. (Solomon and Horton eds.) Elsevier/North Holland. 51-57.

BONE, Q. (1960). The origin of the chordates. *Zoological Journal Linnean Society* 44:252-269

BONE, Q. (1979). The Origin of Chordates (2nd ed.) Carolina Biological Supply Co. Burlington, N.Cal.

BONE, Q. (1981). The neotenic origin of the chordates. *Academia Nazionale dei Lincei. Atti Conv.* 49:465-486.

BRILLOUET , C., LECLERC, M., PANIJEL, J. AND BINGHI, R. (1981). *In vitro* effect of various mitogens on starfish (*Asterias rubens*) axial organ cells. *Cellular Immunology* 57:136-144

BRUNING, J.W., KARDOL, M.J. AND ARENTZEN, R. (1980). Carboxyfluorescein fluorochromasia assays I. Non-radioactively labeled cell mediated lympholysis. *Journal of Immunological Methods* 33:33-44.

BRUNNER, K.T., MAUEL, J., CEROTTINI, J.C. AND CHAPVIS, B. (1968). Quantitative analysis of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* 14:181-196.

BYRD, W.J., JACOBS, D.M. AND ARNOSS, M.S. (1977). Synthetic polyamines added to cultures containing bovine sera reversibly inhibits *in vitro* parameters of immunity. *Nature* 267:621-623

CAMMARATA, M., PARRINELLO, N. AND ARIZZA, V. (1993). In vitro release of lectins from *Phallusia mamillata* hemocytes after their fractionation on a density gradient. *Journal of Experimental Zoology* **266**:319-327

CANTACUZÉNE, J. (1919). Anticorps normaux et expérimentaux chez qualques invertebés marins. *Comptes Rendus des Seances. Societe de Biologie et des Ses Filiades*.**82**:1087.

CARLSON, R.L., EVANS, D.L. AND GRAVES, S.S. (1985). Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*). V. Metabolic requirements of lysis. *Developmental and Comparative Immunology* **9**:271-280.

CARPÉN, O., VIRTANEN, I. AND SAKSELA, E. (1981). The cytotoxic activity of human natural killer cells requires an intact secretory apparatus. *Cellular Immunology* **58**:97-106.

CONWAY MORRIS, S. (1993). The fossil record and the early evolution of the Metazoa. *Nature* **361**:219-225.

COOPER, E.L. (1980). Phylogeny of cytotoxicity. *Endeavour New Series* **4**:160-165.

COOPER, E.L. (1981). Immunity in invertebrates. *Chemical Rubber Company. Critical Reviews in Immunology* **2**:1-32

COOPER, E.L. (1982). General Immunology. Pergamon Press. Oxford. England.

CROWTHER, R.J. AND WHITTAKER, J.R. (1991). Structure of the caudal neural tube in an ascidian larva: vestiges of its possible evolutionary origin from a ciliated band. *Journal of Neurobiology* **23**:280-292.

DAVIDSON, B.S. (1993). Ascidians : producers of amino acid derived metabolites *Chemical Reviews* **93**:1771-1791

DE LEO, G. (1992). Ascidian hemocytes and their involvement in defence reactions. *Bolletino de Zoologica* **59**:195-213.

DE LEO, G., PARRINELLO, N. AND DI BELLA, M.A. (1987) Fine structure of blood system in *Ciona intestinalis* L. (Tunicata) : vessels and hemocytes in pharyngeal wall. *Archives of Biology* **98**:35-52.

DE LEO, G., PATRICOLO, E. AND FRITTITTA, G. (1981). Fine structure of the tunic of *Ciona intestinalis* L. II. Tunic morphology, cell distribution and their functional importance. *Acta Zoologica (Stockholm).* **62**:259-271.

DECKER, J.M., ELMHOLT, A. AND MUCHMORE, A.V. (1981). Spontaneous cytotoxicity mediated by invertebrate mononuclear cells toward normal and malignant vertebrate targets: inhibition by defined mono- and disaccharides. *Cellular Immunology* **59**:161-170.

DELMOTTE, F., BRILLOUET, C., LECLERC, M., LUQUET, G. AND KADER, J. (1986). Purification of an antibody-like protein from the sea-star, *Asterias rubens European Journal of Immunology* **16**:1325-1330.

DOHERTY, P.C. (1993). Cell-mediated cytotoxicity. *Cell* **7**:607-612.

DONGRONG, C., BOXER, L.A. AND PETTY, H.R. (1993). Deposition of reactive oxygen metabolites onto and within living tumor cells during neutrophil-mediated antibody-dependent cellular cytotoxicity. *Journal of Cellular Physiology* **156**:428-436.

DRACH, P. (1948). La notion de procordé et les embranchements de cordés. In : Traité de Zoologie : Anatomie, Systématique, Biologie. (P. -P. Grassé, ed.). Masson, Paris. Vol. 11. 545-551.

DUWE, A.K., WERKMEISTER, J., RODER, J.C., LAUZON, R. AND PAYNE, U. (1985). Natural killer cell-mediated lysis involves a hydroxyl radical mediated step. *Journal of Immunology* **134**:2637-2644.

ERMAK, T.H. (1975). An autoradiographic demonstration of blood cell renewal in *Styela clava* (Urochordata : Ascidiacea). *Experientia* **31**:837-839

ERMAK, T.H. (1976). The hematogenic tissues of tunicates. In : Phylogeny of Thymus and Bone Marrow-Bursa cells (R. K. Wright and E. L. Cooper, eds.). Elsevier/North Holland, Amsterdam. 45-56.

ERMAK, T.H. (1982). The renewing cell populations of ascidians. *American Zoologist* **22**:795-805.

EVANS, D.L. AND COOPER, E.L. (1990). Natural killer cells in ectothermic vertebrates. *Bioscience* **40**:745-749.

EVANS, D.L. AND MCKINNEY, E.C. (1990). Phylogeny of cytotoxic cells. In : Phylogenesis of Immune Functions. (G. W. Warr and N. Cohen.eds.) Chemical Rubber Company Press Boca Raton F.L. 215-239.

EVANS, D.L., GRAVES, S. S., COBB, D. AND DAWE, D.L. (1984b). Non-specific cytotoxic cells in fish (*Ictalurus punctatus*). II. parameters of target cell lysis and specificity. *Developmental and Comparative Immunology* **8**:303-312.

EVANS, D.L., HOGAN, K.T., GRAVES, S.S., CARSON, R.L., FLOYD, E. AND DAWE, D.L. (1984a). Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*). III. Biophysical and biochemical properties affecting cytolysis. *Developmental and Comparative Immunology* **8**:599-610.

FIELD, K.G., OLSEN, G.J., LANE, D.J., GIOVANNONI, S.J., GHISELIN, M.T., RAFF, E.C., PACE, N.R. AND RAFF, R.A. (1988). Molecular phylogeny of the animal kingdom. *Science* **239**:748-753.

FINDLAY, C. AND SMITH, V.J. (1995 submitted). Antibacterial activity in the blood cells of the solitary ascidian *Ciona intestinalis in vitro*. *Journal of Experimental Biology*.

FINDLAY, C. AND TATNER, M.F. (1994). A comparative study of T and B lymphocytes in rainbow trout (*Oncorhynchus mykiss*) following their separation by nylon wool adherence and lectin agglutinination techniques. *Comparative Haematology International* **4**:55-60.

FINSTAD, J. AND GOOD, R.A. (1964). The evolution of the immune response III. Immunological responses in the lamprey. *Journal of Experimental Medicine*. **120**:1151-1167.

FRANCESCHI, C., COSSARIZZA, A., MONTI, D. AND OTTAVIANI, E. (1991). Cytotoxicity and immmunocyte markers in cells from the freshwater snail, *Planorbarius corneus* (L.) (*Gastropoda pulmonata*) : implications for the evolution of natural killer cells. *European Journal of Immunology* **21**:489-493.

FREEMAN, G. (1970). The reticuloendothelial system of tunicates. *Journal of the Reticuloendothelial Society* **7**:183-194.

FRIDOVICH, I. (1978). The biology of oxygen radicals. *Science* **201**:875-880.

FRINDEL, E., MASSE, A., VOLKOV, L., LAVIGNAC, C. AND RIGAUD, M. (1992). Inhibitory effects of Ac SDKP on the mixed lymphocyte reaction (MLR). I. MLR with mouse spleen cells. *Leukemia.* **6**:1043-1044.

FUKE, M AND FUKUMOTO, M. (1993). Correlative fine structural, behavioral, and histochemical analysis of ascidian blood cells. *Acta Zoologica (Stockholm).* **74**:61-71.

FUKE, M.T. (1979). Studies on the coelomic cells of some Japanese ascidians. *Bulletins of the Marine Biological Station. Asamushi Tohoku University* **16**:143

FUKE, M.T. (1980). 'Contact reaction' between xenogeneic or allogeneic coelomic cells of solitary ascidians. *Biological Bulletin* **158**:304-315.

FUKE, M.T. AND NUMAKUNAI, T. (1982). Allogeneic cellular reactions between intra-specific types of a solitary ascidian, *Halocynthia roretzi. Developmental and Comparative Immunology* **6**:253-261.

FUKE, N. AND NAKAMURA, I. (1985). Patterns of cellular alloreactivity of the solitary ascidian, *Halocynthia roretzi,* in relation to genetic control. *Biological Bulletin* **169**:631-637.

FULTON, A.B. (1984). In : The Cytoskeleton. Cellular Architecture and Choreography. Chapman and Hall. New York. London. 62.

GARSTANG, W. (1928). The morphology of the Tunicata, and its bearing on the phylogeny of the Chordata. *Quarterly Journal of Microscopical Science.* **72**:51-187.

GHONEUM, M. AND COOPER, E.L. (1987). Inhibition of frog SK effector-target cell binding. *Developmental and Comparative Immunology* **11**:167-178.

GHONEUM, M., COOPER, E.L. AND SADEK, I. (1990). Variability of NK activity in anuran amphibians. *Developmental and Comparative Immunology* **14**:359-365.

GHONEUM, M., COOPER, E.L. AND SMITH,C.T. (1987). Inhibition of SK cell activity in frogs by certain drugs and sugars. *Developmental and Comparative Immunology* **11**:363-373.

GRAVES, S.S., EVANS, D.L., COBB, D. AND DAWE, D.L. (1984). Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*). I. Optimum requirements for cell lysis. *Developmental and Comparative Immunology* **8**:293-302.

HARRELSON, A.L. AND GOODMAN, C.S. (1988).Growth cone guidnce in insects: Fascilin II is a member of the immunoglobulin superfamily. *Science* **242**:700-708

HAWKINS, C.J., PARRY, D.L. AND PIERCE, C. (1980). Chemistry of the blood of the ascidian, *Podoclavella moluccensis*. *Biological Bulletin* **159**:669-680

HAYDEN, B.J. AND LAUX, D.C. (1985). Cell-mediated lysis of murine target cells by nonimmune salmonid lymphoid preparations. *Developmental and Comparative Immunololgy* **9**:627-639.

HAYWARD, P.J. AND RYLAND, J.S. (1990). In : The Marine Fauna of the British Isles and North-West Europe. Volume 2. Molluscs to Chordates. Clarendon Press. Oxford

HEBEBRAND, L.C., OLSEN, R.G., MATHES, L.E. AND NICHOLS, W.S. (1979). Inhibition of human lymphocyte mitogen and antigen response by a 15,000 dalton protein from feline leukemia virus. *Cancer Research*. **39**:443-447.

HILDEMANN, W.H. AND REDDY, A.L. (1973). Phylogeny of immune responsiveness: marine invertebrates. *Federation Proceedings* **32**:2188-2194.

HIRSCH, J.G. AND FEDORKO, M.E. (1968). Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehdye and osmium tetroxide and post-fixation in uranyl acetate. *Journal of Cell Biology* **38**:615-627

HISERODT, J.C., BRITVAN, L.J. AND TARGAN, S.R. (1982). Characterization of the cytolytic reaction mechanism of the human natural killer (NK) lymphocyte: Resolution into binding, programming and killer cell independent steps. *Journal of Immunology* **129**:1782-1787.

HORTON, J.D. (1994). Amphibians In : Immunology : A comparative approach. (R.J. Turner.ed) John Wiley and Sons, Chichester, UK. 101-136

HUGHES, W.L., COMMEFORD, S.L., GITTIN, D., KRUGER, R.C., SCHLUTZE, B., SHAH, V. AND REILLY, P. (1964) Deoxyribonucleic acid metabolism in vivo 1. Cell proliferation and death as measured by incorporation and elimination of iodoeoxyuridine. *Federation Proceedings* **23**:640-648

JACKSON A.D. AND SMITH, V.J. (1993). LPS-sensitive protease in the blood cells of the solitary ascidian, *Ciona intestinlais* (L). *Comparative Biochemistry and Physiology* **106B**:505-512.

JACKSON, A.D., SMITH, V.J. AND PEDDIE, C.M. (1993). *In vitro* phenoloxidase activity in the blood of *Ciona intestinalis* and other ascidians. *Developmental and Comparative Immunology* **17**:97-108.

JANEWAY, C.J. (1989). Natural killer cells : a primitive immune system. *Nature* **341**:108

JOHNSON, P.T. AND CHAPMAN, F.A. (1970). Comparative studies on the *in vitro* response of bacteria to invertebrate body fluids II. *Aplysia californica* (Sea Hare). and *Ciona intestinalis* (Tunicate). *Journal of Invertebrate Pathology* **16**:259-267

JULIUS, M.H., SIMPSON, E. AND HERZENBERG, L.A. (1973). A rapid method for the isolation of functional thymus-derived murine lymphocytes. *European Journal of Immunology* **3**:645-649

JURD, R.D. (1994). Reptiles and Birds. In : Immunology : A comparative approach. (R.J. Turner, ed.) John Wiley and Sons, Chichester, UK. 137-172.

KATZ, M.J. (1983). Comparative anatomy of the tunicate tadpole, *Ciona intestinalis*. *Biological Bulletin* **164**:1-27.

KELLY, K.L., COOPER, E.L. AND RAFTOS, D.A. (1992). In vitro allogeneic cytotoxicity in the solitary urochordate *Styela clava*. *Journal of Experimental Zoology* **262**:202-208.

KINGSLEY, E.A., BRISCOE, D.A. AND RAFTOS, D.A. (1989). Correlation of histocompatability reactions with fusion between conspecifics in the solitary urochordate *Styela plicata* *Biological Bulletin* **133**:282-289

KLEIN, J. (1989). Are invertebrates capable of anticipatory immune responses? *Scandanavian Journal of Immunology* **29**:499-505.

KLUIN-NELEMANS, H.C., VAN DER HARST, D., VAN DEN BURGH, J. F., VAN LUXEMBERG, S. AND BRAND, A. (1989). Comparison between clonogeneic and cytotoxic assays for measuring LAK activity. *International Journal of Cell Cloning* 7:232-241.

KOWALEVSKY, A. (1866). Entwicklungsgeschichte der einfachen Ascidien. *Mém. Acad. Mikr. Sci. St. Pétersbourg* **10**:1-19.

LARSON, M.K. AND BAYNE, C.J. (1994). Role of echinoid axial organ immunity. *Journal of Experimental Zoology* **270**:474-485.

LAVERACK, M.S. AND DANDO, J. (1987). In : Lecture Notes on Invertebrate Zoology. Third edition. Blackwell Scientific Publications. Oxford.

LAVIGNAC, C., VOLKOV, L., MASSE, A., RIGAUD, M. AND FRINDEL, E. (1992). Inhibitory effects of Ac SDKP on the mixed lymphocyte reaction (MLR). III. Human whole blood cells. *Leukemia.* **6**:1045-1047.

LUQUET, G. AND LECLERC, M. (1983). Spontaneous and induced cytotoxicity of axial organ cells from *Asterias rubens* (Asterid-echinoderm). *Immunology Letters* **6**:339-342.

MANNING, M.J. (1994). Fishes. In : Immunology : A comparative approach. (R. J. Turner.ed.) John Wiley and Sons, Chichester, UK. 69-100.

MANSOUR, M.H. AND COOPER, E.L. (1984). Serological and partial molecular characterization of a Thy-1 homolog in tunicates. *European Journal of Immunology* **14**:1031-1039.

MARCHALONIS, J.J. (1977).In : Immunity in Evolution. Harvard University Press, Cambridge, MA.

MARCHALONIS, J.J. AND SCHULTER, S.F. (1994). Development of an immune system. *Annals of the New York Academy of Sciences* **712**:1-12

MARTOJA, R., GOUZERH, P. AND MONNIOT, F. (1994). Cytochemical studies of vanadium, tunichromes and related substances in ascidians : possible biological significance. *Oceanography and Marine Biology: an Annual Review* **32**:531-556

MCKINNEY, E.C., HAYNES, L. AND DROESE, A. L. (1986). Macrophage-like effector of spontaneous cytotoxicity from the shark. *Developmental and Comparative Immunology* **10**:497-508

METCHNIKOFF, E. (1892). Leçons sur la pathologie comparée de l'inflammation, Masson, Paris; reissued (1968). in English as Lectures on the Comparative Pathology of Inflammation, Dover, New York.

MICHIBATA, H. AND UYAMA, T. (1990). Extraction of vanadium binding substance (vandobin). from a subpopulation of signet ring cells newly identified as vanadocytes in ascidians. *Journal of Experimental Zoology* **254**:132-137

MICHIBATA, H., UYAMA, T. AND HIRATA, J. (1990). Vanandium-containing blood cells (vanadocytes). show no fluorescence due to the tunichrome in the ascidian, *Ascidia sydneienis samea. Zoological Science* **7**:55-61.

MILANSEI, C. AND BURIGHEL, P. (1978). Blood cell ultrastructure of the ascidian *Botryllus schlosseri*. I. Hemoblast, granulocytes, macrophage, morula cell and nephrocyte. *Acta Zoologica (Stockholm).* **59**:135-147.

MILLAR, R.H. (1970). In : Synopses of the British Fauna (New Series). No. 1. British Ascidians. The Linnean Society of London. Academic Press. London. New York.

MILLAR, R.H. (1971). The biology of ascidians. *Advances in Marine Biology* **9**:1-100.

MOODY, C.E., SERREZE, D.V. AND RENO, P.W. (1985). Non-specific cytotoxic activity of teleost leukocytes. *Developmental and Comparative Immunology* **9**:51-64.

MUKAI, H. AND WATANABE, H. (1976). Studies on the formation of germ cells in a compound ascidian *Botryllus primigenus*. *Journal of Morphology* **148**:337-362

OHTAKE,S., ABE, T., SHISHIKURA, F. AND TANAKA, K. (1990). The fine structure and adhesion/aggregation *in vitro* of hemocytes in *Halocynthia roretzi*. *Abstracts I Meeting of Japanese Association of Developmental and Comparative Immunology* **14**:VI

OKA, H. AND WATANABE, H. (1960). Problems of colony specificity in compound ascidians. *Bulletin Marine Biological Stationn of Asamushi*. **10**:153-155

OVERTON, J. (1966). The fine structure of blood cells in the ascidian, *Perophora viridis*. *Journal of Morphology* **119**:305-326.

PALADE, G.E. (1952). A study of fixation for electron microscopy. *Journal of Experimental Medicine*. **95**:289-298

PARRINELLO, N. AND ARIZZA, V. (1988). D-galactose binding lectins from the tunicate *Ascidia malaca*: subunit characterization and hemocyte surface distribution. *Developmental and Comparative Immunology* **12**:495-508.

PARRINELLO, N. AND ARIZZA, V. (1992). Cytotoxic activity of invertebrate hemocytes with preliminary findins on the tunicate *Ciona intestinalis*. *Bollettino di Zoologia* **59**:183-189.

PARRINELLO, N., ARRIZZA, V., CAMMARATA, M. AND PARRINELLO, D.M. (1993). Cytotoxic activity of *Ciona intestinalis* (Tunicata) hemocytes: properties of the in vitro reaction against erythrocyte targets. *Developmental and Comparative Immunology* **17**:19-27

PARRINELLO, N., CAMMARATA, M., ARRIZZA, V., LIPARI, L. AND PICCIURRO, A. (1994). *In vitro* cytotoxic activity against erythrocytes by ascidian hemocytes : Target/ effector interactions. *Developmental and Comparative Immunology*. 18 S1:S89 D.P14.

PARRINELLO, N., PATRICOLO, E. AND CANICATTI, C. (1977). Tunicate immunobiology 1. Tunic reaction of *Ciona intestinalis* L. to erythrocyte injection. *Bollettino di Zoologia* **44**:373-381

PARRINELLO, N., PATRICOLO, E. AND CANICATTI, C. (1984). Inflammatory reaction in the tunic of *Ciona intestinalis* (Tunicata). I. Encapsulation and tissue injury. *Biological Bulletin* **167**:229-237

PETERSEN, J.K. AND RIISGÅRD, H.U. (1992). Filtration capacity of the ascidian *Ciona intestinalis* and its grazing impact in a shallow fjord. *Marine Ecology Progress Series*. **88**:9-17.

PETTY, C.L. AND MCKINNEY, E.C. (1983). Temperature and cellular regulation of spontaneous cytotoxicity in the shark. *European Journal of Immunology* **13**:133-138.

PORCHET-HENNERÉ, E., DUGIMONT, T. AND FISCHER, A. (1992). Natural killer cells in a lower invertebrate, *Nereis diversicolor*. *European Journal of Cell Biology* **58**:99-107

PROVINCIALI, M., STEFANO, G.D. AND FABRIS, N. (1992). Optimization of cytotoxic assay by target cell retention of the fluorescent dye carboxyfluorescein diacetate (CFDA). and comparison with conventional ^{51}Cr release assay. *Journal of Immunological Methods* **155**:19-24.

RAFTOS, D.A. (1991). Cellular restriction of histocompatability responses in the solitary urochordate, *Styela plicata*. *Developmental and Comparative Immunololgy* **15**:93-98

RAFTOS, D.A. (1994). Allorecogntion and humoral immunity in tunicates. *Annals of the New York Academy of Sciences* **712**:227-244

RAFTOS, D.A. AND BRISCOE, D.A. (1990). Genetic basis of histocompatability in the solitary urochordate *Styela plicata Journal of Heredity* **81**:96-100

RAFTOS, D.A. AND COOPER, E.L. (1991). Proliferation of lymphocyte-like cells from the solitary tunicate, *Styela clava*, in response to allogeneic stimuli. *Journal of Experimental Zoology* **260**:391-400.

RAFTOS, D.A. AND RAISON, R.L. (1992). The echinoid immune system revisited. *Immunology Today* **14**:92-93.

RAFTOS, D.A., COOPER, E.L., HABICHT, G.S. AND BECK, G. (1991b). Invertebrate cytokines - Tunicate cell proliferation stimulated by an interleukin-1-like molecule. *Proceedings of the National Academy of Sciences United States of America* **88**:9518-9522.

RAFTOS, D.A., STILLMAN, D.L. AND COOPER, E.L. (1990). *In vitro* culture of tissue from the tunicate *Styela clava. In Vitro Cellular and Developmental Biology* **26**:962-970.

RAFTOS, D.A., STILLMAN, D.L. AND COOPER, E.L. (1991a). Interleukin-2 and phytohaemmagglutinin stimulate proliferation of tunicate cells. *Immunology and Cell Biology* **69**:225-234.

RAFTOS, D.A., TAIT, N.N. AND BRISCOE, D.A. (1987a). Allograft rejection and alloimmune memory in the solitary urochordate, *Styela plicata. Developmental and Comparative Immunology* **11**:343-351.

RAFTOS, D.A., TAIT, N.N. AND BRISCOE, D.A. (1987b). Cellular basis of allograft rejection in the solitary urochordate, *Styela plicata. Developmental and Comparative Immunology* **11**:713-725

RATCLIFFE, N.A. AND ROWLEY, A.F. (1981) In : Invertebrate Blood cells 2. Academic Press, New York.

RATCLIFFE, N.A., ROWLEY, A.F., FITZGERALD, S.W. AND RHODES, C.P. (1985). Invertebrate immunity : basic concepts and recent advances. *International Reviews in Cytology* **97**:183-350.

REDDY, A.L., BRYAN, B. AND HILDEMANN, W.H. (1975). Integumentary allograft versus autograft reactions in *Ciona intestinalis* : a protochordate species of solitary tunicate. *Immunogenetics* **1**:584-590.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**:208-211.

RINEHART, K.L.JR., GLOER, G.B., WILSON, G.R., HUGHES, R.J.JR., LI, L.H., RENIS, H.E. AND MCGOVREN, J. P. (1983). Antiviral and antitumour compounds from tunicates. *Federation Proceedings* **42**:87-90.

RINKEVICH, B. (1992). Aspects of the incompatibility nature in botryllid ascidians. *Animal Biology* **1**:17-28.

RINKEVICH, B., FRANK, U., BAK, R.P.M. AND MILLER,W.E.G. (1994). Alloimmune responses between *Acropora hemprichi* conspecifics: nontransitive patterns of overgrowth and delayed cytotoxicity. *Marine Biology* **118**:731-737.

RINKEVICH, B., SHAPIRA, M., WEISSMAN, I.L. AND SAITO, Y. (1992). Allogeneic responses between three remote populations of the cosmopolitan ascidian *Botryllus schlosseri*. *Zoological Science* **9**:989-994.

ROBINSON, W.E. , AGUDELO, M.I. AND KUSTIN, K. (1984). Tunichrome content in the blood cells of the tunicate, *Ascidia callosa* stimpson, as an indicator of vanadium distribution. *Comparative Biochemistry and Physiology* **78A**:667-673.

ROCH, P.H., CANICATTI, C. AND VALEMBOIS, P. (1989). Interaction between the hemolytic system of the earthworm *Eisenia foetida andrei* on the SRBC membrane. *Biochimica et Biophysica Acta.* **938**:193-198.

ROCH, P.H., VALEMBOIS, P. AND DU PASQUIER, L. (1975). Response of earthworm leukocytes to concanavalin A and transplantation antigens. *Advances in Experimental and Medical Biology.* **64**:44.

RODER, J.C., KIESSLING, R., BIBERFELD, P. AND B. ANDERSON. (1978). Target-effector interacting in the natural killer (NK) system II. The isolation of NK cells and studies on the mechanism of killing. *Journal of Immunology* **212**:2509.

ROITT, I.M., BROSTOFF, J. AND MALE, D.K. (1989 & 1993). In : Immunology. Gower Medical Publishing. 2 nd & 3rd editions. London. New York.

ROSENSHEIN, I.L., SCHULTER, S.F., VASTA, G.R. AND MARCHALONIS, J.J. (1985). Phylogenetic conservation of heavy chain determinants of vertebrates and protochordates. *Developmental and Comparative Immunology* **9**:783-795.

ROWLEY, A.F. (1981). The blood cells of the sea squirt *Ciona intestinalis* : morphology, differential counts and *in vitro* phagocytic activity. *Journal of Invertebrate Pathology* **37**:91-100

ROWLEY, A.F. (1982a). The blood cells of *Ciona intestinalis* : an electron probe X-ray microanalytical study. *Journal of the Marine Biological Association of the United Kingdom* **62**:607-620

ROWLEY, A.F. (1982b). Ultrastructural and cytochemical studies on the blood cells of the sea squirt *Ciona intestinalis* . I. Stem cells and amoebocytes. *Cell and Tissue Research* **223**:403-414.

ROWLEY, A.F. (1983). Preliminary investigations on the possible antimicrobial properties of tunicate blood cell vanadium. *Journal of Experimental Zoology* **227**:319-322.

ROWLEY, A.F., RHODES, C.P. AND RATCLIFFE, N.A. (1984). Protochordate leucocytes : a review. *Zoological Journal Linnean Society* **227**:319-322.

SABATINI, D.D., BENSCH, K.G. AND BARRNETT, R.J. (1963). Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzyme activity by aldehyde fixation. *Journal of Cell Biology* 17:19-28.

SABBADIN, A., ZANIOLO, G. AND BALLARIN, L. (1992). Genetic and cytological aspects of histocompatibility in ascidians. *Bollettino di Zoologica* **59**:167-173.

SARGENT, J.A. (1988). Low temperature scanning electron microscopy : advantages and applications. *Scanning Microscopy* **2**:835-849.

SAWADA, T., ZHANG, J. AND COOPER, E.L. (1993). Classification and characterization of hemocytes in *Steyla clava*. *Biological Bulletin* **184**:87-96

SAWADA, T., ZHANG, J. AND COOPER, E.L. (1994). Sustained viability and proliferation of hemocytes from the cultured pharynx of *Styela clava*. *Marine Biology* **119**:597-603

SCHULTER, S.F. AND EY, P.L. (1989). Purification of three lectins from the haemolymph of the ascidian *Botrylloides leachii*. *Comparative Biochemistry and Physiology* **93B**:145-155.

SCHULTER, S.F., SCHROEDER, J., WANG, E. AND MARCHALONIS, J.J. (1994). Recognition molecules and immunoglobulin domains in invertebrates. *Annals of the New York Academy of Sciences* **712**:74-81.

SCIPPA, S., BOTTE, L. AND DE VINCENTIIS, M. (1982). Ultrastructure and X-ray microanalysis of blood cells of *Ascidiamalaca. Acta Zoologica (Stockholm)* **63**:121-131

SCIPPA, S., BOTTE, L., ZIEROLD, K. AND DE VINCENTIIS, M. (1985). X-ray microanalytical studies on cryofixed blood cells of the ascidian *Phallusiamammillata.* I. Elemental composistion of morula cells. *Cell and Tissue Research* **239**:459-461.

SCIPPA, S., IAZZETTI, G. AND DE VINCENTIIS, M. (1987). Observations on the ascidian *Phallusia mamillata* blood cells during development. *Acta Embryological and Morphological Experiments n.s.* **8**:341-346

SCOFIELD, V.L. AND NAGASHIMA, L.S. (1983). Morphology and genetics of rejection reactions between oozoids from the tunicate *Botryllus schlosseri. Biological Bulletin* **165**:733-744

SCOFIELD, V.L., SCHLUMPBERGER, J.M., WEST, L.A. AND WEISSMAN, I.L. (1982). Protochordate allorecognition is controlled by an MHC-like gene system. *Nature* **295**:499-502.

SEEGER, M.A., HAFFLEY, L. AND KAUFMAN, T.C. (1988). Characterization of amalgam : a member of the immunoglobulin super family from *Drosophila Cell* **55**:489-600

SEGAL, A.W. AND ABO, A. (1993). The biochemical basis of the NADPH oxidase of phagocytes. *Trends in Biochemical Sciences.* **18**:43-47.

SHERIF, M. AND RIDI, R.E. (1992). Natural cytotoxic cell activity in the snake *Psammophis sibilans*. *Immunobiology* **184**:348-358.

SHORTMAN, K., WILLIAMS, N., JACKSON, H., RUSSELL, P., BYTRT, P. AND DIENER, E. (1971). The separation of different cell classes from lymphoid organs. IV. The separation of lymphocytes from phagocytes on glass bead columns and its effect on sub-populations of lymphocytes and antibody-forming cells. *Journal of Cell Biology* **48**:506.

SMITH, C.T., GHONEUM, M. AND COOPER, E.L. (1988). Ultrastructure of cytotoxic cells in frogs. *Acta Zoologica*. **69**:125-133.

SMITH, L.C. AND DAVIDSON, E.H. (1992). The echinoid immune system and the phylogenetic occurrence of immune mechanisms in deuterostomes. *Immunology Today* **13**:356-361..

SMITH, M.J. (1970). The blood cells and tunic of the ascidian *Halocynthia aurantium* (PALLAS). I. Hematology, tunic morphology, and partition of cells between blood and tunic. *Biological Bulletin* **138**:354-378

SMITH, V.J. AND PEDDIE, C.M. (1992). Cell cooperation during host defense in the solitary tunicate *Ciona intestinalis* (L). *Biological Bulletin* **183**:211-219.

SMITH, V.J. AND SÖDERHÄLL, K. (1991). A comparison of phenoloxidase activity in the blood cells of marine invertebrates. *Developmental and Comparative Immunology* **15**:251-261.

SÖDERHÄLL, K., WINGEN, A., JOHANSSON, M.W. AND BERTHEUSSEN, K. (1985). The cytotoxic reactions of hemocytes from the freshwater crayfish, *Astacus astacus*. *Cellular Immunology* **94**:326-332.

SOKAL, R.R. AND ROHLF, F.J. (1981). In : Biometry. W.H. Freeman, New York.

SVANE, I.B. AND HAVENHAND, J.N. (1993). Spawning and dispersal in *Ciona intestinalis*. *Marine Ecology*. **14**:53-66.

TAKAHASHI, H., AZUMI, K. AND YOKOSAWA, H. (1994). Hemocyte aggregation in the solitary ascidian *Halocynthia roretzi* : plasma factors, magnesium ion, and Met-Lys-Bradykinin induce the aggregation. *Biological Bulletin* **186**:247-253

TAM, M.R., REDDY, A.L., KARP, R.D. AND HILDEMANN, W.H. (1976). Phylogeny of cellular immunity among vertebrates. In: Comparative Immunology. (Marchalonis, J.J ed.). Blackwell, Oxford. 98-119.

TANEDA, Y. AND WATANABE, H. (1982b). Studies on colony specificity in the compound ascidian, *Botryllus primigenus* Oka. II. In vivo bioassay for analyzing the mechanism of nonfusion reaction. *Developmental and Comparative Immunology* **6**:243-252.

TANEDA, Y. AND WATANABE, H. (1982c). Effects of X-irradiation on colony specificity in the compound ascidian, *Botryllus primigenus*. *Developmental and Comparative Immunology* **6**:665-673.

TANEDA, Y., SAITO, Y. AND WATANABE, H. (1985). Self or non-self discrimination in ascidians. *Zoological Science* **2**:433-442.

TANEDA, Y. AND WATANABE, H. (1982a). Studies on colony specificity in the compound ascidian, *Botryllus primigenus* Oka. I. Initiation of "non-fusion" reaction with special reference to blood cells infiltration. *Developmental and Comparative Immunology* **6**:43-52.

TAYLOR, M.K. AND COHEN, J.J. (1992). Cell-mediated cytotoxicity. *Current Opinion in Immunology* **4**:338-343.

TEO, S.L. AND RYLAND, J.S. (1994). Toxicity and palatability of some British ascidians. *Marine Biology* **120**:297-303.

THOMAS, J.A. (1931). Sur les réaction de la tunique d'*Ascidia mentula* Müll, à l'inoculation de *Bacterium tumefaciens* Sm. *Comptes Rendus des Seances. Societe de Biologie et de Ses Filiales* **108**:694-696.

TOKIOKA, T. (1971). *Publications of Seto Marine Biological Laboratory* **14**:43-63

TOMONAGA, S., MANSOUR, M.H. AND COOPER, E.L. (1985). An unusual tissue distribution of Thy-1 during evolution. *Federation Proceedings* **44**:790.

TOUPIN, J. AND LAMOUREAUX, G. (1976). Mitogen responsiveness of *Lumbricus terrestris* coelomocytes. In : Phylogeny of Thymus and Bone marrow-bursa cells. (R.K. Wright and E.L. Cooper, eds.). Elsevier, North Holland, Amsterdam. 19-25.

TYSON, C.J. AND JENKIN, C.R. (1974). The cytotoxic effect of haemocytes from the crayfish (*Parachaeraps bicarinatus*). on tumour cells of vertebrates. *Australian Journal of Experimental Biology and Medical Science* **52**:915-923.

VALEMBOIS, P. AND ROCH, P. (1977). Identification par autoradiographic des leucocytes stimulés à la suite de plaies ou de greffes chez un ver de terre. *Biologie Cellulaire* **28**:81.

VALEMBOIS, P., ROCH, P. AND BOILEDEAU, D. (1982). Cellular defense systems of the Platyhelminthes, Nemertea, Sipunculidea, and Annelida. In : The Reticuloendothelial System. A comprehensive treastise. (N. Cohen and M. M. Sigel. eds.). Plenum Press, New York. vol 3. 283-320

VALEMBOIS, P., ROCH, P. AND BOILEDIEU, D. (1980). Natural and induced cytotoxicities in sipunculids and annelids. In: Phylogeny of immune memory. (Manning, M.J. ed.) North Holland Biomedical Press, Elsevier, 47-55.

VASTA, G.R. AND MARCHALONIS, J.J. (1987). Invertebrate agglutinins and the evolution of humoral and cellular recognition factors. In : Invertebrate models. Cell receptors and cell communication. (A. H. Greenberg,ed.) Karger, Basel.104-117.

VASTA, G.R., AHMED, H., FINK, N.E., ELOLA, M.T., MARSH, A.G., SNOWDEN, A. AND ODOM, E.W. (1994). Animal lectins as self/non-self recogntion molecules : Biochemical and genetic approaches to understanding their biological roles and evolution. *Annals of the New York Academy of Sciences* **712**:43-55

VASTA, G.R., HUNT, J., MARCHALONIS, J.J. AND FISH, W.W. (1986). Galactosyl-binding lectins from the tunicate *Didemnum candidum*. Purification and physiochemical characterization . *Journal of Biological Chemistry* **261**:9174-9181.

WADA, H. AND SATOH, N. (1994). Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequences of 18S r DNA. *Proceedings of the National Academy of Sciences United States of America* **91**:1801-1804.

WARR, G.W. (1981). Evolution of the lymphocyte. *Immunology Today* **2**:63-68.

WARR, G.W., DECKER, J.M. AND MANDEL, D.D. (1977). Lymphocyte-like cells from the tunicate *Pyura stolonifera* : binding of lectins, morphological and functional studies. *Australian Journal of Experimental Biology and Medical Science* **55**:151-164

WEISSMAN, I.L., SAITO, Y. AND RINKEVICH, B. (1990). Allorecogntion histocompatability in a protochordate species : Is the relationship to MHC semantic or structural? *Immunological Reviews* **113**:227-241.

WIERDA, W.G., MEHR, D.S., AND KIM, Y.B. (1989). Comparison of fluorochrome labeled and Cr-labeled targets for natural killer cytotoxicity assay. *Journal of Immunological Methods* 122:15-24.

WITTKE, M. AND RENWRANTZ, L. (1984). Quantification of cytotoxic hemocytes of *Mytilus edulis* using a cytotoxicity assay in agar. *Journal of Invertebrate Pathology* 43:248-253.

WRIGHT, R.K. (1974). Protochordate immunity. I. Primary response of the tunicate *Ciona intestinalis* to vertebrate erythrocytes. *Journal of Invertebrate Pathology* 24:29-36

WRIGHT, R.K. (1981) Urochordates. In : Invertebrate Blood cells 2. (N.A. Ratcliffe, A.F. Rowley, eds).Academic Press, New York. 565-626.

WRIGHT, R.K. AND COOPER, E.L. (1975) Immunological maturation in the tunicate *Ciona intestinalis*. *American Zoologist* 15:21-27.

WRIGHT, R.K. AND ERMAK, T.H. (1982). Cellular defense systems of the Protochordata. In : The Reticuloendothelial System. A comprehensive treastise. (N.Cohen and M.M. Sigel. eds.) Plenum Press, New York, vol 3. 283-320

YOKOSAWA, H., HARADA, K., IGARASHI, K., ABE, Y., TAKAHASHI, K. AND ISHII, S. (1986). Galactose-specific lectin in the hemolymph of solitary ascidian, *Halocynthia roretzi*, Molecular, binding and functional properties. *Biochimica et Biophysica Acta* 870:242-247.

YOSHINO, T.P. (1988). Phospholipase C-like activity in phagocytic cells of the Asian clam, *Corbicula fluminea*, and its possible role in cell-mediated cytolytic reactions. *Journal of Invertebrate Pathology* 51:32-40.

ZHANG, H., SAWADA, T., COOPER, E.L. AND TOMONAGA, S. (1992). Electron microscopic analysis of tunicate (*Halocynthia roretzi*) hemocytes. *Zoological Science* 9:551-562

ZYCHLINSKY, A., JOAG, S. AND YOUNG, J.D.E. (1988). Cytolytic mechanisms of the immune system. *Current Opinion in Immunology* 1:63-69.

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In Vitro Spontaneous Cytotoxic Activity Against Mammalian Target Cells by the Hemocytes of the Solitary Ascidian, *Ciona intestinalis*

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ABSTRACT Blood cell–mediated cytotoxic activity against mammalian target cells by the hemocytes of the solitary ascidian *Ciona intestinalis* was investigated in vitro by fluorochromasia. Salt-conditioned target cells were labeled with carboxyfluorescein diacetate and challenged with mixed and separated hemocytes. The assay provided optimal conditions for the functioning of the effector hemocytes while maintaining low background leakage from the target cells. Comparison of different hemocyte populations, separated by density gradient centrifugation, revealed that only cell bands containing the phagocytic and nonphagocytic amoebocytes exhibited cytotoxicity. Experiments to characterize cytolysis demonstrated that activity increased with the effector to target cell ratio, occurred within 15 min, and was maximal at an incubation temperature of 20°C. Both human (K562) and mouse [YAC-1, P815, WEHI (3B) and L929] target cell lines were killed by the ascidian effector hemocytes. This paper demonstrates a population of nonspecific cytotoxic effector cells in the blood of *C. intestinalis* that are able to spontaneously kill a range of mammalian targets in vitro.

It is well documented that ascidians mount rejection responses to tissue grafts (Reddy et al., '75; Raftos, '87a) and that botryllid ascidians exhibit nonfusion reactions between allogeneic individuals (see review by Rinkevich, '92). Both tissue graft rejection and nonfusion reactions are associated with infiltration by the blood cells and subsequent cytotoxic events (Raftos, '87a; Rinkevich, '92). However, there have been few detailed in vitro analyses of cytotoxic responses by ascidian hemocytes.[1]

In mammals, spontaneous extracellular killing is primarily a function of the natural killer cells. Other mechanisms of nonphagocytic killing require stimulation either by antigen for antibody-dependent cell-mediated cytotoxicity, or by lymphokines for nonspecific macrophage activation. Natural killer cell (NK) activity, the ability to lyse mammalian target cells (such as K562 and YAC-1) without prior sensitization, has been detected in virtually all species of vertebrates examined and consequentially the phylogenetic emergence of the natural killer cell has attracted much interest (see review by Cooper, '80). A recent report (Sherif and Ridi, '92) describes natural cytotoxic cell activity by the thymocytes, plenocytes, and peripheral blood mononuclear cells of the snake *Psammophis sibilans*, while Ghoneum et al. ('90) report on NK activity in a variety of anuran amphibians. In teleost fish, NK-like activity is mediated by lymphoid-type cells called nonspecific cytotoxic cells (NCC) (Evans et al., '84). By contrast, nonspecific spontaneous cytotoxicity in elasmobranchs appears to be mediated by macrophage-like cells (McKinney et al., '86). Elasmobranchs have two cytotoxic cell populations: macrophage-like cells which exhibit spontaneous cytotoxicity requiring no in vitro activation, and the effectors of antibody-dependent cell-mediated cytotoxicity which are probably nonphagocytic granulocytes (see review by Evans and McKinney, '90).

Blood cell–mediated cytotoxic responses in vitro have been reported in five different invertebrate phyla: Sipunculoidea (Valembois et al., '80), Annelida (Valembois et al., 80; Decker et al., '81), Mollusca (Wittke and Renwrantz, '84; Decker et al., '81), Arthropoda (Tyson and Jenkin, '74; Söderhäll et al., '85), and Echinodermata (Bertheussen, '79; Decker et al., '81). It is not known whether these invertebrate hemocytes recognize and destroy foreign cells in a manner similar to the cytotoxic effects exerted by vertebrate natural killer cells, cytotoxic T lym-

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[1]In this paper, the term "hemocyte" can be read as "blood cell" and is not intended to infer a blood cell derived from a hemocoel.

phocytes, or activated macrophages. Additionally, there has been no investigation of in vitro cytotoxic activity against mammalian tumor cells by hemocytes from the protochordates, despite the close phylogenetic relationship of these invertebrates to the vertebrates (Bone, '79).

In vitro cytotoxic responses occur between some, but not all, allogeneic combinations of hemocytes from the ascidian *Halocynthia roretzi* (Fuke and Numakunai, '82). But, because of the mutual death of apposed cells (Fuke and Numakunai, '82), there are limited possibilities for quantitative assessment of the mechanism and kinetics behind these responses. Taneda and Watanabe ('82) demonstrated the involvement of a variety of blood cells in colonial ascidian histoincompatibility reactions, and Raftos et al. ('87b) have shown that lymphocyte-like cells specifically accumulate around allografts (but not autografts) immediately prior to rejection. Raftos et al. ('87b) suggested that urochordates can mount rapid nonadaptive responses involving many cell types and slower proliferative reactions requiring specific immunocytes (LLCs) (Raftos et al., '87b), and Kelly et al. ('92) considered that in vitro allogeneic cytotoxic responses and allogeneic transplantation rejection reactions may be mediated by the same cellular and molecular recognition mechanisms. The cellular mechanisms underlying both of these cytotoxic functions have not yet been fully investigated and characterization of the effector cells involved requires clarification.

Cytotoxicity examined in vitro constitutes a useful model with which to assess the recognitive capabilities of invertebrate hemocytes. This study examines cell-mediated cytotoxic activity in the solitary ascidian, *Ciona intestinalis*, towards mammalian target cells in vitro utilizing a modification of the fluorochromasia cytotoxicity assay described by Bruning et al. ('80). This modification entails salt conditioning the target cells in a 740 mOsm kg^{-1} buffer prior to labeling, thereby permitting the subsequent assay of cytotoxic activity in a high salt medium (940 mOsm kg^{-1}) optimal for the effectors.

MATERIALS AND METHODS

Ascidians

Specimens of *C. intestinalis* measuring between 5–10 cm long were collected from the Sound of Mull, Scotland. They were kept in aquaria with constantly circulating seawater (940–960 mOsm kg^{-1}; 10°C) and fed twice weekly with either a laboratory-grown culture of marine algae (*Rhodomonas sp.*) or live plankton samples collected with a fine net plankton tow from St. Andrews Bay, Scotland. The animals were bled according to the method previously described by Smith and Peddie ('92) using ice-cold marine anticoagulant (MAC) (0.1 M glucose; 15 mM trisodium citrate; 13 mM citric acid; 10 mM EDTA; 0.45M NaCl; pH 7.0) as diluent.

Target cells

The following cell lines were used as targets: WEHI, a mouse myelomonocytic leukemic cell (strain 3B); K562, a human erythromyeloid leukemia cell; L929, an adherent murine fibroblast; YAC-1, a murine lymphoma cell induced by Moloney virus in A/Sn mouse; and P815, a methylcholantrene-induced mastocytoma cell from DBA/2 mouse. The WEHI 3B, L929, and P815 cells were cultured in Dulbecco Eagle's medium supplemented with 10% fetal bovine serum, while the YAC-1 and K562 cells were cultured in RPMI 1640, also with 10% fetal bovine serum. Preliminary trials showed an increased subsequent rate of labeling in target cells which were allowed to stabilize in a higher osmolality saline. Therefore, prior to labeling, each target cell line at logarithmic growth phase was washed from the culture medium by centrifugation for 5 min at 400*g*, resuspended in 10 ml low salt marine saline (MS I) (740 mOsm kg^{-1}) (12 mM $CaCl_2 \cdot 6H_2O$; 11 mM KCl; 26 mM $MgCl_2 \cdot 6H_2O$; 45 mM tris; 38 mM HCl; 0.3 M NaCl; pH 7.4), and incubated for 30 min at 20°C.

Target cell labeling

The target cells were labeled with 5-carboxyfluorescein diacetate (CFDA) (Sigma, Poole, Dorset, England). A stock solution of 10 mg CFDA in 1 ml AnalaR acetone (BDH, Poole, Dorset) was prepared and stored in the dark at −20°C. The target cells in MS I (see above) were centrifuged at 400*g* for 5 min, resuspended in 5 ml of labeling solution (15 μl of the CFDA stock solution in 5 ml MS I), and incubated for 15 min at 37°C in the dark. The labeled targets were then washed twice in MS I and resuspended in a marine saline of higher osmolality (MS) (940 mOsm kg^{-1}) (12 mM $CaCl_2 \cdot 6H_2O$; 11 mM KCl; 26 mM $MgCl_2 \cdot 6H_2O$; 45 mM tris; 38 mM HCl; 0.4 M NaCl; pH 7.4) at a concentration of 2×10^6 ml^{-1}. Staining of the target cells was assessed using the fluorescent attachment of a Leitz Diaplan phase contrast microscope. Any cultures with less than 90% positively staining cells were rejected.

Effector cells

The cytotoxic blood cell populations of *C. intestinalis* were enriched using the density gradient

centrifugation method described by Smith and Peddie ('92). This technique enriches six distinct cell populations; the signet ring cells, the non-phagocytic hyaline amoebocytes, the phagocytic amoebocytes, the morula cells, the pigment cells, and the stem cells (Smith and Peddie, '92). Each of the separated cell populations, or for some experiments (see below) mixed blood cells in MAC, were washed twice in MS at 800*g* for 10 min, resuspended in MS at the required concentration, and kept on ice until needed.

Cytotoxicity assay

Cytotoxic activity was detected by incubating selected mammalian target cells with the hemocytes from *C. intestinalis*. Labeled target cells (25 μl at a concentration of 2×10^6 ml^{-1}) and effector cell suspensions [25 μl, usually at a concentration of 2×10^7 ml^{-1} depending on the effector to target cell (E:T) ratio required] were added to each well of U-bottomed 96 well microtitre plates for fluorometric use (Dynatech, Billinghurst, Sussex). The trays were then covered and, unless otherwise stated, incubated in the dark for 60 min at 20°C. Control conditions were designed to match those of the positive assay as closely as possible to avoid the effects of quenching by the effector cells (see Bruning et al., '80). For controls, therefore, 25 μl of heat-inactivated effector cells (15 min, 46°C in water bath) were substituted for the effector cells kept on ice (Bruning et al., '80). At the end of the incubation period, the microtitre trays were centrifuged (250*g*, 5 min) and the supernatant decanted. The cell pellets were then gently resuspended in 50 μl MS and the fluorescence was measured using a microplate attachment to a luminescence spectrometer (Perkin Elmer LS50) in fluorescence mode (excitation wavelength 490 nm, emission wavelength 518 nm). Typically, the control and experimental wells yielded fluorescent intensities of circa 80 units and circa 58 units, respectively, when measured against a blank of unstained targets in MS. Each assay consisted of at least four well replicates of each treatment and was repeated at least three times. Incubation in the high salt media in itself was not lethal to the target cells as clonogenicity assays showed that these target cells were capable of growth when returned to their original culture medium of Dulbecco Eagle's medium supplemented with 10% fetal bovine serum (data not shown).

Experimental assays

Initially, to establish the presence of cytotoxic activity by the blood cells of *C. intestinalis*, mixed blood cells were assayed for activity at an effector to target cell ratio of 5:1 against WEHI 3B. Subsequent assays were performed with separated cell populations enriched by density gradient centrifugation (Smith and Peddie, '92). Each cell population was removed from the gradient, washed, and resuspended in MS to give a final effector to target cell (WEHI 3B) ratio in the wells of 5:1 and assayed for cytotoxic activity as above.

Using the enriched effector cell preparation (see below) the conditions for target cell lysis were examined. The optimal osmolality of the incubation saline was found by running the assay in salines of 0.05, 0.1, 0.2, 0.3, or 0.4 M NaCl to give osmolalities of 325, 370, 560, 735, and 940 mOsm kg^{-1}, respectively (all other components of the saline were prepared as above). Using the optimal saline, the assay was performed first with effector to target cell ratios of 5, 10, 15, 20, 25, or 35:1, second at a range of incubation temperatures (5, 10, 20, 25, 30, or 35°C), and third over incubation periods of 15, 45, 75, 90, or 105 min. Finally, the cytotoxic activity of the effector cells against a range of target cells typically used in NK assays was screened.

Analysis of results

The percentage specific release of CFDA (%SR) for each assay was calculated from the fluorescent intensities of the control (F_c) and experimental wells (F_e) with each of the four well replicates (Bruning et al., '80) as follows:

$$\%SR = (1 - F_e/F_c) \times 100$$

Data from all experiments and/or treatments were compared using the Student's *t* test. In the experiments designed to determine the cell population responsible for the cytotoxic activity and to examine the effect of osmolality, temperature, or incubation time, effector cells from the same animal were assayed at each variable; therefore the test for paired variants was used (Sokal and Rohlf, '81). In the assays to determine the importance of the effector to target cell ratio, effector cells from different animals were used for each assay and the *t* test for unpaired variants was used (Sokal and Rohlf, '81). Differences were considered significant if $P < 0.05$.

RESULTS

Cytotoxic activity by mixed and separated hemocytes

The percentage specific release from the WEHI (3B) cells following incubation with mixed hemocytes from *C. intestinalis* (2 hr, 20°C) in a saline of

940 mOsm kg^{-1} was 42.66 ± 8.67 (n = 8). This cytotoxic activity was completely ammeliorated by heat treatment (46°C, 15 min) of the effector hemocytes. Comparison of the cytotoxic activity by each of the separated cell bands showed that the cytotoxic cells were enriched on bands containing the nonphagocytic and phagocytic amoebocytes (Fig. 1). Statistical analysis by paired *t* test revealed that the %SR with effectors from the population of nonphagocytic hyaline amoebocytes was significantly larger than with the phagocytic amoebocytes ($P = 0.007$) (Fig. 1). The lower bands of cells, namely the morula cells, the pigment cells, and the stem cells, did not exhibit any significant cytotoxic activity (Fig. 1). The signet ring cells were seldom present in sufficient numbers to achieve an equivalent E:T ratio, although a few experiments failed to show cytotoxic activity by these cells (data not shown). For all further experiments, activity was examined using cytotoxic cell-enriched populations of nonphagocytic and phagocytic amoebocytes.

Cytotoxic activity in media of increasing osmolality

Experiments to determine the optimum osmolality for activity showed that the cytotoxic effector cells functioned significantly better in a medium of 940 mOsm kg^{-1} (0.4 M NaCl) than in media of 735, 560, 370, or 325 mOsm kg^{-1} ($P = 0.05$, 0.006, 0.023, and 0.027, respectively) (Fig. 2). Preliminary experiments showed that the background leakage from the mammalian target cells was minimized by conditioning in a medium of 735 mOsm kg^{-1} (MS 1) for 30 min at 20°C prior to labeling.

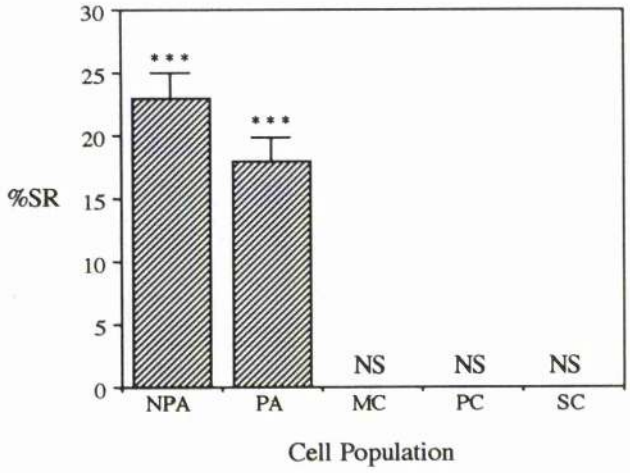

Fig. 1. Percentage specific release of carboxyfluorescein diacetate (CFDA) (%SR) from the target cells (WEHI 3B) after incubation with different enriched populations of hemocytes harvested from a density gradient separation of the blood of *Ciona intestinalis*. Incubation time 40 min, effector to target cell (E:T) ratio 5:1. Eight repetitions with different animals were performed for this experiment and the error bars represent the standard error of the mean. NS, no significant release of CFDA when compared with the controls. ***$P > 0.001$; NPA, nonphagocytic amoebocytes; PA, phagocytic amoebocytes; MC, morula cells; PC, pigment cells; SC, stem cells.

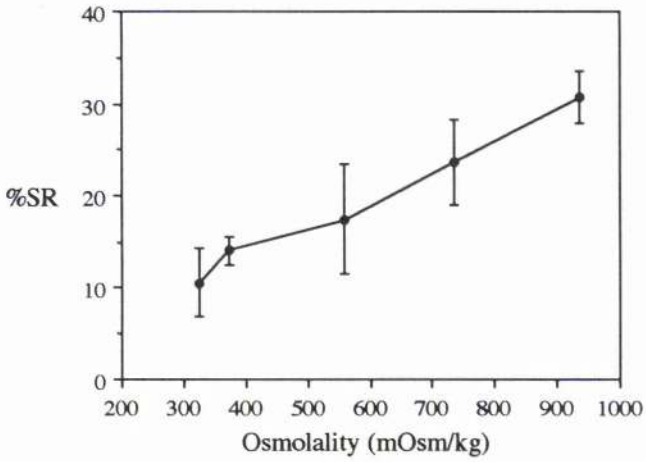

Fig. 2. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) when incubated with enriched effector hemocytes from *C. intestinalis* at an E:T ratio of 10:1 for 40 min at 20°C in media of different osmolalities. Values represent the means of three separate experiments, bars represent standard error of the mean.

Cytotoxic activity and effector:target cell ratio

By measuring the percentage specific release of CFDA from the targets following incubation with the effectors at E:T ratios of 5, 10, 15, 20, 25, or 35:1, the cytotoxic activity was shown to increase with the effector:target cell ratio (Fig. 3). The %SR

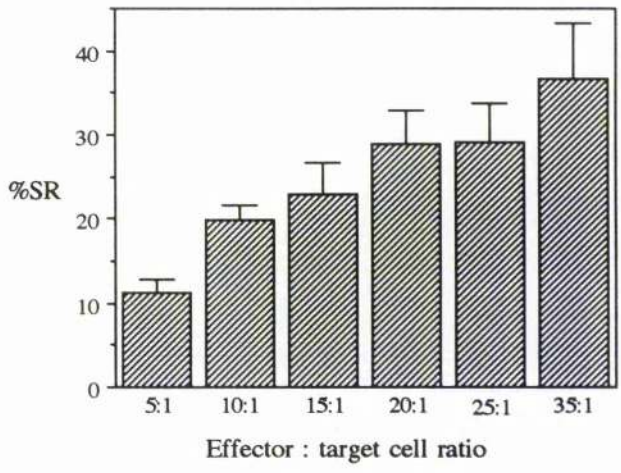

Fig. 3. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) when incubated with enriched effector hemocytes from *C. intestinalis* for 40 min at 20°C at different effector to target cell ratios. Values represent the means of three separate experiments, bars represent standard error of the mean.

increased significantly from 11.36 ± 1.47 to 19.81 ± 1.80 when the E:T ratio was increased from 5:1 to 10:1 ($P = 0.001$) (Fig. 3). Likewise when the E:T ratio was increased from 10:1 to 20:1 the %SR significantly rose from 19.81 ± 1.80 to 28.94 ± 4.03 ($P = 0.030$) (Fig. 3). The %SR for the E:T ratio of 35:1 increased from 28.94 to 36.69 ± 6.65 but this increase was not statistically significant at the 5% level (Fig. 3). Thereafter, an effector to target cell ratio of 10:1 was used. This ratio was more manageable in terms of effector cell number and consistently gave reliable significant results.

Effect of incubation temperature on cytotoxic activity

The measurement of the cytotoxic response at different incubation temperatures revealed that the %SR of CFDA was significantly higher at 20°C (23.50 ± 1.10) than at 15°C, 10°C, or 5°C ($P = 0.001$, 0.013, and 0.004, respectively) (Fig. 4). At 25°C and 35°C, the %SR declined to 20.02 ± 1.88 and 18.92 ± 2.81, respectively, although these values were not significantly lower than the %SR at 20°C due to the larger standard errors at 25°C and 30°C ($P > 0.05$) (Fig. 4). An incubation temperature of 20°C was used in all other experiments.

Optimal time for cytotoxic activity

The time study of the killing by the cytotoxic effector cells showed that the cytotoxic activity by the hemocytes of *C. intestinalis* was rapid; the percentage specific release of CFDA was 19.94 ± 4.26 after 15 min incubation (Fig. 5). After 45 min the %SR had significantly increased from 19.94 ± 4.26 to 28.44 ± 4.09 ($P = 0.028$), and at 75 min reached a maximum of 36.22 ± 4.33. No further increase in activity was recorded after this time (Fig. 5).

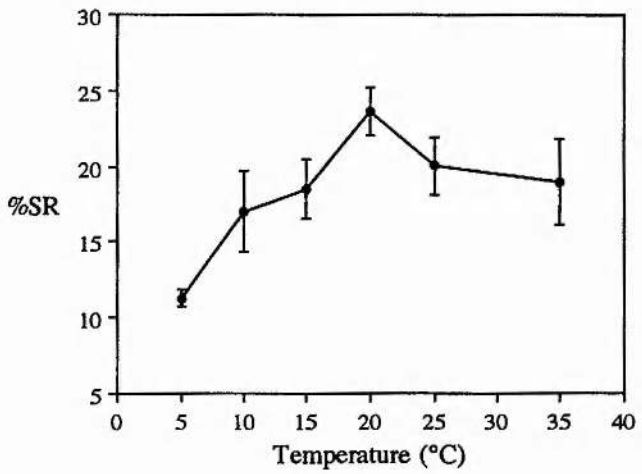

Fig. 4. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) when incubated with enriched effector hemocytes from *C. intestinalis* at an E:T ratio of 10:1, for 40 min at different temperatures. Values represent the means of three separate experiments, bars represent standard error of the mean.

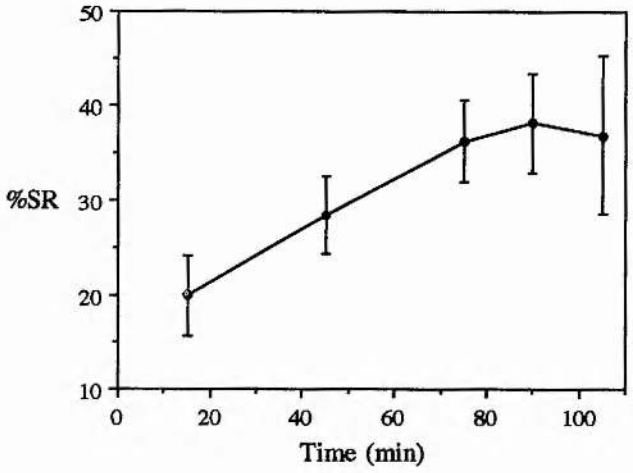

Fig. 5. Percentage specific release of CFDA (%SR) from the target cells (WEHI 3B) when incubated with enriched effector hemocytes from *C. intestinalis* at an E:T ratio of 10:1 at 20°C for increasing incubation periods. Values represent the means of three separate experiments, bars represent standard error of the mean.

Activity against different target cells

Finally, in experiments to determine the specificity of the cytotoxic reaction, the effector cells from *C. intestinalis* were found to exhibit cytotoxic activity against all the cell lines tested (Table 1). The values shown are not statistically comparable because the experiments were performed on separate occasions with different effector cell preparations (Table 1). However, the activity against the adherent cell line, L929, was noticeably lower than against the other nonadherent cell lines (Table 1).

TABLE 1. The percentage specific release of CFDA (%SR) from different mammalian target cell lines when incubated with enriched effector hemocytes from C. intestinalis *for 60 min 20°C at an E:T ratio of 10:1*[1]

Target cell line	Percentage specific release ± SEM
WEHI 3B	30.71 ± 2.90
K562	23.25 ± 6.84
L929	14.86 ± 4.56
P815	29.65 ± 1.96
YAC-1	42.28 ± 3.13

[1]Each experiment was repeated at least four times, results are means ± standard error of the mean. WEHI, a mouse myelomonocytic leukemic cell (strain 3B); K562, a human erythromyeloid leukemia cell; L929, an adherent murine fibroblast; YAC-1, a murine lymphoma cell induced by Moloney virus in A/Sn mouse; and P815, a methylcholantrene-induced mastocytoma cell from DBA/2 mouse.

DISCUSSION

The present study establishes that hemocytes from the solitary tunicate *Ciona intestinalis* effect cytotoxic activity against mammalian target cells in vitro and that this activity is lost when the effector cells are heat treated. The effector cells are enriched in the populations of phagocytic and nonphagocytic amoebocytes separated on a continuous density gradient. Cytotoxic activity was found to be rapid, temperature dependent, and effective against a range of human and mouse target cell lines. This is the first report of in vitro cytotoxic activity by the hemocytes of an ascidian against mammalian tumor cells.

The only previous study, to our knowledge, which has investigated cytotoxic activity by the blood cells of marine invertebrates against mammalian tumor cells examined activity by blood cells from an annelid, an echinoderm, and a mollusc (Decker et al., '81). The assay was performed in a medium which was considered by the authors as not ideal for the effector cells (Decker et al., '81). In our study, media of differing osmolality were tested to achieve the optimal functioning of the effector cells while maintaining low leakage of CFDA from the control target cells over the incubation period. For this purpose, fluorochromasia has many advantages over the ^{51}Cr release assays for cytotoxicity. Namely, it allows for rapid labeling of the target cells (10 min as opposed to 1 hr), fast measurement of activity (3 min for 96 wells instead of 5 hr for 60 samples), direct visual quantification of labeling, and enables the use of small sample volumes (50 μl instead of 200 μl) (Bruning et al., '80). Carboxyfluorescein diacetate passively crosses the target cell membranes and is converted by intracellular esterases to a polar fluorescent product that is retained only by cells with intact plasma membranes. Dead or dying cells with compromised membranes rapidly leak the dye allowing quantitative assays of cell viability. In our study, background leakage was minimized by conditioning the targets in a medium of 740 mOsm kg^{-1} before labeling and maximal cytolysis by the effector cells was obtained by incubation in a medium of 940 mOsm kg^{-1}. As expected for an osmoconforming marine invertebrate, the effector cells functioned best in a medium closest in osmolality to that of the aquarium seawater (940–960 mOsm kg^{-1}). This adaptation of the fluorochromasia method (Bruning et al., '80) provides, for the first time, a method by which the cytotoxic activity of marine invertebrate effector cells against mammalian target cells can be reliably determined. Using this technique, we are able to define other optimum requirements for nonspecific cytotoxic activity against WEHI (3B) in vitro and investigate the activity against a range of mammalian target cells.

Spontaneous cytotoxic cells characterized for other invertebrates have been shown to be amoebocytic and sometimes phagocytic. For example, Bertheussen ('79) found that the effector cells in the echinoid *Strongylocentrotus droebachiensis* were phagocytic amoebocytes, while Söderhäll et al. ('85) demonstrated cytotoxic activity towards mammalian target cells by both the phagocytic semigranular cells and the nonphagocytic granular cells from the freshwater crayfish *Astacus astacus*. However, more research into the characterization of the amoebocytic effector cells described in our study and in other invertebrates is necessary to clarify the phylogeny of nonspecific spontaneous cytotoxic macrophages.

In the present investigation, cytotoxic activity was found to increase with the effector to target cell ratio over the range of ratios examined. In vertebrates, killing similarly increases with E:T ratio (Evans et al., '84; Sherif and Ridi, '92), but we are unaware of any studies of the effect of increased E:T ratio in invertebrate cytotoxicity.

This study also established that incubation temperature influences the cytotoxicity and that maximal activity occurs at 20°C. In sharks, spontaneous cytotoxic activity was also found to be temperature dependent and was significantly higher at 23°C than at 30°C (Petty and McKinney, '83). Unfortunately no data are available for temperatures lower than 23°C (Petty and McKinney, '83). Similarly, in fish and anuran amphibians, the optimal in vitro temperature conditions for cytotoxicity are between 18 and 25°C (Evans and Cooper, '90). Otherwise, there is a paucity of information available on lower vertebrate and invertebrate cytotoxic activity with relation to temperature. Decker et al. ('81) found that activity in the starfish and the limpet was maximal at both 10 and 20°C but decreased at 37°C. Surprisingly, Kelly et al. ('92) were unable to detect significant differences between allogeneic cytotoxicity in *Styela clava* hemocytes incubated at 4, 15, and 25°C. In the present study the temperature dependence of cytotoxic activity indicates a metabolic requirement by the hemocytes of *C. intestinalis*.

The kinetics of the cytolytic activity by the hemocytes of *C. intestinalis* are inconsistent with the few previous analyses on the time course of invertebrate cytotoxicity against mammalian target cells. With *Astacus astacus*, a minimum of one hour is required for cytolysis to become detectable (Söder-

häll et al., '85) and, in the study by Decker et al. ('81), cytotoxic activity against P815Y mastocytoma cells was measured over 24 hr with no indication of the time course of activity. In our investigation, cytolysis was detected within 15 min of incubation. Possibly, the disparity between our results and those obtained by other workers is a reflection of the differing assay systems. Alternatively, it may be an indication of differences in the efficiency of killing between cytotoxic cells of the various invertebrate phyla.

Cytotoxic activity by the ascidian effector cells was assayed against both mouse [L929, YAC-1, P815, and WEHI (3B)] and human (K562) target cells. All of our targets were susceptible to cytolysis by the hemocytes from *C. intestinalis,* although activity was found to be less against the adherent cell line (L929) than the other, nonadherent, cell lines. While lysis of a variety of target cell lines by ascidian effector cells indicates that cytotoxic activity is nonspecific, it is not clear whether determinants on both adherent and nonadherent targets are recognized by one effector cell or whether more than one type of effector cell exists.

This is the first report of nonspecific in vitro cytotoxic activity against mammalian tumor cells by the hemocytes of a protochordate. We describe certain optimal requirements for the cytolytic process and show that activity resides in the populations of phagocytic and nonphagocytic amoebocytes. The effector cells appear to require no in vitro activation and effect rapid cytolysis of a wide range of mammalian target cells. Investigations in vivo of hemocyte function in solitary tunicates have revealed adaptive graft rejection in *Styela plicata* which is mediated by cells that bear morphological resemblances to vertebrate lymphocytes (Raftos et al., '87b). Additionally, lymphocyte-like cells in *Styela clava* have been found to proliferate in response to allogeneic stimuli (Raftos and Cooper, '91). In vitro, allogeneic cultures of mixed hemocyte types in *S. clava* exhibit enhanced cytotoxic activity over autogeneic controls (Kelly et al., '92). As yet it is difficult to draw analogies between the cell types involved in allogeneic responses in *Styela* spp. and the effectors of cytotoxic activity described in this paper. Further investigations are necessary to determine whether a second lymphocyte-like population of cytotoxic effector cells proliferate in response to foreign challenges in *C. intestinalis,* as shown for *S. clava* by Raftos et al. ('87b).

In mammals, proposed molecular mediators of cellular cytotoxicity include perforin (or cytolysin), granule-associated serine esterases, other granule-associated proteins, and ATP (see review by Taylor and Cohen, '92). Whether protochordate cytotoxic hemocytes rely on the same or similar mediators remains unknown. We are currently examining the biochemical mechanisms by which the hemocytes from *C. intestinalis* effect cytotoxic activity with a view to clarifying the extent of functional homology between the cytotoxic cells reported here and vertebrate cytotoxic cells.

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LITERATURE CITED

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

Bone, Q. (1979) The Origin of Chordates, 2nd ed. Carolina Biological Supply Co., Burlington, NC.

Bruning, J.W., M.J. Kardol, and R. Arentzen (1980) Carboxyfluorescein fluorochromasia assays I. Non-radioactively labeled cell mediated lympholysis. J. Immunol. Methods, *33:*33–44.

Cooper, E.L. (1980) Phylogeny of cytotoxicity. Endeavour, *4:*160–165.

Decker, J.M., A. Elmholt, and A.V. Muchmore (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.

Evans, D.L., and E.L. Cooper (1990) Natural killer cells in ectothermic vertebrates. Bioscience, *40:*745–749.

Evans, D.L., S.S. Graves, D. Cobb, and D.L. Dawe (1984) Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*) II. Parameters of target cell lysis and specificity. Dev. Comp. Immunol., *8:*303–312.

Evans, D.L., and E.C. McKinney (1990) Phylogeny of cytotoxic cells. In: Phylogenesis of Immune Functions. G.W. Warr and N. Cohen, eds. CRC PR, Boca Raton FL, pp. 215–239.

Fuke, M.T., and T. Numakunai (1982) Allogeneic cellular reactions between intra-specific types of a solitary ascidian, *Halocynthia roretzi.* Dev. Comp. Immunol., *6:*253–261.

Ghoneum, M., E.L. Cooper, and I. Sadek (1990) Variability of NK activity in anuran amphibians. Dev. Comp. Immunol., *14:*359–365.

Kelly, K.L., E.L. Cooper, and D.A. Raftos (1992) In vitro allogeneic cytotoxicity in the solitary urochordate *Styela clava*. J. Exp. Zool., *262*:202–208.

McKinney, E.C., L. Haynes, and A.L. Droese (1986) Macrophage-like effector of spontaneous cytotoxicity from the shark. Dev. Comp. Immunol., *10*:497–508.

Petty, C.L., and E.C. McKinney (1983) Temperature and cellular regulation of spontaneous cytotoxicity in the shark. Eur. J. Immunol., *13*:133–138.

Raftos, D.A., N.N. Tait, and D.A. Briscoe (1987a) Allograft rejection and alloimmune memory in the solitary urochordate, *Styela plicata*. Dev. Comp. Immunol., *11*:343–351.

Raftos, D.A., N.N. Tait, and D.A. Briscoe (1987b) Cellular basis of allograft rejection in the solitary urochordate, *Styela plicata*. Dev. Comp. Immunol., *11*:713–725.

Raftos, D.A., and E.L. Cooper (1991) Proliferation of lymphocyte-like cells from the solitary tunicate, *Styela clava*, in response to allogeneic stimuli. J. Exp. Zool., *260*:391–400.

Reddy, A.L., B. Bryan, and W.H. Hildemann (1975) Integumentary allograft versus autograft reactions in *Ciona intestinalis:* A protochordate species of solitary tunicate. Immunogenetics, *1*:584–590.

Rinkevich, B. (1992) Aspects of the incompatibility nature in botryllid ascidians. Anim. Biol., *1*:17–28.

Sherif, M., and R.E. Ridi (1992) Natural cytotoxic cell activity in the snake *Psammophis sibilans*. Immunobiology, *184:* 348–358.

Smith, V.J., and C.M. Peddie (1992) Cell cooperation during host defense in the solitary tunicate *Ciona intestinalis* (L). Biol. Bull., *183*:211–219.

Söderhäll, K., A. Wingen, M.W. Johansson, and K. Bertheussen (1985) The cytotoxic reactions of hemocytes from the freshwater crayfish, *Astacus astacus*. Cell. Immunol., *94*:326–332.

Sokal, R.R., and F.J. Rohlf (1981) Biometry. W.H. Freeman, New York.

Taneda, Y., and H. Watanabe (1982) Studies on colony specificity in the compound ascidian, *Botryllus primigenus* Oka. I. Initiation of "non-fusion" reaction with special reference to blood cell infiltration. Dev. Comp. Immunol., *6*:43–52.

Taylor, M.K., and J.J. Cohen (1992) Cell-mediated cytotoxicity. Curr. Opin. Immunol., *4*:338–343.

Tyson, C.J., and C.R. Jenkin (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.

Valembois, P., Ph. Roch, and D. Boiledieu (1980) Natural and induced cytotoxicities in sipunculids and annelids. In: Phylogeny of Immune Memory. M.J. Manning, ed. North Holland Biomedical Press, Elsevier, pp. 47–55.

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

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Mechanism of Cytotoxic Activity by Hemocytes of the Solitary Ascidian, *Ciona intestinalis*

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ABSTRACT The mechanism of cytotoxicity by the hemocytes from the solitary ascidian, *Ciona intestinalis,* against the target cell WEHI, a mouse myelomonocytic leukemic cell (strain 3B), was investigated in vitro. Experiments using chelators of Mg^{2+} and Ca^{2+} show that the cytotoxic activity by these hemocytes involves at least two stages distinguishable by their divalent cation requirements. In particular removal of both Ca^{2+} and Mg^{2+} by EDTA inhibits effector to target cell conjugate formation and target cell lysis, whereas selective removal of Ca^{2+} by EGTA inhibits target cell lysis but does not reduce the formation of effector to target cell conjugates. Evidence that cytotoxic activity is reduced by sodium azide, cytochalasin B, colchicine, vinblastine sulfate, and monensin, indicates that the response involves energy metabolism, requires an intact cytoskeleton, and entails active secretory processes in the effector cells. In addition, cytotoxic activity is probably not mediated by superoxide anions or hydrogen peroxide, because the antioxidant enzymes, superoxide dismutase, and catalase did not affect target cell lysis. It is therefore likely that cytotoxic activity by the hemocytes from *C. intestinalis* is mediated by mechanisms similar to those of vertebrate cytotoxic cells.

Non-specific spontaneous cytotoxic activity against mammalian tumor cells is well documented for many species of vertebrates (Evans and McKinney, '90). Recently, research has been directed towards determining the phylogenetic origins of this fundamental immune response (see review by Evans and Cooper, '90). Since anatomical, embryological, and molecular phylogenetic studies place ascidians close to the origin of the vertebrate line (Bone, '79; Field et al., '88; Conway-Morris, '93), studies of ascidian immune responses may reveal similarities with lower vertebrate immune systems.

The mechanisms of cytotoxic activity by vertebrate natural killer (NK) cells or cytotoxic lymphocytes have been extensively investigated. Non-specific cytotoxic cells (NCC) in fish have requirements for electron transport systems, effector cell motility, Mg^{2+} and Ca^{2+} (for binding and cytolysis, respectively), and intact secretory apparatus (Carlson et al., '85). In mammals, precise cytotoxic killing mechanisms are still the subject of great debate (see review by Taylor and Cohen, '92), although studies have also revealed dependence upon intact secretory apparatus, divalent cations, and a functional cytoskeleton (Carpén et al., '81). Teleost NCC and mammalian NK cells therefore seem to have similar metabolic requirements for cytotoxic activity (Evans and McKinney, '90). Studies on human neutrophil mediated cytotoxicity have linked the generation of reactive oxygen metabolites with antibody-dependent target cell destruction (Dongrong et al., '93). However, natural killer cell activity does not appear to be mediated by superoxide radicals or hydrogen peroxide (Duwe et al., '85).

The existence of non-specific cytotoxic cells in various invertebrate groups has often been described but few have investigated possible killing mechanisms. The most detailed by Boiledieu and Valembois ('77), reports that the cytotoxic activity of sipunculid leucocytes in vitro requires effector to target cell contact, divalent calcium ions, and intact microtubule assemblages. Limited evidence exists for molluscs that hemocyte-mediated cytotoxicity involves reactive oxygen intermediates and/or the action of lysosomal enzymes (see review by Adema et al., '91).

Fuke ('80), Fuke and Numakunai ('82), and Kelly et al. ('92) have shown that during cytotoxic events between allogeneic or xenogeneic ascidian

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hemocytes, which represent more appropriate targets, both effector and target cells lyse upon contact, thereby confounding investigation of the mechanism(s) of killing. We have recently shown that a cell population present in the blood of the solitary ascidian *Ciona intestinalis* mediates cytotoxic activity towards a range of human and mouse target cell lines in vitro (Peddie and Smith, '93). This activity was found to increase with the effector to target cell ratio, occur within 15 minutes, and to be temperature-dependent (Peddie and Smith, '93). In this present study, using a mammalian target cell line, we examine the cellular mechanisms involved in the cytotoxic activity by the hemocytes of *C. intestinalis.*

MATERIALS AND METHODS

Animals

Specimens of *C. intestinalis,* measuring between 5–10 cm long, were collected from Croab Haven, Argyll, Scotland. They were kept in aquaria with constantly circulating seawater (32 ± 2‰; 10°C) and fed twice weekly with either a laboratory grown culture of marine algae (*Rhodomonas sp.*) or live plankton samples collected with a fine net plankton tow from St. Andrews Bay, Scotland. The animals were bled into ice cold marine anticoagulant (MAC) according to the methods previously described by Smith and Peddie ('92).

Effector cell preparation

The blood cells of *C. intestinalis* were separated on a 60% Percoll continuous density gradient as described by Smith and Peddie ('92). The bands containing the enriched cytotoxic effector cells were washed twice in marine saline (MS) (940 mOsM kg^{-1}) (12 mM $CaCl_2.6H_2O$; 11 mM KCl; 26 mM $MgCl_2.6H_2O$; 45 mM tris; 38 mM HCl; 0.4 M NaCl; pH 7.4) at 800*g* for 10 min, resuspended in MS at 2×10^7 ml^{-1}, and stored on ice until use (Peddie and Smith, '93).

Target cell preparation

The target cells from a mouse myelomonocytic leukemic line (WEHI, strain 3B) were prepared and labeled with 5-carboxyfluorescein diacetate (CFDA) (Sigma, Poole, Dorset, England) as described previously (Peddie and Smith, '94). Briefly, the target cells were labeled with CFDA in low salt marine saline (MS I) (740 mOsm kg^{-1}) (12 mM $CaCl_2.6H_2O$; 11 mM KCl; 26 mM $MgCl_2.6H_2O$; 45 mM tris; 38 mM HCl; 0.3 M NaCl; pH 7.4) for 15 min at 37°C (Peddie and Smith, '93), washed in MS I, and resuspended in MS at a concentration of 2×10^6 ml^{-1}. Staining of the target cell was assessed using the fluorescent attachment o Leitz Diaplan phase contrast microscope. Any cul tures with less than 90% positively staining cell were rejected. Clonogeneic assays confirmed tha the target cells remained viable and capable o colony formation for the duration of the assay.

Cytotoxicity assay

Cytotoxic activity was assayed by incubating th target cells with enriched effector cells from *C intestinalis* as described in Peddie and Smith ('93) Labeled target cells (20 µl at a concentration of $\times 10^6$ ml^{-1}) were incubated with effector cell sus pensions (20 µl at a concentration of 2×10^7 ml^{-1} and 10 µl of divalent cation chelator, inhibitor, an tioxidant enzyme, or MS, in 96 well microtitr plates (U-bottomed for fluorometric use Dynatech Billinghurst, Sussex). For negative controls, 20 µ of heat inactivated effector cells (15 min, 46°C i water bath) were substituted for the effector cell kept on ice (Bruning et al., '80). All trays were the incubated in the dark for 45 min at 20°C. Afte incubation, the trays were centrifuged (250*g*, 5 min and the cell pellets gently resuspended in 50 µ MS. The fluorescence was measured using microplate attachment to a luminescence spectrom eter (Perkin-Elmer, Beaconsfield, Bucks, England LS50) in fluorescence mode (excitation wavelengt 490 nm, emission wavelength 518 nm). Each assa consisted of at least four well replicates of eac treatment and was repeated at least three times.

Ion chelators

The effect of divalent cation chelators on effec tor to target cell binding and cytotoxic activity b the hemocytes of *C. intestinalis* was investigate by the inclusion of EDTA or EGTA with Mg^{2+} EDTA or EGTA were dissolved in Mg^{2+} and Ca^2 depleted-MS (MSD) at a stock concentration of 10 mM. Cytotoxicity was then measured with MSI at EDTA concentrations of 10, 1, or 0.1 mM, o with MSD plus 5 mM Mg^{2+} at EGTA concentra tions of 10, 1, or 0.1 mM. Positive controls wer performed with MSD plus 5 mM Ca^{2+} and 5 mM Mg^{2+}. Live and heat killed effector cells were in cubated in the appropriate saline for 15 minute prior to the addition of the target cells.

To assess the importance of effector to targe cell binding in the cytotoxic activity, the numbe of effector to target cell conjugates formed i EDTA (10 mM), EGTA (10 mM) plus Mg^{2+} (5 mM) or MS were counted. For this, agarose was pre

pared by dissolving a 1:5 blend of agarose (Sigma types I and IV; Sigma Chemical Co., Poole Dorset, England) in boiling water to make a 2% solution which was stored at 4°C in 2 ml aliquots. As required, each aliquot was melted, cooled to 45°C, supplemented with 1 ml of triple strength marine saline to give the correct salt concentration, and then held at 39°C in a water bath. Meanwhile, target (150 µl of 10^6 ml^{-1}) and effector cells (150 µl of 10^7 ml^{-1}) were incubated together for 5 minutes at 20°C, then gently centrifuged (200*g*) for 5 minutes and the supernatant discarded. The target and effector cell pellet was then gently resuspended in 50 µl MS and subsequently dispersed in 50 µl of the prepared molten agarose. This cell suspension was quickly spread over agarose pre-coated glass slides and after air drying for 2 minutes, stored in dishes filled with MS. Using light microscopy, the proportion of 100 target cells which had conjugated effector cells was recorded for each slide. Three replicate slides were prepared for each treatment.

Inhibitors of cellular processes

The effect of inhibitors of cellular processes upon the cytotoxic activity was determined by treating both the live and the heat-killed effector cells with various concentrations of the inhibitors for 15 minutes. All the inhibitors, with the exception of monensin, were present during the subsequent cytotoxicity assay. Monensin, which acts irreversibly, was removed by washing the treated effector cells.

Vinblastine sulfate (Sigma) (1 mg ml^{-1}) was dissolved in MS and incubated with the effector cells at concentrations of 50, 100, or 200 µg ml^{-1}. Colchicine (Sigma) (4 mg ml^{-1}) was also dissolved in MS and was incubated with the effector cells at concentrations of 50, 100, or 400 µg ml^{-1}. Cytochalasin B (from *Helmithosporium dematioideum,* Sigma) was dissolved in dimethyl sulfoxide (DMSO) (Sigma) (1 mg ml^{-1}) and used at final concentrations in MS of 0.1, 1, or 10 µg ml^{-1}. Sodium azide (NaN_3) (BDH, Poole, Dorset) was prepared in MS (1 M) and incubated with the effector cells at final concentrations of 10, 1, or 0.1 mM. Monensin (Sigma) was dissolved in absolute ethanol at a concentration of 1 mg ml^{-1} and incubated with the effectors at concentrations in MS of 2.5, 10, or 50 µg ml^{-1} before washing. Preliminary investigations confirmed that DMSO and ethanol, at the concentrations used, had no effect on target cell viability or effector cell cytotoxicity.

Antioxidant enzymes

To ascertain the role of reactive oxygen metabolites in cytotoxic activity, superoxide dismutase and catalase were used as scavengers of superoxide anions and hydrogen peroxide, respectively. Superoxide dismutase (SOD) (from bovine erythrocytes E.C. 1.15.1.1, Sigma) was used at final concentrations of 50, 100, and 280 units ml^{-1}, while catalase (from bovine liver E.C. 1.11.1.16, Sigma) was used at final concentrations of 100, 200, and 1,150 units ml^{-1}. Assays for cytotoxic activity were carried out in the presence of SOD or catalase either alone or in combination. For positive controls, the antioxidant enzymes were replaced with MS. For every assay, the effect of the enzyme on the target cells alone was controlled by inclusion in the wells containing target cells and heat killed effector cells.

Analysis of results

The percentage specific release of CFDA (%SR) for each assay was calculated from the fluorescent intensities of the control (F_c) and experimental wells (F_e) with each of the four well replicates (Bruning et al., '80) as follows:

$$\%SR = (1 - F_e/F_c) \times 100.$$

Data was analysed using a two-way analysis of variance, first, to find if the treatment had a significant effect upon the %SR, and second, to find if the effect was dose dependent (Sokal and Rohlf, '81). In assays for binding of effector and target cells, control and experimental results were compared with the t-test for paired variants (Sokal and Rohlf, '81). Differences were considered significant for both analyses when $P \geq 0.5$.

RESULTS

The cytotoxic activity by the hemocytes of *C. intestinalis* against the target cells was significantly inhibited by both EDTA and EGTA (P = 0.006 and 0.003, respectively) (Figs. 1, 2). In the experiments to measure effector to target cell conjugate formation and related cytotoxic activity, EDTA, a chelator of both magnesium and calcium ions, reduced both effector to target cell binding (P = 0.002) and cytotoxic activity (P = 0.010) (Table 1). By contrast, EGTA (a chelator of calcium ions only) plus Mg^{2+} had no effect on effector to target cell binding (P = 0.882) but significantly inhibited cytotoxic activity (P = 0.016) (Table 1). The ability of Ca^{2+} to facilitate binding was not investigated.

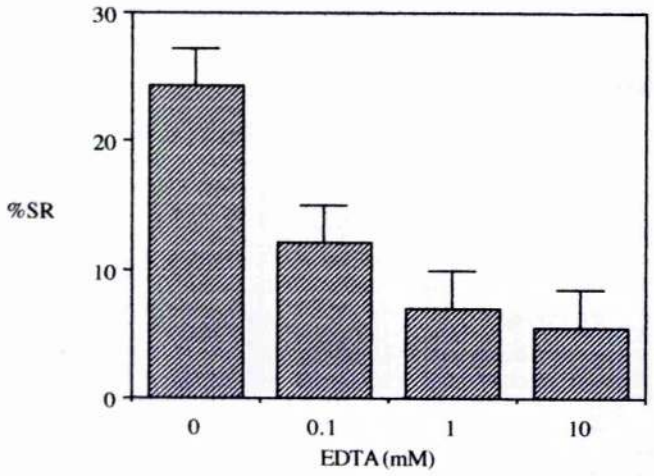

Fig. 1. The effect of calcium and magnesium ions on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector hemocytes from *C. intestinalis*. Live and heat killed effector cells were incubated in Mg^{2+}- and Ca^{2+}-depleted MS at EDTA concentrations of 10, 1, or 0.1 mM for 15 minutes prior to the addition of the target cells. Positive controls were performed with MS. Values represent the mean of four separate experiments, bars represent the standard error.

TABLE 1. The effect of calcium and magnesium ions on the percentage conjugate formation between effector and target cells and the related percentage specific release of carboxyfluorescein diacetate (CFDA) from the target cells[1]

Saline	Percentage conjugates	Percentage specific releas
10 mM EDTA	4.6 ± 1.1	5.6 ± 2.2
10 mM EGTA + 5 mM Mg^{2+}	19.8 ± 4.8	8.6 ± 1.3
5 mM Ca^{2+} + 5 mM Mg^{2+}	20.7 ± 7.7	24.3 ± 2.2

[1]Target (150 µl of 10^6 ml^{-1}) and effector cells (150 µl of 10^7 ml^{-1}) wer incubated together for 5 minutes at 20°C with EDTA, EGTA + Mg^{2} and for controls with MS, centrifuged and the pellet was resuspende in 50 µl MS and then with 50 µl of the prepared molten agarose (se Materials and Methods). The pellet was then quickly spread ove agarose pre-coated glass slides. After air drying for 2 minutes th slides were then stored in dishes filled with saline. The proportion c 100 target cells which had conjugated effector cells on each slide wer recorded under a light microscope. Three replicate slides were pre pared for each treatment. Values represent the mean with standar error of four separate experiments.

Sodium azide (NaN_3) was used to investigate the energy requirements of tunicate cytotoxic cells during target cell lysis. Sodium azide interferes with electron transfer and affects the active move-

ment of cells, cell surface dynamics, and cellula secretion processes (Carlson et al., '85). In this study NaN_3, was found to significantly reduce target cel lysis by *C. intestinalis* hemocytes ($P = < 0.001$) in a dose-dependent way ($P = 0.001$) (Fig. 3).

Cytochalasin B was used to investigate the rol of actin-containing microfilaments and cell motil

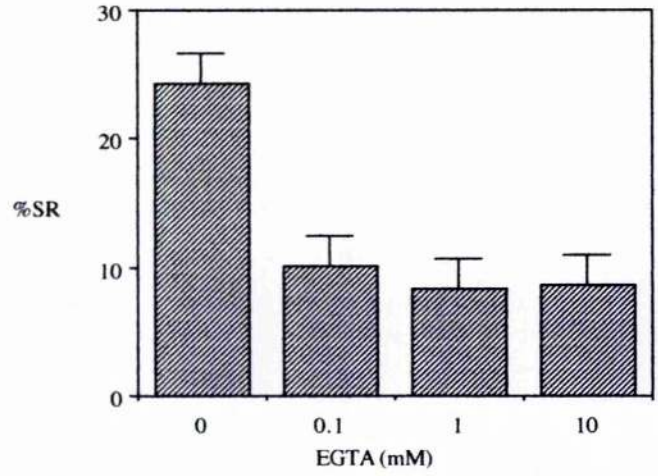

Fig. 2. The effect of calcium ions on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector hemocytes from *C. intestinalis*. Live and heat killed effector cells were incubated with MS + 5 mM Mg^{2+} but no Ca^{2+} ions at EGTA concentrations of 10, 1, or 0.1 mM for 15 minutes prior to the addition of the target cells. Positive controls were performed with MS. Values represent the mean of four separate experiments, bars represent the standard error.

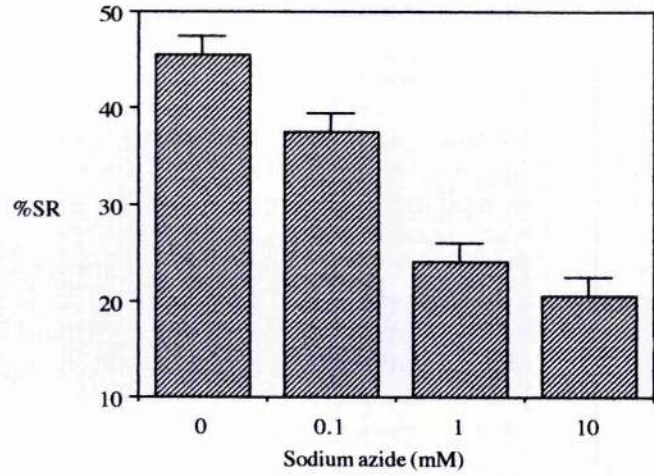

Fig. 3. The effect of sodium azide on the percentage spe cific release (%SR) of carboxyfluorescein diacetate (CFDA from the target cells (WEHI 3B) after incubation with en riched effector hemocytes from *C. intestinalis*. Sodium azid was incubated with the effector cells at final concentration of 10, 1, or 0.1 mM prior to the addition of the target cell and remained during the assay. Positive controls were per formed with MS. Values represent the mean of six separat experiments, bars represent the standard error.

ity in target cell lysis. Cytochalasin B has been demonstrated to inhibit amoeboid movement (which is insensitive to colchicine) by competing with cellular proteins for the fast assembly ends of microfilaments (Fulton, '84). Cytochalasin B significantly inhibited cytotoxic activity by *C. intestinalis* hemocytes at all concentrations ($P = 0.014$) although inhibition was not dose-dependent ($P = 0.205$) (Fig. 4).

Colchicine and vinblastine sulfate were used to disrupt cellular microtubules, thereby assessing the role of microtubule dependent movement in cytotoxic activity. Significant inhibition of cytotoxic activity ($P = 0.001$) in a dose-dependent manner ($P = 0.004$) was observed following addition of vinblastine sulfate to the effector cells (Fig. 5). Colchicine produced similar inhibition ($P = 0.01$) although the reduction was not dose-dependent ($P = 0.334$) (Fig. 6).

Experiments to ascertain whether inhibition of cellular secretion effects the cytotoxicity using carboxylic ionophore, monensin, showed that target cell lysis was significantly inhibited by monensin ($P = < 0.001$) in a dose dependent manner ($P = 0.003$) (Fig. 7).

The role of superoxide anions and hydrogen peroxide was investigated using the antioxidant enzymes, SOD and catalase. No significant inhibition of cytotoxic activity by the hemocytes from *C. intestinalis* was observed following treatment with SOD ($P = 0.148$), catalase ($P =$

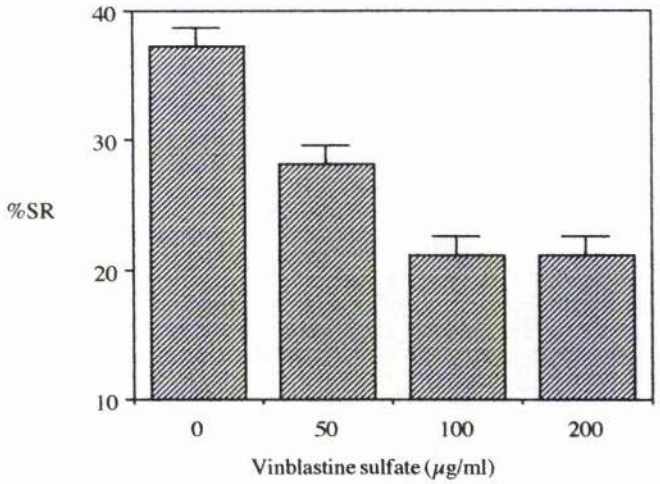

Fig. 5. The effect of vinblastine sulfate on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector hemocytes from *C. intestinalis*. Vinblastine sulfate was incubated with the effector cells at final concentrations of 50, 100, or 200 μg ml^{-1} prior to the addition of the target cells and remained during the assay. Positive controls were performed with MS. Values represent the mean of three separate experiments, bars represent the standard error.

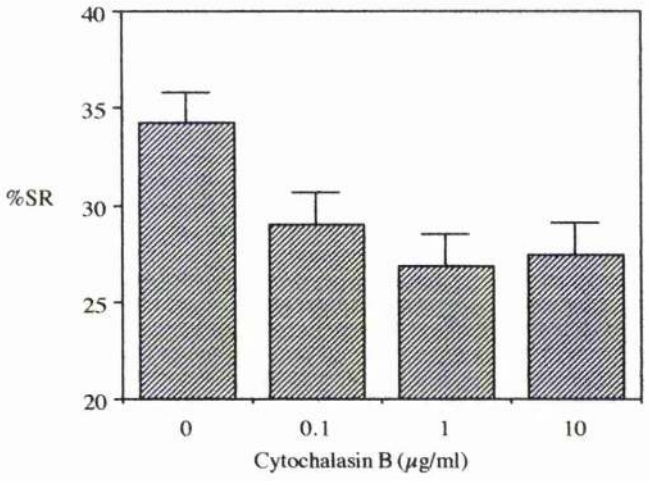

Fig. 4. The effect of cytochalasin B on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector hemocytes from *C. intestinalis*. Cytochalasin B at final concentrations of 0.1, 1, or 10 μg ml^{-1}. Live and heat killed effector cells were incubated for 15 minutes prior to the addition of the target cells and remained during the assay. Positive controls were performed with MS. Values represent the mean of six separate experiments, bars represent the standard error.

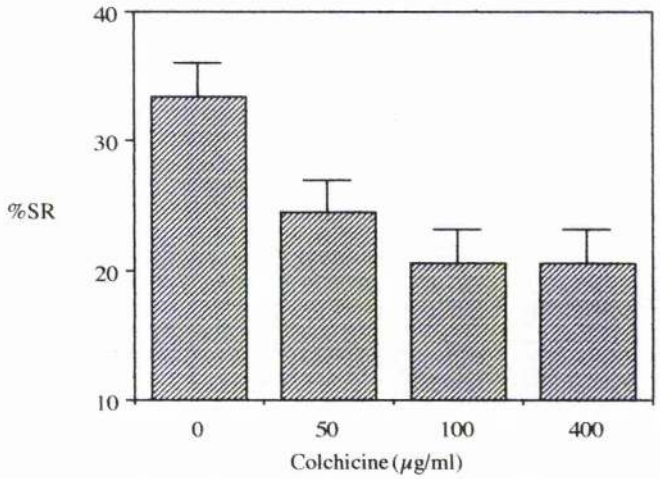

Fig. 6. The effect of colchicine on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector hemocytes from *C. intestinalis*. Colchicine was incubated with the effector cells at concentrations of 50, 100, or 400 μg ml^{-1} prior to the addition of the target cells and remained during the assay. Positive controls were performed with MS. Values represent the mean of three separate experiments, bars represent the standard error.

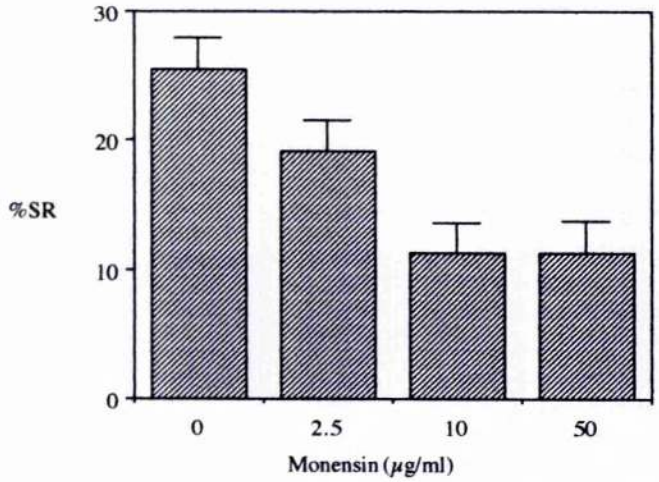

Fig. 7. The effect of monensin on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector hemocytes from *C. intestinalis*. Monensin was incubated with the effectors at concentrations of 2.5, 10, or 50 μg ml^{-1} before washing the treated effector cells prior to the addition of the target cells. Values represent the mean of six separate experiments, bars represent the standard error.

0.846), or SOD and catalase ($P = 0.196$) at any of the concentrations tested (data for SOD at 280 units ml^{-1} and catalase at 1,150 units ml^{-1} shown in Fig. 8).

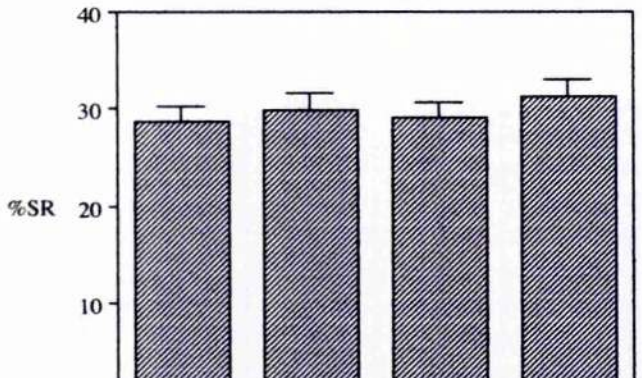

Fig. 8. The effect of antioxidant enzymes on the percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) after incubation with enriched effector hemocytes from *C. intestinalis*. SOD at 280 units ml^{-1} and catalase at 1,150 units ml^{-1} (final concentrations) were incubated with the effector cells for 15 minutes prior to the addition of the target cells and remained during the assay. For positive controls, the antioxidant enzymes were replaced with MS. Values represent the mean of six separate experiments, bars represent the standard error.

DISCUSSION

In this study, we demonstrate that cytotoxic a
tivity by hemocytes from *C. intestinalis* toward
mammalian tumor cells in vitro is reduced by ch
lation of divalent cations and by inhibition of ce
lular processes. By contrast, the inclusion
antioxidant enzymes to remove superoxide anion
and hydrogen peroxide does not affect cytotox
activity.

These results indicate that cytotoxic activity b
the hemocytes of *C. intestinalis* involves at lea
two stages distinguishable by their divalent ca
ion requirements. The first stage entails recogn
tion and binding of the effector to the target cell
dependent only upon the presence of magnesiu
ions, while target cell lysis is calcium dependen
Similar two stage divalent cation dependencies hav
been reported in mammalian (Hiserodt et al., '82
anuran (Ghoneum and Cooper, '87), and teleo
(Carlson et al., '85) cytotoxic cells.

The finding that cytotoxic activity is inhibite
by sodium azide, a reversible inhibitor of energ
metabolism which blocks electron transfer, den
onstrates that cellular energy metabolism is r
quired for target cell lysis by *C. intestinal*
hemocytes. Disruptors of the cytoskeleton wer
also found to inhibit the cytotoxic activity. Cy
tochalasin B, which disrupts the microfilament
functioning in cell movement (Fulton, '84), ma
inhibit cytotoxic activity by preventing contact fo
mation between the effector and the target cell
Vinblastine sulfate and colchicine are known t
disrupt microtubule assemblies and prohibit m
crotubule dependent movement by blood cel
(Fulton, '84). However, intact microtubule assem
blies are also commonly thought to be necessar
for cellular secretion (Fulton, '84). Therefor
vinblastine sulfate and colchicine may inhibit cyt
toxic activity by the hemocytes from *C. intestinal*
through either mechanism. Monensin is known t
specifically block cellular secretion by causing
partial Na/K equilibrium within cells and to in
terrupt the vesicular traffic of Golgi-derive
vesicles to cell membranes (Carpén et al., '81). I
the present study, monensin was found to inhib
the lysis of the target cells in a dose-depender
manner indicating that target cell killing is e
fected through the secretion of one or more fa
tors from the effector cell. Recently, Parrinello
al. ('93) have found that Ca^{2+} dependent factor
present within sonicated hemocyte debris from
intestinalis lyse sheep erythrocytes. Whether sim
lar lytic factors actively secreted from intact

intestinalis hemocytes cause the lysis of target cells observed in the present study is unknown and requires further investigation.

Finally, antioxidant enzymes were used to test whether oxidative damage by super oxide radicals and/or hydrogen peroxide mediate cytotoxic activity by the hemocytes of *C. intestinalis*. However, inclusion of SOD and catalase in the cytotoxicity assay, even at very high concentrations, produced no inhibition of cytotoxic activity by the hemocytes of *C. intestinalis*. It seems unlikely, therefore, that superoxide anions and hydrogen peroxide are involved in the mechanism of cytotoxic activity by the hemocytes from *C. intestinalis*. Natural killer cell activity in humans, unlike other mammalian antibody-dependent cytotoxic activities (Dongrong et al., '93), is also unaffected by the antioxidant enzymes catalase and superoxide dismutase (Duwe et al., '85).

This present study demonstrates that cytotoxic activity by the hemocytes of *C. intestinalis* involves energy metabolism, effector to target cell binding, a requirement for divalent calcium in the post-binding lytic stage and the active secretion of cytotoxic factor(s) from the effector cells. We also show that the phenomenon is probably not mediated by superoxide anion or hydrogen peroxide production. Therefore, this study indicates that the mechanism(s) of non-specific cytotoxic activity employed by the hemocytes from *C. intestinalis* are similar to those by mammalian NK and teleost non-specific cytotoxic blood cells. Further characterization of these ascidian cytotoxic cells and the possible secreted cytotoxic factor(s) should reveal more information relevant to the phylogeny of non-specific cytotoxic blood cells throughout the animal kingdom.

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LITERATURE CITED

Adema, C.M., W.P.W. Van der Knaap, and T. Sminia (1991) Molluscan hemocyte-mediated cytotoxicity: The role of reactive oxygen intermediates. Rev. Aquatic Sci., *4: 201–223.*

Boiledieu, D., and P. Valembois (1977) The mechanism of leukocyte cytotoxicity studied by time-lapse microcinematography and its inhibition. An example of in vitro specific recognition in invertebrates. In Developmental Immunobiology. J.B. Solomon and J.D. Horton, eds. Elsevier, North Holland, Amsterdam, pp. 51–57.

Bone, Q. (1979) The Origin of Chordates, Ed. 2. Carolina Biological Supply Co., Burlington, NC.

Bruning, J.W., M.J. Kardol, and R. Arentzen (1980) Carboxyfluorescein fluorochromasia assays I. Non-radioactively labeled cell mediated lympholysis. J. Immunol. Methods, *33:*33–44.

Carlson, R.L., D.L. Evans, and S.S. Graves (1985) Non-specific cytotoxic cells in fish (*Ictalurus punctatus*) V. Metabolic requirements of lysis. Dev. Comp. Immunol., *9:*271–280.

Carpén, O., I. Virtanen, and E. Saksela (1981) The cytotoxic activity of human natural killer cells requires an intact secretory apparatus. Cell. Immunol., *58:*97–106.

Conway Morris, S. (1993) The fossil record and the early evolution of the metazoa. Nature, *361:*219–225.

Dongrong, C., L.A. Boxer, and H.R. Petty (1993) Deposition of reactive oxygen metabolites onto and within living tumor cells during neutrophil-mediated antibody-dependent cellular cytotoxicity. J. Cell. Physiol., *156:* 428–436.

Duwe, A.K., J. Werkmeister, J.C. Roder, R. Lauzon, and U. Payne (1985) Natural killer cell-mediated lysis involves a hydroxyl radical mediated step. J. Immunol., *134:*2637–2644.

Evans, D.L., and E.L. Cooper (1990) Natural killer cells in ectothermic vertebrates. Bioscience, *40:*745–749.

Evans, D.L., and E.C. McKinney (1990) Phylogeny of cytotoxic cells. In Phylogenesis of Immune Functions. G.W. Warr and N. Cohen, eds. CRC Press, Boca Raton, FL, pp. 215–239.

Field, K.G., G.J. Olsen, D.J. Lane, S.J. Giovannoni, M.T. Ghiselin, E.C. Raff, N.R. Pace, and R.A. Raff (1988) Molecular phylogeny of the animal kingdom. Science, *239:*748–753.

Fuke, M.T. (1980) "Contact reaction" between xenogeneic or allogeneic coelomic cells of solitary ascidians. Biol. Bull., *158:*304–315.

Fuke, M.T., and T. Numakunai (1982) Allogeneic cellular reactions between intra-specific types of a solitary ascidian, *Halocynthia roretzi.* Dev. Comp. Immunol., *6:*253–261.

Fulton, A.B. (1984) The Cytoskeleton. Cellular Architecture and Choreography. Chapman and Hall, New York, 80 pp.

Ghoneum, M., and E.L. Cooper (1987) Inhibition of frog SK effector-target cell binding. Dev. Comp. Immunol., *11:*167–178.

Hiserodt, J.C., L.J. Britvan, and S.R. Targan (1982) Characterization of the cytolytic reaction mechanism of the human natural killer (NK) lymphocyte: Resolution into binding, programming and killer cell independent steps. J. Immunol., *129:*1782–1787.

Kelly, K.L., E.L. Cooper, and D.A. Raftos (1992) In vitro allogeneic cytotoxicity in the solitary urochordate *Styela clava.* J. Exp. Zool., *262:*202–208.

Parrinello, N., V. Arrizza, M. Cammarata, and D.M. Parrinello. (1993) Cytotoxic activity of *Ciona intestinalis* (Tunicata) hemocytes: Properties of the in vitro reaction

against erythrocyte targets. Dev. Comp. Immunol., *17*:19–27.

Peddie, C.M., and V.J. Smith (1993) In vitro spontaneous cytotoxic activity against mammalian target cells by the hemocytes of the solitary ascidian, *Ciona intestinalis*. J. Exp. Zool., *267*:616–623.

Smith, V.J., and C.M. Peddie (1992) Cell cooperation during host defense in the solitary tunicate *Ciona intestinalis* (L) Biol. Bull., *183*:211–219.

Sokal, R.R., and F.J. Rohlf (1981) Biometry. W.H. Freeman New York.

Taylor, M.K., and J.J. Cohen (1992) Cell-mediated cytotoxicity. Curr. Opin. Immunol., *4*:338–343.

Blood Cell–Mediated Cytotoxic Activity in the Solitary Ascidian *Ciona intestinalis*

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Nonfusion reactions between allogeneic colonial ascidians and rejection responses to tissue grafts by solitary ascidians are associated with infiltration of the area by blood cells and subsequent *in vivo* cytotoxic activity.[1,2] *In vitro* cytotoxic activity by circulating blood cells has been investigated in mammals, lower vertebrates, and some invertebrates. However, there have been few detailed *in vitro* analyses of cytotoxic responses by ascidian hemocytes. This study examines *in vitro* blood cell–mediated cytotoxic activity in the solitary ascidian, *Ciona intestinalis,* towards mammalian tumor cells using a modification of the fluorochromasia cytotoxicity assay.[3]

MATERIALS AND METHOD

The cytotoxic blood cell populations were enriched by density gradient centrifugation of blood harvested from *C. intestinalis.*[4] Target cells were allowed to stabilize in 10 ml of low-salt marine saline (MS I) (740 mOsm) (12 mM $CaCl_2 \cdot 6H_2O$; 11 mM KCl; 26 mM $MgCl_2 \cdot 6H_2O$; 45 mM Tris; 38 mM HCl; 0.3 M NaCl; pH 7.4) for 30 min at 20°C before labeling with 5-carboxyfluorescein diacetate (CFDA) (Sigma).[3] The labeled targets were then washed and resuspended in MS (940 mOsm) (12 mM $CaCl_2 \cdot 6H_2O$; 11 mM KCl; 26 mM $MgCl_2 \cdot 6H_2O$; 45 mM Tris; 38 mM HCl; 0.4 M NaCl; pH 7.4). Cytotoxic activity was detected by incubating labeled target cells (2×10^6 ml^{-1} in MS) with effector suspensions (2×10^7 ml^{-1} in MS depending on the effector to target cell (E:T) ratio) in a 96-well microtiter plate for fluorometric use (Dynatech, Billinghurst, Sussex, England). For controls, heat-inactivated effector cells (15 min, 46°C in water bath) were substituted for untreated effector cells.[3] At the end of the incubation period, the microtiter plate was centrifuged (250 g, 5 min), and the pellets were resuspended in MS. The remaining fluorescence was measured using a microplate attachment to a luminescence spectrometer (Perkin Elmer LS50) in fluorescence mode (excitation wavelength, 490 nm; emission wavelength, 518 nm). The percentage specific release of CFDA (%SR) for each assay was calculated, as shown below, using the fluorescence data from the control (F_c) and experimental wells (F_e) with each of four well replicates.[3]

$$\%SR = (1 - F_c/F_e) \times 100$$

The assay was performed at increasing effector to target cell ratios and using a range of mammalian tumor cells previously used in assays for vertebrate natural killer cell activity.

RESULTS

This study demonstrates a population of nonspecific cytotoxic effector cells in the blood of *C. intestinalis* that kill mammalian target cells *in vitro.* Preliminary experiments showed that the assay provided optimal conditions for the functioning of the effector cells while maintaining low background leakage from the target cells. Hemocyte populations, enriched by density gradient centrifugation, exhibited cytotoxic activity that increased with the effector to target cell ratio (FIG. 1). Both human (K562) and mouse (P815, WEHI [3B] and L929) target cell lines were killed by the tunicate effector cells, indicating nonspecific activity (FIG. 2).

DISCUSSION

Preliminary results are presented that show that hemocytes, enriched by continuous density gradient centrifugation of the blood from the solitary tunicate, *Ciona intestinalis,* effect spontaneous, nonspecific cytotoxic activity against mammalian

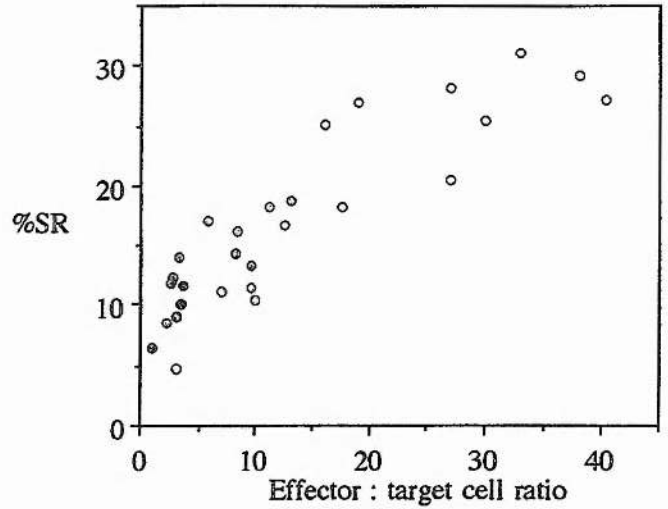

FIGURE 1. Percentage specific release (%SR) of carboxyfluorescein diacetate (CFDA) from the target cells (WEHI 3B) when incubated with enriched effector hemocytes from *Ciona intestinalis* at different effector to target cell ratios. Target cells were incubated with enriched effector cells from *Ciona intestinalis* for 40 min at 20°C. Each experiment was repeated four times.

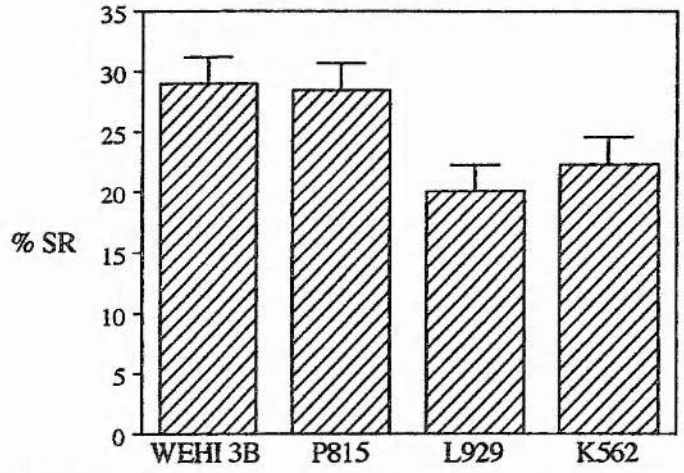

FIGURE 2. Percentage specific release (%SR) of CFDA from different mammalian target cell lines when incubated with enriched effector hemocytes from *Ciona intestinalis.* Target cells were incubated with enriched effector cells from *Ciona intestinalis* for 60 min at 20°C at an E:T ratio of 10:1. Each experiment was repeated twice; results are means ± SEM. WEHI, a mouse myelomonocytic leukemic cell (strain 3B); K562, a human erthyromyeloid leukemia cell; L929, an adherent murine fibroblast; P815, a methylcholantrene-induced mastocytoma cell from DBA/2 mouse.

tumor cells *in vitro.* This activity may represent a primordial form of the nonspecific spontaneous cytotoxic activity described in lower vertebrates.[5] The method developed here could be applied to investigate blood cell–mediated cytotoxicity in a range of marine invertebrates.

REFERENCES

1. Raftos, D. A., N. N. Tait & D. A. Briscoe. 1987. Allograft rejection and alloimmune memory in the solitary urochordate, *Styela plicata.* Dev. Comp. Immunol. **11:** 343–351.
2. Rinkevich, B. 1992. Aspects of the incompatibility nature in botryllid ascidians. Anim. Biol. **1:** 17–28.
3. Bruning, J. W., M. J. Kardol & R. Arentzen. 1980. Carboxyfluorescein fluorochromasia assays I. Non-radioactively labeled cell-mediated lympholysis. J. Immunol. Methods **33:** 33–44.
4. Smith, V. J. & C. M. Peddie. 1992. Cell cooperation during host defense in the solitary ascidian *Ciona intestinalis* (L). Biol. Bull. **183:** 211–219.
5. McKinney, E. C., L. Haynes & A. L. Droese. 1986. Macrophage-like effector of spontaneous cytotoxicity from the shark. Dev. Comp. Immunol. **10:** 497–508.