THE DEVELOPMENT OF *IN VITRO* TECHNIQUES TO FACILITATE THE STUDY OF HEMOPOIESIS IN THE RAINBOW TROUT (*Oncorhynchus mykiss*)

by

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ABSTRACT

As a first step in improving knowledge of factors controlling and influencing blood cell formation in fish, three general types of cell cultures have been developed as tools to study hemopoiesis in rainbow trout. The first of these is long-term hemopoietic cultures from the major hemopoietic organs, the spleen and head kidney. These cultures are initiated from spleen or kidney tissue and form a complex stromal layer of cells on the tissue culture surface. Like mammalian long-term bone-marrow cultures, these cultures produce numerous types of progeny cells for a period of several months, without the addition of exogenous growth factors other than serum. The products are difficult to characterize due to the lack of definitive markers for fish leukocytes, but the primary products of spleen cultures seem to be macrophages and dendritic cells, while the kidney produces a broader array of cells, including macrophages, granulocytes and occasionally lymphocytes. The dendritic cell progeny of spleen cultures are morphologically similar to dendritic cells found in mammals, and exhibit similar patterns of movement.

From these spleen cultures, two cell lines of different types have been developed. The first of these, RTS11, is representative of progeny cells, and is a non-adherent cell line consisting mainly of small, round cells with a small percentage of larger, granular, macrophage-like cells. The round cells appear to be an earlier stage of monocyte/macrophage development. They respond with increased growth to several crude extracts of rainbow trout origin, including PHA-LCM and cell-line conditioned medium, but not to cell-line conditioned medium of mammalian cells known to secrete cytokines influencing mammalian hemopoiesis. The second cell line, RTS34st, is representative of the stromal cell layer found in long-term hemopoietic cultures. It is made up of fibroblastic and epithelial cells, and is able to provide a hemopoietic inductive microenvironment (HIM) capable of supporting *in vitro* hemopoiesis. When suspensions of head kidney leukocytes or RTS11 cells are added, they adhere selectively to the fibroblastic stromal cell population, where they form proliferative foci, increase in number, and release non-adherent cells into the medium.

Both of the cell lines developed produce conditioned medium that stimulates ^{3}H-thymidine incorporation by freshly isolated rainbow trout leukocytes. Their response to fish extracts and production of factors stimulating trout leukocyte proliferation suggests that fish may respond to fish specific factors. These cell lines may thus be a potential source of novel fish cytokines or growth factors.

Finally, cultures in semi-solid media were initiated with isolated head kidney leukocytes. Of four semi-solid media tested, colony formation was best in 1.05% methylcellulose. Colony formation was greatly stimulated by rainbow trout serum, which promoted the growth of macrophage-like cell colonies, suggesting that trout serum may also contain growth factors stimulating hemopoiesis. The methylcellulose colony assay developed is potentially useful in scoring the types and numbers of cells formed in response to growth factors and cytokines, and will be useful in screening the activity of crude extracts to help identify important modulators of hemopoiesis in rainbow trout.

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"All progress must come from deep within and cannot be pressed or hurried by anything. *Everything* is gestation and then bringing forth." *Rainer Maria Rilke*

Completing this Ph.D. was, for me, like scaling a mountain while terrified of heights. When I came to the University of Waterloo as an undergraduate "mature student", I had to fight many fears. Being cursed with a touch of agoraphobia, simply sitting in a lecture hall filled with hundreds of undergraduate students was a challenge. For me, the important result of 9 years of university education is not the document that you are holding, but rather, progressing to the point of being able to stand in front of a similar group and lecture.

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DEDICATION

This work is dedicated, with much love, to my children, Mark, Eric and Katie. Always believe in the beauty of your dreams.

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ABBREVIATIONS USED THROUGHOUT THIS DOCUMENT

1.14	hybridoma secreting antibody to rainbow trout IgM
^{3}H- thymidine	tritiated 3-methyl thymidine
5637	human bladder carcinoma cell line
A23187	calcium ionophore
Ado	adenosine
APC	antigen presenting cell
ATCC	American Type Culture Collection
C1A	clone 1A, a rainbow trout peripheral blood cell line
CD	cluster of differentiation
CFC assay	*in vitro* colony forming cell assay
CFDA-AM	5-(6)-carboxyfluorescein diacetate acetoxy methyl ester
CM	conditioned medium
Con A	concanavalin A
CSF	colony stimulating factor
dbcAMP	dibutyryl cyclic AMP
DC	dendritic cell
DC-FHDA	2',7'-dichlorofluorescin diacetate
dFBS	dialyzed fetal bovine serum
DiI-Ac-LDL	1, 1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGC	eosinophilic granule cells
EM	extracellular matrix
FBS	fetal bovine serum
fig.	figure(s)
FITC	fluorescein isothiocyanate
GM	normal growth medium, as defined
GM-CSF	granulocyte macrophage colony stimulating factor
HIM	hemopoietic inductive microenvironment
HMC	Hoffman modulation contrast
HS	horse serum
IgM	Immunoglobulin M
IL-3	interleukin 3, or multi-CSF
Ino	inosine
LC	Langerhans cell
LPS	lipopolysaccharide
LTBMC	long term bone marrow culture
mag.	magnification
M-CSF	macrophage colony stimulating factor
mc	methylcellulose
MS222	tricaine methanesulfonate
NCC	natural cytotoxic cell
NK	natural killer cell
PBS	phosphate buffered saline with calcium and magnesium
PBS-	phosphate buffered saline without calcium and magnesium
PHA	*Phaseolus vulgaris* agglutinin, or phytohemmaglutinin

PHA LCM	Phytohemmaglutinin leukocyte conditioned medium
PMA (TPA)	phorbol -12-myristate 13-acetate
PNA	peanut agglutinin
PWM	pokeweed mitogen
RA	retinoic acid
RBC	red blood cell
RTS11	rainbow trout spleen myelomonocytic cell line
RTS34	rainbow trout stromal and macrophage cell line
RTS34st	rainbow trout isolated stromal cells
SEM	scanning electron microscopy
TEM	transmission electron microscopy
WEHI-3	mouse myelomonocytic cell line
WGA	wheat germ agglutinin

Chapter 1

General Introduction

1. HEMOPOIESIS

A: WHAT IS HEMOPOIESIS?

Hemopoiesis is the process of blood cell formation. A healthy human must produce approximately 3.7×10^{11} blood cells per day just to maintain steady state levels (Kuby, 1991). Remarkably, these huge numbers of differentiated hemopoietic cells are supplied in the correct proportions to meet the organism's requirements in health and disease throughout life from a small number of pluripotent stem cells. In mammals these originate in the bone marrow and occur with a frequency of approximately 1 in 10^4 marrow cells (Kuby, 1991).

The hemopoietic stem cell has two important properties: it is able to both renew itself and to produce committed progenitor cells that differentiate along many pathways to give rise to cells with very different morphologies and functions (summarized in fig. 1.1). Briefly, stem cells give rise to progenitor cells, which are morphologically indistinguishable but are committed to a particular developmental pathway, and incapable (in most cases) of self-renewal. Several intervening stages of precursor cells occur as the cells gradually acquire the characteristics that make them recognizable, fully functional differentiated products. Prior to the acquisition of morphologically recognizable characteristics, the progenitor cells are recognizable only by their expression of surface antigens, and the precise number of stages occurring is unclear. The final products of hemopoiesis include erythroid cells (red blood cells), lymphoid cells (T and B lymphocytes, NK cells) and myeloid cells (granulocytes, macrophages, platelets and dendritic cells).

In vivo studies in adult mammals have shown that developing hemopoietic cells are always found in close association with stromal cells. Stroma, from the Greek word, stornynai, meaning bed covering (Mish, 1993) is the supporting framework of the bone marrow. The hemopoietic stroma comprises numerous, varied cell types: endothelial cells, fibroblastic cells, epithelial cells, macrophages, and adipocytes may be included. In addition to providing support, stromal cells have been shown to produce a variety of

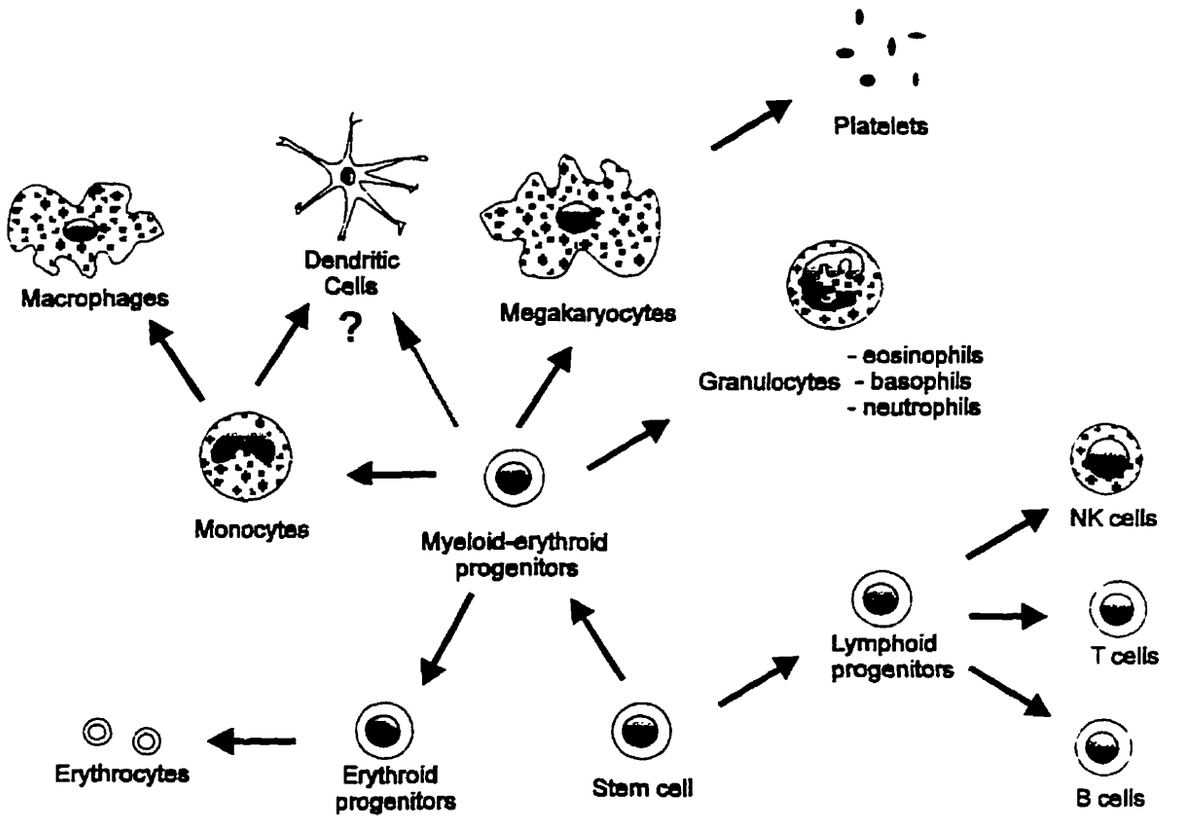

Figure 1.1. Schematic representation of the process of hemopoiesis in the mammal. Multipotent stem cells differentiate into committed progenitor cells, which in turn undergo several stages (not shown for the sake of simplicity) of lineage-restricted precursor cells and several multiplication divisions to become terminally differentiated functional blood cells. This process occurs in the bone marrow in close association with stromal cells. The dendritic cell origin is still under debate, although there is agreement that they either arise from or share a common precursor with the monocyte (Inaba et al, 1993; Peters et al, 1993).

cell-bound and secreted factors that locally regulate the process of blood cell production. Thus, the hemopoietic stroma is often referred to as the hemopoietic inductive microenvironment (HIM) (Curry et al, 1967). Developing blood cells are influenced by both microenvironmental and humoral factors (fig. 1.2).

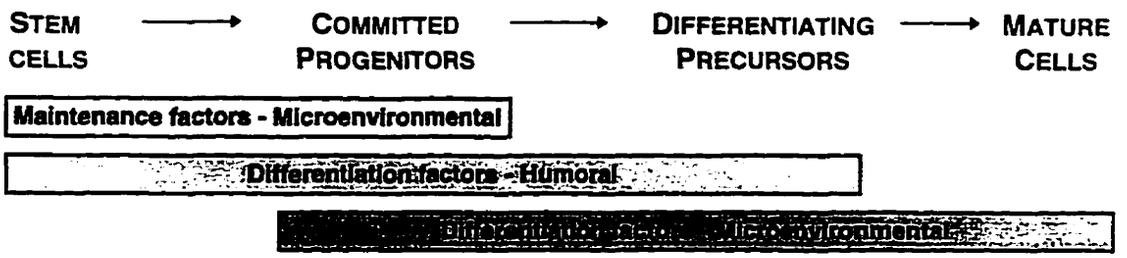

Figure 1.2 Developing blood cells are under the influence of both soluble (humoral) signals and interactions with stromal cells (microenvironmental factors). These factors act to maintain the stem cell population as well as to promote the development of mature cells. Each compartment of cells is subjected to a different array of signals, and the ranges of influence of each of these types of signals overlap as shown.

2. CULTURE METHODS FOR THE STUDY OF THE PROCESS OF HEMOPOIESIS

A: *IN VIVO* METHODS

Modern hematology originated with the experiments of Till and McCulloch (1961). Their demonstration *in vivo* of the reconstitution of an irradiated mouse's immune system by a few transplanted cells from another genetically identical mouse was the first demonstration of the existence of stem cells. The observation that the hemopoietic recovery of the irradiated mice could be anticipated by the appearance of nodules in the spleen led to the development of the spleen-colony forming assay.

The spleen-colony forming assay remains a valuable general purpose assay for multipotent progenitors in the whole animal, and the only assay that directly reflects the *in vivo* situation. However, the requirement for enormous numbers of experimental animals, and the difficulty of interpreting results at the level of an individual cell's response makes it desirable to use alternate approaches for many types of studies.

B: *IN VITRO* CULTURE SYSTEMS

A variety of *in vitro* systems are available to facilitate various aspects of the study of hemopoiesis in mammalian systems. These include colony forming cell assays, long-term bone marrow culture and a variety of leukocyte and stromal cell lines. Many of the landmark discoveries of immunology have been made using these *in vitro* techniques (Bibel, 1988).

Colony forming cell assays

The study of soluble factors affecting blood cell proliferation was greatly facilitated by the development of *in vitro* methods of measuring the colony-forming ability of bone marrow cells (Bradley & Metcalf, 1966: Ichikawa et al, 1966). The *in vitro* colony-forming cell (CFC) assay allows cells that can produce a clone of mature progeny in semisolid media to be detected and quantified. In these assays, a small number of cells are suspended in a semisolid media, such as agar, agarose, or methylcellulose. These cultures must be supplemented with serum or a serum substitute, and a source of growth factors such as conditioned medium from cell lines or tissue extracts. With the addition of the appropriate growth factor, precursor cells proliferate and, in approximately two weeks, form clones or colonies of progeny cells, with distinctive, recognizable morphologies. The identity and number of the colonies of progeny cells formed identifies the growth factor contained in the crude extract. CFC assays have led directly to the identification and isolation of a variety of glycoprotein growth factor molecules that can stimulate hemopoietic cell proliferation and differentiation and have also provided much information about normal and abnormal hemopoiesis.

There are, however, several limitations to CFC assays. Firstly, *in vivo*, cells develop at high density in association with a variety of other cell types. In these assays cells are removed from their natural environment and normal associations with other cells. Secondly, growth of the cells is dependent on high levels of exogenously added sources of growth factors. Finally, the majority of progenitor cells detected in this way do not display self-renewal, indicating that the assays are missing an important component(s). Although still an important and widely used technique, these limitations make the CFC assay system less than ideal for the study of normal, steady-state hemopoiesis.

Long term bone marrow culture

In the late 1970's, long-term bone-marrow culture (LTBMC) was developed from the mouse. Stem cells, as assayed by the *in vivo* spleen-colony forming assay, persisted for many weeks even though no growth factors were added to these cultures (Dexter et al, 1977). This system thus allowed the self-renewal of the stem-cell population and provided an important new experimental model of the interactions of hemopoietic

progenitor cells with the bone marrow stroma. To date, it remains the only *in vitro* technique that supports the maintenance of stem cells (Spooncer et al, 1993).

In long-term bone marrow culture, an adherent layer consisting of all cell types found in bone marrow covers the culture surface. Primitive precursor cells migrate underneath this adherent layer, where they proliferate and differentiate, in intimate contact with stromal cells. When examined with a phase contrast or dissecting microscope, these clones of hemopoietic cells situated beneath the stromal layer have a cobblestone appearance. As progeny cells mature, they migrate through the stromal layer, forming proliferative clusters on top, and finally become non-adherent and free-floating in the culture medium (Mauch et al, 1980; Toksoz et al, 1980).

Long-term bone-marrow culture requires the sacrifice of an animal for the initiation of each culture, and is subject to variability between individuals. Its other disadvantage is shared by clonal culture methods. LTBMC is highly dependent on high concentrations of serum, generally fetal bovine serum (FBS) for myeloid cultures, and horse serum for lymphoid cultures. FBS is a complex mixture of over 500 proteins, and varies in composition from batch to batch (Honn et al, 1975; Olmstead et al, 1967). Batches of serum used in either LTBMC or clonal culture assays have to be screened prior to use to ensure their ability to support hemopoiesis.

Leukocyte cell lines

Several types of cell lines derived from leukocytes have been developed. These include cell lines consisting of differentiated leukocytes that retain some of the functions of *in vivo* cells, such as the production and secretion of cytokines and growth factors. The WEHI-3 cell line is a good example of this type: these mouse cells are myelomonocytic and constitutively secrete large amounts of IL-3 (Lee et al, 1982). These cell lines are useful sources of growth factors, and also as sources of large numbers of pure, differentiated cells for use in experiments.

A second type is the growth factor dependent cell line, which requires an exogenous source of hemopoietic growth factors for survival and proliferation, but in general is not

capable of differentiation. These cell lines represent various stages of hemopoietic stem cell differentiation, and provide excellent experimental systems for the investigation of signal transduction mechanisms (Ihle & Askew, 1989).

Other cell lines of hemopoietic origin do not require exogenous sources of growth factors for survival and proliferation, and can be induced to develop into apparently normal, mature cells in response to a signal. Signaling molecules can include one or more of a variety of growth factors, PMA, retinoic acid, vitamin D, or even non-physiological stimuli such as the organic compound DMSO. Some of these cell lines are multipotent, and can be induced to develop into more than one mature cell type. An example of this type of cell line is HL-60, a human promyelocytic leukemia cell line that differentiates into granulocytes upon exposure to PMA, but becomes macrophage-like with exposure to DMSO (Collins et al, 1977). Cell lines of this type are particularly advantageous for the study of the molecular mechanisms that are activated by hemopoietic growth factors and lead to differentiation.

In general, leukocyte cell lines provide excellent model systems of leukocyte functions, responses and interactions with other cells. They provide a convenient source of large numbers of homogeneous cells with less variability than their freshly isolated counterparts, without the need to sacrifice an animal to obtain them.

Stromal cell lines

Stromal cell lines of mammalian origin have been invaluable for defining the interactions between hemopoietic cells and their environment (Deryugina & Müller-Sieburg, 1993). Several adherent cell lines from cultures of mammalian bone marrow (Itoh et al, 1989; Aizawa et al, 1994) and spleen (Piersma et al, 1984) can influence hemopoiesis *in vitro*. They have been used as replacements for the stromal layer formed by freshly isolated bone marrow in LTBMC; for example, the S17 stromal cell line can maintain pluripotent stem cells in culture to the same or a greater degree than primary stroma (Wineman et al, 1993), and have been shown to support hemopoiesis to the same level (Breems et al, 1994). This substantially reduces the use of experimental animals, and in the case of human cell culture, reduces the requirement for large and variable grafts (Sutherland et al, 1991).

Stromal cell lines have been used to analyze what factors are important for adhesion of hemopoietic cells to stromal cells, and to study maintenance and differentiation signals (fig. 1.2) that the microenvironment provides to developing cells. Stromal cell lines have also served as a source of many novel cytokines, which have been isolated and identified from cultured cells (Deryugina & Müller-Sieburg, 1993).

3. OTHER IMPORTANT TOOLS FOR THE EXAMINATION OF HEMOPOIESIS

A: MONOCLONAL ANTIBODIES TO CELL SURFACE ANTIGENS

The identification of leukocytes and their developmental stages in humans and rodents has been greatly advanced by the availability of hundreds of monoclonal antibodies to cell surface markers, the cluster of differentiation (CD) antigens. Different CD markers may be characteristic of different cell lineages, different developmental stages, or different states of activation (Roitt, 1993).

As stem cells and progenitor cells (see fig. 1.1) do not have a distinct, recognizable appearance, distinguishing between them requires other methods. The CFC assay has historically been used to identify these cells on the basis of the type of progeny colonies that the cells produce. This is tedious and time consuming, and as previously mentioned, the stem cells themselves differentiate but do not self-renew in the CFC assay, making this an impractical method for studying the very early developmental stages. Monoclonal antibodies are thus invaluable tools in the identification of progenitor cells that often have no other distinguishing characteristics, for distinguishing between morphologically similar cells, and for positive identification of cells at any stage of differentiation or function.

Table 1.1: The effect of cytokines on mammalian hemopoietic cells.

Target cells acted on in bone marrow	Cytokine										
	IL-3	GM-CSF	G-CSF	M-CSF	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	Epo
Pluripotent stem cell	+	+	-	-	-	-	-	-	-	-	-
Myeloid stem cell	+	+	-	-	-	-	+	-	-	-	-
Granulocyte-monocyte progenitor	+	+	+	+	-	-	-	-	-	-	-
Monocyte progenitor	+	+	-	+	-	-	-	-	-	-	-
Neutrophil progenitor	+	+	+	-	-	-	-	-	+	-	-
Eosinophil progenitor	+	+	-	-	-	+	-	-	-	-	-
Basophil progenitor	-	+	-	-	+	-	-	-	-	-	-
Mast cell	+	+	-	-	+	-	-	-	-	+	-
Megakaryocyte	+	+	-	-	-	-	-	-	-	-	+/-
Erythroid progenitor	+/-	+/-	-	-	-	-	-	-	-	-	+
B cell progenitor	-	-	-	-	+	-	-	+	-	-	-
T cell progenitor	-	-	-	-	-	-	-	+	-	-	-

(from Kuby, 1991) (+) acts on target cell to stimulate proliferation and differentiation, (-) no effect of the cytokine on the indicated cell. See fig. 1.2 for the position of the various cells in the overall process.

B: CYTOKINES

A cytokine is one of a large group of small proteins and glycoproteins involved in local signaling between cells during immune responses. All of the colony-stimulating factors (CSF's), cytokines which range from 15-70 kD in size, are involved in directing the division and differentiation of hemopoietic cells. They are effective at very low concentrations, in the 10^{-10} to 10^{-12} M range. Many of the CSF's with direct stimulatory effects upon mammalian hemopoietic cells (see table 1.1) have been expressed as recombinant molecules and pure forms are commercially available.

While IL-3 and GM-CSF have a similar spectrum of effects, IL-3 acts early in differentiation, perhaps even at the level of the stem cell, while GM-CSF acts at a slightly later stage. A range of other cytokines show no or very little effect on hemopoietic progenitor cells when used on their own, but have strong stimulatory

Table 1.2: Crude extracts with CSF activity.

Source	Cell origin/tissue source	Effective concentration (v/v)	Major activity
mouse lung conditioned medium[1]	lipopolysaccharide (LPS) injected mouse lung	10-15%	G-CSF, GM-CSF
L929-cell conditioned medium (LCM) [2]	Mouse fibroblasts	10-15%	M-CSF
5637 conditioned medium[3]	human bladder carcinoma	10%	G-CSF, GM-CSF, IL-1, IL-6
WEHI-3 conditioned medium[4]	mouse myelomonocytic leukemia	10-15%	IL-3
PWM-SCM[5]	pokeweed mitogen stimulated spleen cells	10-20%	GM-CSF
PHA-LCM[6]	phytohemmaglutinin stimulated peripheral blood leukocytes	10-20%	G-CSF, GM-CSF, IL-1, IL-6

[1] Burgess et al, 1977; [2] Stanley & Heard, 1977; [3] Myers et al, 1984; [4] Lee et al, 1982; [5]Metcalf & Johnson, 1978; [6] Metcalf, 1984

effects when used in combination with the CSF's. These include IL-11, and stem cell factor (SCF) also known as c-kit ligand or steel factor (Heyworth & Spooncer, 1993).

Other cytokines may have modulatory effects on the hemopoietic process, but these effects are not as significant.

Since the commercially available, purified growth factors are prohibitively expensive, crude sources of growth factors are still commonly used for routine assays. These include conditioned medium from a variety of cell lines, and extracts prepared from leukocytes and other tissues (table 1.2). Cytokines are usually interactive in their effects, and conditioned medium offers the second advantage of providing an array of cytokines. Determining the correct combination of recombinant cytokines to effectively support growth can be financially and practically difficult and is necessary only to answer very specific questions.

4. RAINBOW TROUT HEMOPOIESIS

A: THE FISH IMMUNE SYSTEM

Fish are of great interest to the comparative immunologist because they are the earliest vertebrates to clearly demonstrate both cellular immunity and humoral immunity with specificity and memory. In fishes, knowledge of even the specific location of production of the various blood cells is incomplete and tentative. There has been little effort to identify possible hemopoietic stem cells in fish, unlike the situation with amphibians, birds and mammals (Rowley et al, 1988), and little work concerned with developmental pathways and factors regulating hemopoiesis.

B: RAINBOW TROUT BLOOD CELLS, STRUCTURE AND FUNCTION

The composition of rainbow trout blood (table 1.3) is similar to that of mammals, with a few notable differences. First of all, rainbow trout red blood cells (RBCs) are oval, flattened cells containing a central nucleus. Secondly, rainbow trout blood does not contain platelets; the clotting function is instead performed by another variously shaped, nucleated cell, the thrombocyte. Finally, although some literature mentions eosinophils

Table 1.3: Cells found in the blood of rainbow trout.

Cell type	Cell size (μm)	Abundance	Description
red blood cells	7-10 x 13-16	98-99% of all blood cells	ellipsoid; homogeneous cytoplasm; elliptical nucleus ~ 3 x 4 μm with dense chromatin; variation in size partially due to presence of different developmental stages
lymphocytes	7-10 some 10-15	89-98% of leukocytes	spherical; round nucleus with compact chromatin, sometimes indented; thin rim of non-granulated cytoplasm
thrombocytes	5-8	1-6% of leukocytes	varied shapes (spheroid, elongated, spindle, fusiform); round to oval clefted nucleus
neutrophil (heterophil)*	9-13	1-9%	round; multi-lobed eccentric nucleus with 2-5 lobes; granular cytoplasm
monocyte	9-25	0-20% usually rare	large, ovoid; irregular, bean-shaped or oval nucleus, cytoplasm may have vacuoles or inclusions but generally non-granulated

Compiled from Yasutake & Wales (1983); Rowley, A. F. (1990); Rowley et al (1988); Blaxhall & Daisley (1973); Fänge, R. (1994); Tatner & Manning (1984).
*Heterophil is an alternate designation for the neutrophil

and basophils, it is generally accepted that there is a single type of granulocyte, the neutrophil, present in rainbow trout blood (Rowley et al, 1988). The blood of other species of fish may contain eosinophils and basophils, sometimes in large proportions.

Rainbow trout lymphoid cells include lymphocytes, plasma cells and natural cytotoxic cells (NCC), an analogue of mammalian NK cells. Fish are the first animals with clearly defined functional lymphocytes, and after a period of controversy, have been shown to have separate T and B cell populations (Clem et al, 1996).

In addition to the circulating blood cells, rainbow trout tissues contains macrophages and eosinophilic granule cells (ECG). Macrophages in rainbow trout are very similar to those of mammals, and originate from circulating monocytes. They are the predominant phagocytic cells in trout, capable of killing a wide range of pathogens (Secombes & Fletcher, 1992), and of processing and presenting antigen to lymphocytes (Vallejo et al, 1992). Rainbow trout macrophages undergo activation in response to stimuli, demonstrated by respiratory burst, RNA synthesis and increased enzymatic activity (Chung & Secombes, 1987). They also produce and respond to cytokines (Secombes, 1994; Secombes et al, 1996). Macrophages are particularly abundant in the spleen and pronephros. This suggests they may have a role in controlling hemopoiesis, as has been demonstrated in mammals (Crocker & Milon, 1992).

While circulating eosinophils are not found in trout blood, a fixed cell type with similar characteristics known as the eosinophilic granule cell (EGC) is present in some tissues. These cells are migratory and occur in the skin, gut, gill and around the central nervous system of rainbow trout (Powell et al, 1993). These cells seem to be similar in function to mammalian mast cells, and are involved in immune responses to parasitic and other pathogenic infections.

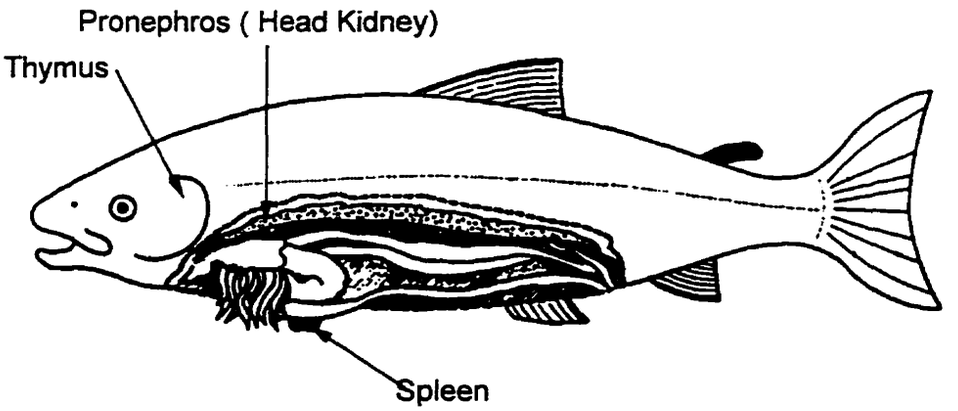

Figure 1.3: Location of the major hemopoietic organs in the rainbow trout, a typical teleost fish. The kidney (stippled) is located along the entire backbone of the fish, and hemopoietic cells are concentrated in the pronephros, but occur throughout the kidney. The thymus is active in blood cell production only in young fish, as it involutes at the time of sexual maturity.

C: RAINBOW TROUT HEMOPOIETIC ORGANS

In fish, the process of hemopoiesis does not occur in the bone marrow. Cooper et al, (1980) speculated that the movement of the process of hemopoiesis to the bone marrow occurred when animals moved out of a water environment, and on to the land. The move of hemopoiesis to the bone marrow may have provided the developing blood cells with the protection of the relatively radiation resistant bone. Since the water column provides protection against radiation, hemopoiesis in fish occurs in other organs. In teleosts, such as the rainbow trout, these include the anterior kidney (pronephros), thymus and the spleen, as shown in fig. 1.3. The thymus and pronephros will be briefly mentioned, while the spleen, which is the focus of this study, will be discussed in more detail.

Thymus

The thymus begins as an active hemopoietic organ, which at five months of age is densely packed with thymocytes, some of which emigrate to the peripheral lymphoid organs. The thymus, however, atrophies at sexual maturity, with an increase in the amount of connective tissue and decrease in the number of leukocytes, leaving the pronephros and spleen as the primary sources of blood cells in the adult animal (Tatner & Manning, 1982).

Pronephros

The pronephros, or anterior or head kidney, of the rainbow trout is not a discreet organ, but is contiguous with the mid- and tail kidney. The kidney is considered the primary hemopoietic organ, most similar to mammalian bone marrow, and produces high numbers of lymphocytes, red blood cells and other leukocytes throughout the life of the fish (Rowley, 1988).

Spleen

Location

In fishes, as in other non-mammalian vertebrates, an elongated spleen extending the full length of the gut is considered the ancestral form (Tischendorf, 1985). In the rainbow trout the spleen is dorso-ventrally compressed relative to this ancestral form and is located associated with the gut, close to the stomach (fig. 1.3).

Structure

The fish spleen consists of the same basic elements but is relatively simple in structure in comparison to that of the mammal. The organ is surrounded by a thin connective tissue capsule (Yasutake & Wales, 1983). It lacks the prominent organization into red and white pulp that is seen in the mammalian spleen. Fish also lack germinal centres, which are aggregates of reticular cells, lymphocytes, macrophages and plasma cells, which are important in antigen trapping and presentation in mammals.

Some species of fish have large accumulations of cells forming melanomacrophage centres, which comprise the same cells as germinal centers (Kennedy-Stoskopf, 1993), and are sometimes considered their primitive analogue. The macrophages contained in these centres contain pigments, including melanin, hemosiderin, and lipofuscin (Agius, 1985), which increase with age and give them a black coloration. In the trout, these are diffusely located and do not occur in discreet melanomacrophage centres as seen in many other teleosts (Roberts, 1975).

Circulation

Fish blood flows through the body in a low pressure, single circulatory vascular system. Arteries and veins run adjacent to one another, and arterioles and capillaries with thick walls and narrow lumens (ellipsoids) branch off into the parenchyma (Fänge & Nilsson, 1985). Here the blood percolates through a loose network of reticular fibers and cells (Ellis, 1976). This structure makes the concentration of cell into germinal centres unnecessary, as antigen trapping and the development of immunological memory take place throughout the spleen (Manning, 1991).

Hemopoiesis

The spleen in the adult rainbow trout (at about twelve months of age) contains roughly equal numbers of erythrocytes and lymphocytes (Tatner & Manning, 1984). As the fish ages there is a tendency for the proportion of leukocytes to increase. Of the leukocytes found in the spleen, 87% or more are lymphocytes, up to 12% are macrophages, and thrombocytes and other leukocytes are scarce or absent (Tatner & Manning, 1984).

The relative importance of the spleen's contribution to blood formation varies among species - in many, splenectomony does not adversely effect circulating blood cell levels or antibody production (Ferren, 1967), while in others it has a drastic effect (Yu et al, 1970). The overall cell composition of the rainbow trout spleen is the same as seen in the kidney (Peters & Schwartzer, 1985). While the function of the spleen in the rainbow trout has not been examined by splenectomony, most studies consider the spleen an accessory hemopoietic organ.

Other functions

Phagocytosis and destruction of aged red blood cells is an important function, as it is in mammals. Filtration and retention of antigen is also an important function (Ellis, 1980; Mass & Bootsma, 1982).

5. CULTURE SYSTEMS USED WITH FISH

Colony forming cell assays

While clonal culture systems have been invaluable in elucidating the roles of cytokines and growth factors active with mammalian leukocytes, they have only occasionally been used with fish. Soft agar culture was used to grow rainbow trout leukocytes, and lymphocytic and other unidentified colonies formed (Finegan & Mulcahy, 1987). Caspi et al (1980, 1982) grew lymphocyte colonies from carp using soft agar as the semi-solid medium. Low melting temperature agarose was used by Moritomo et al, 1993 to demonstrate the proliferative response of carp granulocytes to carp serum. Fibrin clot cultures have been successfully applied by a single group (Estepa & Coll,1992; 1993) to examine the colonies formed by hemopoietic cells from the rainbow trout pronephros in response to stimulation with lipopolysaccharide (LPS), and the lectins Concanavalin A (Con A) and phytohemagglutinin (PHA). While methylcellulose is perhaps the most popular choice for clonal culture of mammalian hemopoietic cells, there has been only one report of its use with fish cells (Kodama et al, 1994). Using pronephric cells suspended in methylcellulose, they showed that rainbow trout serum was a source of macrophage colony-stimulating activity.

These results show that clonal culture of fish leukocytes is an effective method for assaying what factors control the proliferation and differentiation of fish hemopoietic cells. The successful application of colony assays to the identification and isolation of growth factors from mammals suggests that it may be a profitable approach to these same issues in the rainbow trout.

Long term cultures

During the course of this work, another group reported the development of long-term cultures from the rainbow trout pronephros (Diago et al, 1993).

Leukocyte cell lines

Other than those from the channel catfish (Clem et al, 1996), few leukocyte cell lines have been reported. Channel catfish leukocyte cell lines include monocyte-like (Vallejo et al, 1991), T-cell (Lin et al, 1992) and B-cell (Miller et al, 1994) lines. Channel catfish seem to be uniquely amenable to the development of leukocyte cell lines, because few have been reported from other fish species.

Macrophage cell lines from carp (Weyts et al, 1997) and goldfish (Wang et al, 1995) have recently been reported. No other fish leukocyte cell lines are currently available.

Stromal cell lines

While a few cell lines have been developed from fish spleen or kidney, none of these has been characterized as a stromal cell line capable of supporting hemopoiesis or of producing cytokines or soluble regulatory factors.

6. OTHER TOOLS AVAILABLE FOR THE STUDY OF HEMOPOIESIS IN FISH

A: MONOCLONAL ANTIBODIES TO FISH CELL SURFACE ANTIGENS

The study of fish hemopoiesis and immunology is seriously hampered by the paucity of monoclonal antibodies to cell surface markers (Coll & Dominguez, 1995). The few monoclonal antibodies available that bind with rainbow trout leukocyte cell surface antigens are summarized in table 1.4. Most of these antibodies are specific for IgM and are therefore most useful for the identification of B cells. The lack of antibodies to other unique leukocyte markers could be due to their low antigenicity relative to the more common leukocyte surface antigens (Schots et al, 1992).

B: FISH CYTOKINES

The presence of cytokines in fish has been investigated using several approaches, and it is becoming clear that cytokines play an important role in modulating the immune system of fish, as they do in mammals. The first sequence data from fish cytokine genes, TGF-β and IL-1β of the rainbow trout, has just become available (Secombes

Table 1.4: Monoclonal antibodies to rainbow trout leukocyte surface antigens

Antibody	Specificity	Reference	
1.14	B cells (IgM)	DeLuca *et al*, 1983	✓
2H9, 2A1	L chain IgM	Sanchez and Dominguez, 1991,	✓
3B10, 4D11	H chain IgM	Sanchez *et al*, 1993; Sanchez *et al*, 1993b	✓
several	H chain IgM	Thuvander *et al*, 1990	
2IG6	leukocytes other than thrombocytes	Lloyd-Evans et al, 1994	✓
2PBL4X	carbohydrate moiety on most leukocytes	Greenlee and Ristow, 1993	
hyb106-9	granulocytes & thrombocytes	Slierendrecht et al, 1995	

✓ indicates that monoclonal antibody was obtained from the indicated author for use in this study.

et al, 1997). and no purified fish reagents or antibodies to any fish cytokine are yet available.

Macrophage-colony stimulating activities have been demonstrated in rainbow trout (Kodama et al, 1994) and carp sera (Moritomo et al, 1994) and in carp macrophage supernatants (Yoshikawa et al, 1994). These are the only reports of cytokines that modulate hemopoiesis in fish.

7. AIMS OF THIS WORK

The study of hemopoiesis in the rainbow trout is hampered by the lack of appropriate tools. In addition to a serious lack of monoclonal antibodies specific to leukocyte surface antigens, methods commonly used to study mammalian hemopoiesis have seen only limited application in rainbow trout.

The aim of this study was to adapt some of the main methods used to elucidate mammalian hemopoiesis to use with fish. The first of these methods was long-term-cultures with the hemopoietic organs of the adult rainbow trout, the spleen and pronephros (particularly the spleen) similar to long-term bone marrow cultures. In addition, the establishment of cell lines of leukocytes and of stromal cells from rainbow trout spleen were undertaken. Finally, attempts were made to apply culture in semi-solid medium to the clonal growth of rainbow trout hemopoietic cells.

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Chapter 2

Development of long-term rainbow trout spleen cultures that are hemopoietic and produce dendritic cells

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ABSTRACT

Different tissue dissociation protocols and serum supplements resulted in a variety of primary cultures from rainbow trout spleens. These were classified as either hemopoietic or nonhemopoietic. In hemopoietic cultures, a complex stromal layer developed on the cell culture surface; round cells appeared on the stromal layer; and ultimately some round cells were released into the medium as a non-adherent population. Many non-adherent cells were able to reattach in new culture vessels. Most of these had the characteristic shape and unique motility of dendritic cells. Cultures of these cells were subcultivated 2-4 times but continuous cell lines did not arise. Hemopoietic cultures resulted with collagenase dissociation and maintenance in high fetal bovine serum (FBS) concentrations. Nonhemopoietic cultures contained adherent cells, including macrophages and fibroblasts, but did not develop a non-adherent population. These cultures resulted with 10 % FBS or horse serum and after explant outgrowth or dissociation with trypsin. Both culture types should be useful for studying the regulation of hemopoiesis and the functions of specific immune cells in fish.

1. INTRODUCTION

Primary cell cultures of hemopoietic organs have been valuable research tools for studying hemopoiesis in mammals. One of the most common types is long-term bone marrow cultures (LTBMC) or liquid cultures (Dexter & Testa, 1976; Dexter et al, 1977 Allen et al, 1990), which can be maintained for up to 52 weeks or longer (Spooncer et al, 1993). These cultures consist of an attached stromal cell layer and produce a non-adherent cell population of hemopoietic products. The stromal layer supplies the microenvironment that supports both the hemopoietic stem cell self-renewal and differentiation and the proliferation and development of the mature cells. The most common differentiated product varies with the species. For murine LTBMC, these are granulocytes, macrophages and megakaryocytes (Quesenberry 1989).

In teleosts, hemopoiesis does not take place in the bone marrow, but instead occurs in the anterior kidney and spleen (Zapata, 1979; Fangë, 1992). Long-term cultures in liquid medium have been developed recently from the renal hemopoietic tissue of the rainbow trout, *Oncorhynchus mykiss* (Diago et al, 1993). A stromal layer develops that is similar to the stromal layer of LTBMC. In this system, myelopoiesis is defined as the appearance on the stromal layer of foci of round cells, some of which are subsequently released into the medium, (Diago et al, 1993). Unlike the situation for human or mouse LTBMC, the cells produced by this culture system are difficult to identify because of the scarcity of monoclonal antibodies to fish leukocyte surface markers (Coll & Dominguez, 1995).

In this paper, we describe the development of long-term hemopoietic cultures from the rainbow trout spleen, which is thought to be an accessory hemopoietic organ as in other teleosts (Ferren,1967; Chiller et al,1969), and identify one of the culture products as dendritic cells, which have not been described previously in fish. Mammalian dendritic cells act as accessory cells for several immune responses (Steinman et al., 1986). Therefore, the rainbow trout spleen cell cultures should be useful for studying the role of dendritic cells in the immune responses of fish as well as for studying the production of fish immune cells.

2. MATERIALS AND METHODS

Fish

Spleens were obtained from rainbow trout (*Oncorhynchus mykiss*) weighing between 100- 250 grams. Fish were anaesthetized in an aqueous solution of 1:10,000 tricaine methanesulfonate, MS222 (Syndel, Vancouver, BC). After blood was removed by caudal puncture to reduce the blood volume in the spleen, fish were killed by a blow to the head. Spleens were aseptically removed through a ventral incision and adherent tissue was dissected away. On 61 separate occasions, cell cultures were prepared from spleens by the methods outlined as follows.

Cell suspensions

Cell suspensions were prepared in sterile phosphate buffered saline that lacked calcium and magnesium (PBS^-) and contained the antibiotics Gentamicin, 50 µg/ml and Fungizone, 100 µg/ml (Life Science Technologies, Burlington, ON). A single spleen was placed in a 10 cm petri dish with 3 to 5 ml of PBS^-. The spleen was cut into small pieces (approximately 1 mm^3) with a sterile scalpel and scissors. In a few cases these pieces were cultured without further treatment (explant outgrowth). In most cases the spleen fragments were dissociated further as outlined below. All dissociation protocols gave suspensions of single cells, cell clumps and small fragments of undissociated tissue, although the relative proportions of these varied.

For collagenase treatment, the spleen fragments were resuspended in an equal volume of phosphate buffered saline with calcium and magnesium (PBS) at a final concentration of 500 µg/ml of either collagenase A, H (Boehringer Mannheim, Dorval, Que) or type I (Sigma, St. Louis, MO). The incubation was carried out at either 5 °C or room temperature, and the length of incubation varied from 30 min to 24 h. After longer incubations, mixtures of tissue and enzyme were easily pipetted up and down, further dissociating the spleen fragments. In all cases, growth medium with histidine and cysteine, which stopped the action of collagenase, was added and the suspension was centrifuged at 1,000 rpm for 5 min in a table top centrifuge (IEC HN-SII, International Equipment Co., Needham Heights, MA). The pellet of cells was resuspended in growth medium, which varied as outlined in the next section.

For trypsin treatment, the spleen fragments were resuspended in an equal volume of PBS^- with a final concentration of 0.1 % (w/v) bovine trypsin (Sigma) and incubated at room temperature for 30 to 120 min or at 5 °C for 12 to 18 h. If possible, the fragments were further dissociated at the end of the incubation by repeatedly pipetting the solution up and down. The addition of medium with serum stopped the action of trypsin. Cells were collected by centrifugation and resuspended in different growth media.

For mechanical disruption, a cell dissociation sieve-tissue grinder kit (Sigma) was used. The spleen fragments were placed into the cup and forced with a glass pestle through a 40 mesh screen into PBS-. The cells were collected by centrifugation and resuspended in growth medium.

Cell plating conditions

The cell suspensions in different growth media were added to 12.5 or 25 cm^2 flasks (Falcon, Oxnard, CA). The basal medium was Leibovitz's L-15 with 2 mM glutamine, Gentamicin (50 µg/ml) and Fungizone (100 µg/ml) (Canadian Life Technologies, Burlington, ON), supplemented with 10, 20, or 30 % (v/v) of either fetal bovine serum (FBS) (catalog number 200-6140) or horse serum (HS) (catalog number 16050) from Canadian Life Technologies (Burlington, ON). Flasks were incubated at 22 °C. After two weeks, the medium was completely removed, the surface of the flask was rinsed with medium, and fresh medium that had penicillin (100 I.U. /ml) and streptomycin (100 µg/ml) as the antibiotics rather than Gentamicin and Fungizone was added. At this point the only cells that were present were those attached to the surface of the culture flask.

Maintenance of primary cultures

Culture flasks were maintained at 22 °C and medium was replenished in one of two ways. In some cultures, non-adherent cells appeared with time and were released into the medium. For these cultures, half the medium was removed weekly and replaced with the appropriate fresh medium. For the other cultures, the total volume of medium was replaced monthly with fresh medium.

Subcultivation and cryopreservation

Both non-adherent and attached cells of primary cultures were transferred to new flasks. Non-adherent cells were collected by centrifugation, resuspended in fresh medium, and added to new culture flasks. For cells attached to the growth surface, passaging was done with trypsin. At the first and all subsequent passages the contents of one flask were passaged to two new flasks. Cells were cryopreserved in dimethyl sulfoxide (DMSO) as described previously for cell lines (Bols & Lee, 1994). For the attached cells, this was done after approximately 5 passages, and for the non-adherent cells, this was done on cells taken directly from the primary cultures.

Growth of non-adherent cells

The ability of the non-adherent cells to grow was monitored in two ways. When the medium was changed weekly on primary cultures, aliquots of the removed medium were counted with a Coulter Counter (Coulter Electronics, Hialeah, FL). Alternatively, non-adherent cells were collected from primary cultures by centrifugation, resuspended in fresh medium, and plated in 12 well Costar plates, where they attached to the culture surface. Their response to different concentrations of fetal bovine serum was determined using a ^{3}H-thymidine incorporation assay, as previously described (Lee et al, 1988).

Morphology and cytochemistry

A Nikon Diaphot inverted microscope (Nikon Canada, Toronto, ON) with phase optics and a Labovert microscope with Hoffman modulation contrast were used to observe and photograph living cultures in flasks. For general morphology and enzyme cytochemistry, non-adherent cells were deposited onto microscope slides using a cytocentrifuge (Johns Scientific Inc., Toronto, ON). Wrights and Giemsa stain (Sigma) in distilled water, 1:3:50 parts, was used to demonstrate general morphology, and non-specific esterase was demonstrated using alpha naphthyl acetate as a substrate (Sigma procedure 91).

Cell movement

Cell movement was studied in two ways. Cultures were observed with the Nikon inverted phase contrast microscope and photographed every 30 sec for up to 7 minutes. In addition, a Sony CCD camera that was attached to either the Nikon inverted phase contrast microscope or the Labovert microscope with Hoffman modulation contrast was used to capture video images of living cultures at 30 sec intervals for up to 10 min. Northern Exposure Image Analysis Software (ImagExperts Inc., Mississauga, ON) was used to construct a time lapse video of these images.

Phagocytic assay

The particles for phagocytosis were carboxylated fluorescent latex beads that were 1.0 µm in diameter (Polysciences Inc., Warrington, PA) and observations were made with a Nikon Diaphot inverted fluorescent microscope with phase optics. A suspension of beads was prepared by adding 2 µl of the commercial bead preparation (2.5 % solids latex) to 5 ml of growth medium. This suspension replaced the regular growth medium in cultures to be studied. At various times for up to 24 h after the addition of the beads, cultures were observed before and after the cultures had been rinsed 4 times with PBS to remove non-ingested beads. Each microscope field was examined by both phase optics, which allowed the cells to be identified, and by fluorescence (V filter module), which revealed the beads.

Scanning electron microscopy

Cultures were fixed for 10 minutes in modified Karnovsky's fixative, pH 6. 8, then rinsed gently 3 times with 0.1 M sodium cacodylate, then immersed in -20^{o}C 70% ethanol. A heated scalpel was used to cut out small pieces of the tissue culture flask, which were then dehydrated in ascending ethanol concentrations to absolute ethanol, critical point dried in a Denton Vacuum Inc. DCP-1 Critical Point Drying Apparatus and mounted on aluminum stubs. Cells were coated with 150 angstroms of gold in a gold sputter apparatus (Polaron Equipment Ltd. Unit PS3) and examined with a Hitachi S-570 scanning electron microscope at 15 kV.

3. RESULTS

HEMOPOIETIC CULTURES

Only collagenase dissociation and a medium supplement of 20-30 % FBS led to hemopoietic cultures with a complex stroma that released cells into the medium. For the successful development of these cultures, collagenase A was better than collagenase H or type I and an incomplete dissociation was better than complete dissociation into single cells. Incubation with collagenase A for 18 h at 5 °C gave good results. This yielded suspensions with many single cells and clumps of cells, along with large numbers of red blood cells. Two weeks after the suspension was plated in L-15 with 20 to 30 % FBS, the medium was changed completely for the first time. Subsequently, two cell populations were distinguished in these cultures. These were an adherent layer of cells, which became established first, and a non-adherent cell population, which developed later and was released into the medium as the culture matured.

Adherent cells

The adherent cells became arranged on the growth surface either as single cells or as islands of cells, which were multi-layered and heterogeneous and are referred to as the stromal layer. By four weeks after plating, the stromal layer had developed and covered approximately 50 % of the growth surface. Despite prolonged maintenance, the stromal layer never grew to confluency and never covered more than 75 % of the surface. The remainder of the surface contained single cells and these were of two different types (fig. 2.1).

One cell type is referred to as dendritic cells. These cells only became apparent 14 to 16 days after the initiation of a culture. The cells had a distinct irregular shape, with many thin cellular processes, such as spiny dendrites and bulbous pseudopods (fig. 2.1A to 2.1F and 2.2). The cells never completely flattened onto the culture surface. At the ends of some of the cellular processes, flaps of cytoplasm extended out over the surface or up into the medium. These are veils or lamellipodia (fig. 2.1E). The time-lapse photography (fig. 2.2) and video revealed that the processes were dramatically and rapidly motile. Over a 7-10 minute observation period at 15 or 30 second intervals,

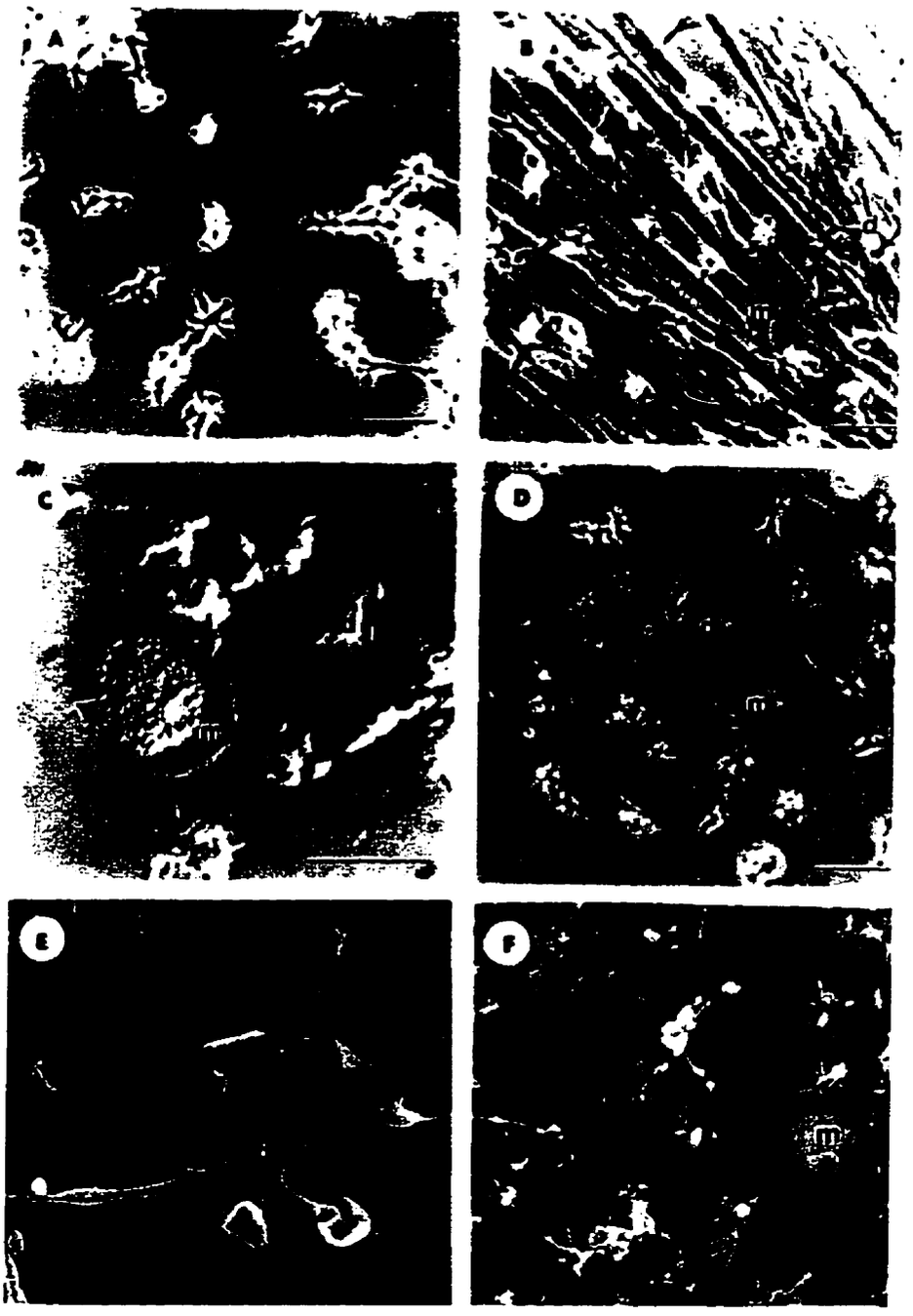

Figure 2.1: Adherent cells in hemopoietic spleen cell cultures. Dendritic cells (d) and macrophages (m) were found on the growth surface (A) and on top of cells in the stromal layer (B). By phase contrast (A), Hoffman modulation contrast (C), and scanning electron microscopy (E and F), dendritic cells had a distinct morphology, with numerous processes, often ending in veils (double arrowheads) (E and F). By phase contrast (D), HMC (C), and SEM (F), macrophages on the growth surface were circumferentially spread and had a characteristic "fried egg" appearance, with a rim of clear cytoplasm. Single arrowheads indicate the large number of vacuoles (single arrowheads) clustered around the nucleus (C, D). Macrophages on top of the stromal layer often were polygonal in shape and appeared darker under phase contrast (B) than those attached directly to the growth surface (D). Size bars represent 25 μM.

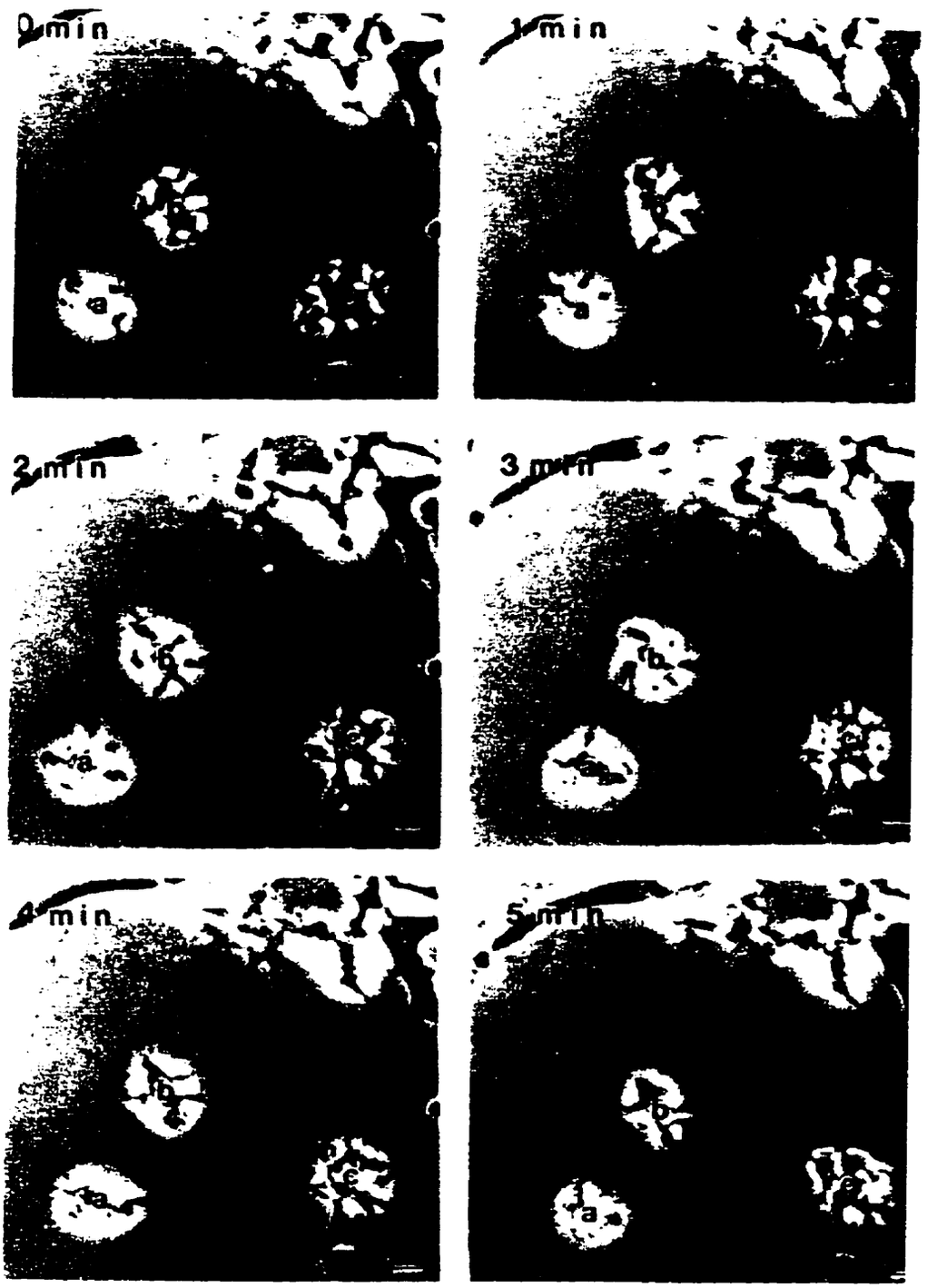

Figure 2.2: Time-lapse photography of dendritic cells. When photographed at 1 minute intervals, dendritic cells (a, b and c) rapidly extended, retracted, and reoriented cell processes, constantly changing their overall shape. Movement of surrounding adherent cells was not evident in this time frame. Phase contrast, size bars represent 25 μM.

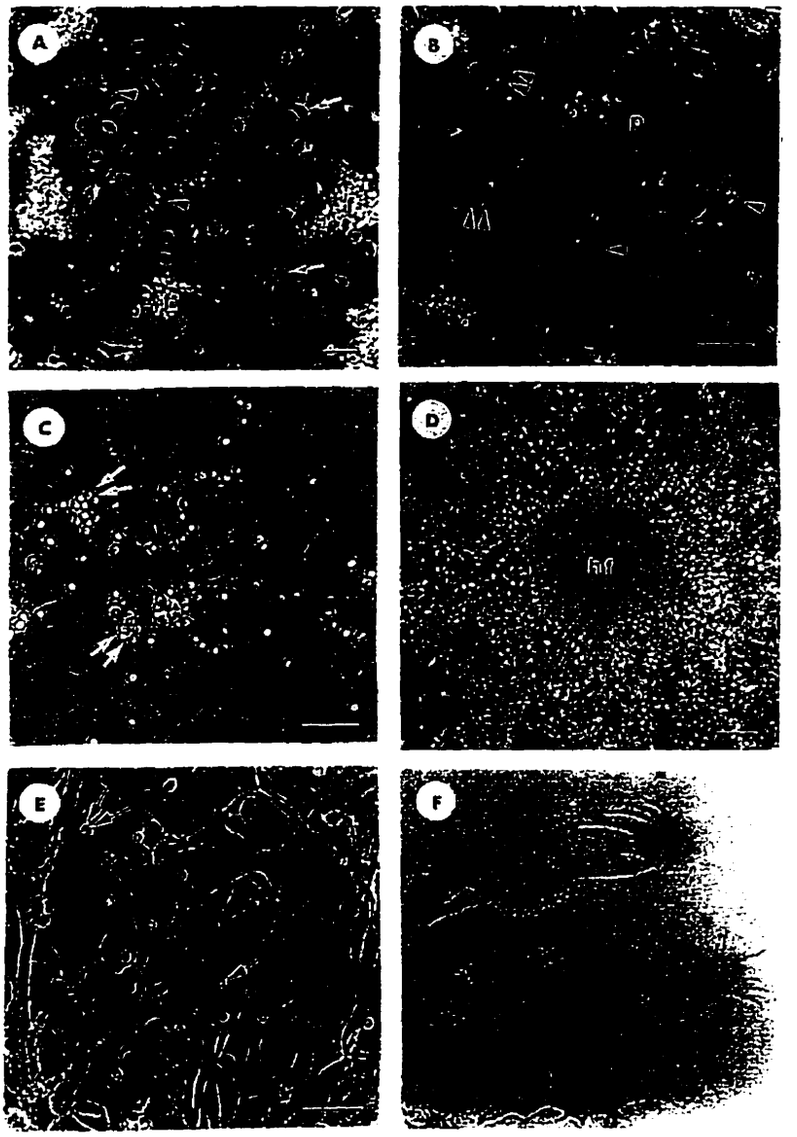

Figure 2.3: Development of hemopoietic spleen cell cultures over time. Three days after culture initiation (A), the culture surface had attached macrophages (single arrowhead) and the medium contained loosely associated spleen fragments (f) and red blood cells (white arrows). Two weeks after culture initiation (B), the medium was changed completely, removing all non-adherent cells. The remaining attached cells included macrophages (single arrowheads), dendritic cells (double arrowheads), and large polygonal cells (p). Two to 24 weeks after the first media change (C), a complex stromal layer had formed, and hemopoietic foci (double white arrows) appeared. Macrophages and dendritic cells were still evident on this stromal layer. In some cultures (D), hemopoietic foci were large and formed mountains of cells (hf). In some cultures (E), the decline of hemopoiesis was signaled by the appearance of epithelial "crowder" cells, which appeared to push aside the productive stromal layer. In this figure, the non-adherent cells, both round (single black arrow) and irregular (double black arrows), have been produced from an area of the flask that has not been overtaken by these "crowder" cells. Cytocentrifuge preparation of non-adherent cells from spleen cultures (F) stained with Wright/Giemsa revealed cells with irregular morphology and eccentric nuclei. A-E: Phase contrast, size bars indicate 25 μM. F: Mag. 400x.

growth surface first and maintained themselves on the surface as single cells rather they were constantly extending, contracting and reorienting, but at the same time the main body of a cell did not change its position. Hoffman modulation contrast (HMC) microscopy and SEM showed the surface of the cells to be smooth (fig. 2.1C and E). The cells were poorly phagocytic, having phagocytized few or no latex beads 16 h after their introduction. The results of tests for non-specific esterase activity were equivocal.

The second cell type is referred to as macrophages. These cells attached to the than assembling into either clumps or monolayers. Their usual shape was circular. By phase contrast microscopy, they had ruffled edges, vacuolated cytoplasms and commonly were very flat (fig. 2.1D). Under Hoffman modulation contrast, which highlights internal structures, the vacuoles were centrally distributed, which was characteristic only of these cells (fig. 2.1C). When these cells were observed under phase contrast at hourly intervals for seven hours, some flat cells were observed to round up and some round cells became flattened. The cell surface was smooth in the very flat circular cells or folded into ridges on the round cells, as judged by SEM (fig. 2.1F, 2.4B). These cells were very phagocytic. In early primary cultures they appeared to be engorged with debris, including red blood cells, and within 2 hours of the introduction of latex beads to old cultures, these cells had phagocytized many beads, whereas other cell types had phagocytized few or none. Finally, these cells stained positive for non-specific esterase.

The stroma contained different cell types and was multi-layered. Initially, macrophages, bipolar cells and large polygonal cells appeared on the growth surface (fig. 2.3 A & B). These cells proliferated to form multi-layered structures in which individual cells were often difficult to discern, but generally were polygonal. Macrophages appeared to be underneath and on top of the stromal layer. When the stromal layer was disturbed with a cell scraper, the top layers often detached and rolled up as a sheet to reveal an underlying layer of macrophages and giant cells. These macrophages had the same appearance as the non-stromal macrophages, but the macrophages on top of the stromal layer appeared darker by phase contrast and usually were polygonal rather than circular. Cells with dendritic-like morphology were also found on top of the stromal layer .

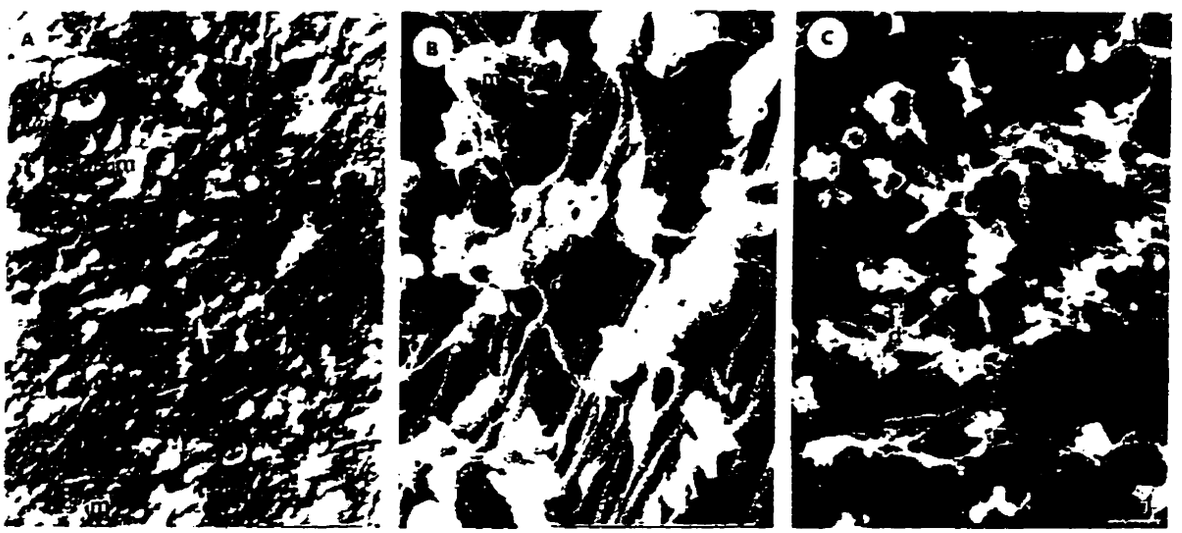

Figure 2.4: Associations between potentially non-adherent cells and the stromal layer. With HMC (A), round cells of a uniform size (white arrows) were occasionally observed clustered around macrophages (m). By SEM (B and C), loosely adherent cells (white arrows) were often found associated with dendritic cells. Size bars represent 25 μM.

From 2 to 24 weeks after the first change of medium, round phase bright cells appeared loosely attached on top of the stroma. Usually these were present in small clusters (fig. 2.3C), some of which developed into huge masses or "mountains" of cells (fig. 2.3D). These are referred to as hemopoietic foci. In Hoffman micrographs, cells of a similar size and shape were seen associated with macrophages (fig. 2.4A). A focus of round, phase bright cells persisted for weeks before declining in size and disappearing. While some foci were disappearing, new ones appeared on other regions of the stroma. Eventually, cultures lost all hemopoietic foci. In many cultures, this was signaled by the appearance of polygonal or epithelial-like cells. The hemopoietic foci disappeared as these cells grew to form a complete monolayer over the culture surface (fig. 2.3E).

Non-adherent cells

Two types of non-adherent cells became apparent 1 or 2 weeks after the appearance of hemopoietic foci. One type was small, round and phase bright, whereas the second was slightly larger, had a very irregular shape, and a darker phase contrast

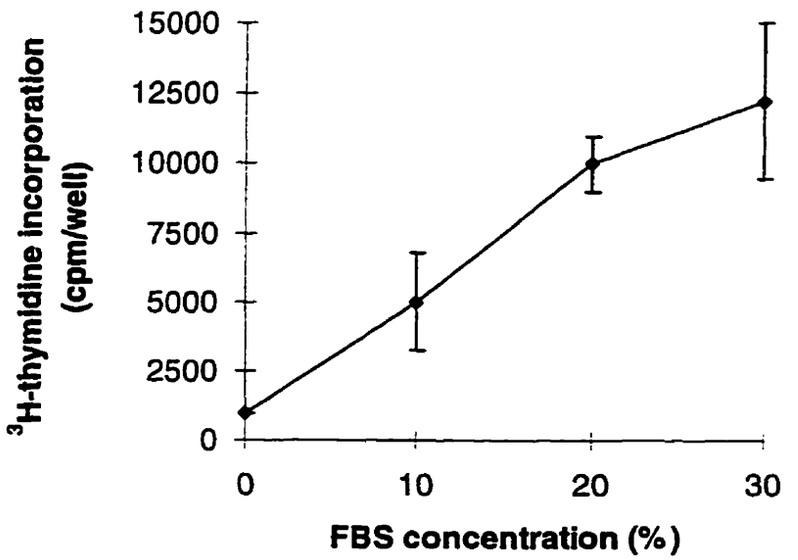

Figure 2.5: Response of the non-adherent cell population to fetal bovine serum. Supernatant containing non-adherent cells was removed from a culture and divided into wells of a 12 well Costar plate. After the cells had attached, cultures received either L-15 without serum or L-15 with 10, 20 or 30% fetal bovine serum. Seventy two hours later, [3H]-thymidine was added and incorporation into acid-insoluble material was assayed after 9 hours. Results shown are those of a single representative experiment and represent the mean of triplicate wells with error bars representing the standard deviation.

appearance (Fig 2.3E). Wright Giemsa staining of cytocentrifuge preparations of the non-adherent cells revealed several different cell types but about 50 % of the cells had a very distinctive appearance (fig. 2.3F). They had an irregular shape with jagged cytoplasmic extensions, and a variably shaped, eccentric nucleus (fig. 2.3F). Cells were continuously released into the medium (fig. 2.3E). In a single culture flask, the number of cells released per week remained constant for up to 6 weeks (data not shown) although the number of cells released varied between individual cultures. Eventually, the non-adherent cell number declined until few or no cells were produced. When non-adherent cells were transferred to new flasks, a fraction of the cells attached to the culture surfaces. Some attached immediately and had the characteristics of macrophages. By three days, many more attached cells were evident and most had the features of dendritic cells. In these new cultures, the adherent cells responded to

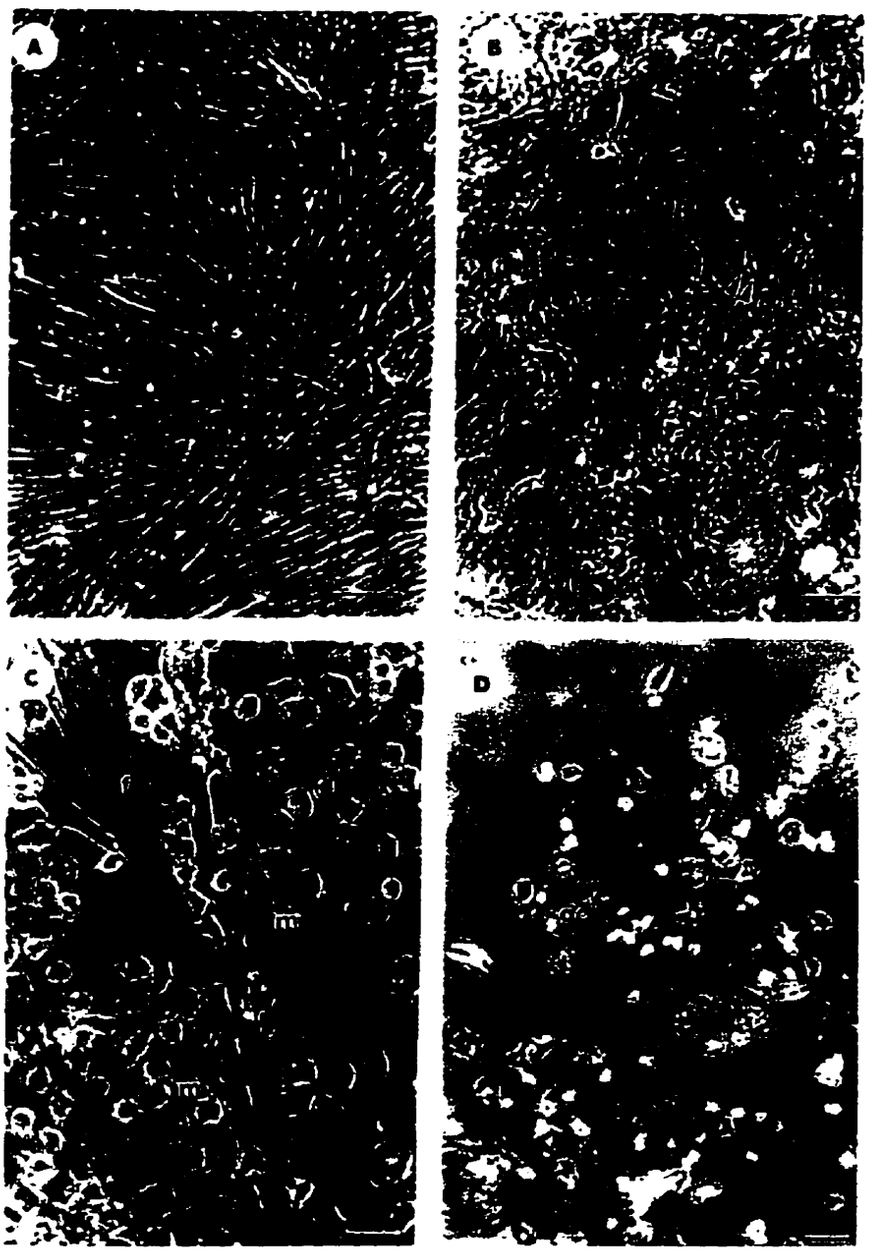

Figure 2.6: Non-hemopoietic spleen cell cultures. When the spleen was dissociated with trypsin and the medium supplemented with 30% FBS (A), criss-crossing fibroblasts dominated the culture. With collagenase dissociation and a 10% FBS supplement (B), a complex stromal layer with macrophages (single arrowhead) and dendritic cells (double arrowheads) formed, but no hemopoietic foci were observed. With collagenase dissociation and a 30% horse serum supplement (C), macrophages (m) and, occasionally, fibroblasts (f) appeared. After some time in culture (D), the macrophages formed multinucleated giant cells (gc) . Phase contrast, size bars represent 25 μM.

increasing serum concentrations with increased DNA synthesis (fig. 2.5), increased in number with time, and were successfully subcultivated several times to new flasks before proliferation ceased. However, non-adherent cells were not produced in these new cultures.

Subcultivation of adherent cells

Adherent hemopoietic cultures could be transferred to new flasks by subcultivating with trypsin. After 3 -5 subcultivations the cultures appeared to lose the capacity to form hemopoietic foci.

NON HEMOPOIETIC CULTURES

Different combinations of dissociation protocols and serum supplements led to four types of cultures, but in all cases the attached cells failed to develop into a stroma that released cells into the medium. A culture with predominantly polygonal or epithelial-like cells developed from the outgrowth of spleen fragments. When further dissociation was done either with bovine trypsin or by mechanical disruption and the medium was supplemented with FBS, cultures of predominately bipolar or fibroblast-like cells resulted. In these cultures the cells were arranged either side-by-side or in a criss-crossing pattern (fig. 2.6A). When dissociation was done with collagenase and the medium was supplemented with only 10 % FBS, a stroma developed but in contrast to cultures with 20-30 % FBS, round phase bright cells rarely appeared on top of this stroma (fig. 2.6B). When the medium was supplemented with 10 to 30 % horse serum (HS), cultures with mainly large, flat cells developed (fig. 2.6C), regardless of the dissociation method. These cells had the characteristics of macrophages. Initially these cells were uniformly spaced on the growth surface with little contact between the cells, but with time the cells formed multinucleated giant cells (fig. 2.6D). Occasionally, fibroblasts were observed in cultures with HS (fig. 2.6C), but were rare.

With time, usually within 4 weeks of culture initiation, confluent monolayers formed in all nonhemopoietic cultures, except for those in HS, and could be subcultivated repeatedly. From the explant outgrowth cultures, an epithelial cell line was developed. The cells have predominantly a polygonal shape, have been passaged 20 times, and

were used in recent experiments on glutamine-free growth (Bols et al, 1994). A fibroblastic cell line was developed from cultures initiated with collagenase digestion and maintained in medium with 10 % FBS. The cells have a bipolar shape and have been passaged 6 times, with no noticeable change in viability and proliferative capability over this time. Both cell lines have been frozen in cryovials in liquid nitrogen for up to 2 years, and upon being thawed, yielded viable cultures.

4. DISCUSSION

Diverse cell cultures were derived from the rainbow trout spleen. The trout spleen primary cell cultures were of two broad types. In one, the sequence of events, which sometimes took as long as six months, was the development of a complex stromal layer on the growth surface, followed by the appearance of round cells on top of the stromal layer, and the release of non-adherent cells into the medium. This pattern of development is similar to both long-term cultures of the rainbow trout kidney (Diago et al, 1993) and mammalian bone marrow (Spooncer et al, 1993). Two products of the trout spleen cultures appear to be dendritic cells and macrophages, as discussed below. Therefore, like rainbow trout kidney cell cultures, which appeared to produce granulocytes (Diago et al, 1993), the trout spleen cultures were myelopoietic. The second type of trout spleen primary cell culture did not yield a stromal layer that supported the development of round, non-adherent cells and is regarded as nonhemopoietic.

HEMOPOIETIC CELL CULTURES

Dendritic cells were identified among the adherent cells of rainbow trout spleen cell cultures on the basis of their similarities with two distinctive features of mammalian dendritic cells : morphology and motility. The appearance of trout spleen dendritic cells was strikingly similar to published phase contrast photographs of mouse spleen dendritic cells (Steinman & Cohn, 1973) and photographs of dendritic cells from the rat peritoneum (van Vugt et al., 1991), mouse bone marrow (Inaba et al., 1992) and mouse liver (Lu et al., 1994) after cytocentrifugation and Giemsa staining. Like dendritic cells from rodents and man (Steinman et al, 1986), trout dendritic cells were irregular in shape, with processes that included spiny dendrites, bulbous pseudopods, and thin lamellipodia. Like murine (Steinman & Cohn, 1973) and human (Freudenthal & Steinman,1990) dendritic cells, the processes on trout dendritic cells were rapidly and constantly moving. In addition, the weak phagocytic ability and equivocal nonspecific esterase activity of trout dendritic cells were features that previously were observed in mammalian dendritic cells (Steinman et al, 1986). With the culture methods of this study, many functional characteristics of mammalian dendritic cells, such as acting as

accessory cells for several immune responses (Steinman et al, 1986), can now be examined in trout.

Dendritic cells appeared to arise from rainbow trout spleen cell cultures in two different ways. In the initial primary culture, small numbers of dendritic precursors in the spleen cell suspension appeared to attach and proliferate. In the second case, dendritic precursors, possibly at an earlier stage than above, appeared to be released into the medium from hemopoietic foci of the primary cell culture and upon transfer to secondary flasks were able to give rise to adherent progeny. In mammals a non-adherent progenitor is thought to give rise to adherent, proliferating dendritic cells (Inaba et al, 1992). For both primary and secondary cultures of the rainbow trout spleen, dendritic cells were not immediately apparent but appeared 14 to 16 days after plating of the primary culture and 3-5 days after plating of the secondary cultures. In mammalian bone marrow cultures, dendritic cells are not initially evident and are usually seen 4 to 7 days after culture preparation (Bowers & Berkowitz, 1986; Gieseler et al, 1991; Inaba et al, 1992).

Macrophages were identified both underneath and on top of islands of stromal cells, as well as between the stromal-cell islands. Identification was on the basis of morphology, phagocytic ability and positive staining for non-specific esterase activity. These characteristics have been commonly accepted as criteria for rainbow trout macrophages (Estepa & Coll, 1993). The frequent occurrence in trout spleen cell cultures of macrophages contrasts with the long-term cultures of the trout kidney, where few macrophages were found (Diago et al, 1993). However, in mammalian LTBMC, macrophages were present and found to overlap and underlie the stromal-cell islands (Allen et al,1990). The macrophages underneath the stromal layer were associated frequently with developing granulocytes and erythrocytes (Allen et al,1990). In trout spleen cell cultures, a macrophage underneath the stroma was occasionally seen associated with a distinct group of round cells, but the identity of these cells currently is unknown.

As well as being present initially, macrophages appeared to be produced by trout spleen cell cultures. Among the non-adherent cell population, some cells were capable of attaching to the growth surface and these cells had the characteristics of

macrophages. Dendritic cells, macrophages and granulocytes are thought to share a common progenitor in mammals and to represent three distinct pathways of myeloid differentiation (Inaba et al, 1993). However, under some culture conditions, dendritic cells have been reported to convert to macrophages (Gieseler et al, 1991). Whether this occurs in the rainbow trout spleen cell cultures is unknown at this time.

Hemopoietic cultures developed only after incomplete dissociation of the trout spleen by collagenase digestion. Dissociation into cell clusters was effective but complete dissociation into single cells was ineffective. This was also noted in LTBMC from mouse (Allen et al,1990). Possibly, prolonged digestion destroyed either essential cells or an essential microenvironmental architecture that was unable to reform. The requirement for dissociation by collagenase rather than by trypsin could be explained by collagenase releasing preferentially a specific cell type or cellular ensemble or alternatively being less toxic to such cells. For the mouse spleen, collagenase treatment released a population of large macrophages that was not otherwise obtained and facilitated the release of dendritic cells (Steinman et al, 1986). Commercial collagenases contain a mixture of activities that include collagenases, clostripain, proteases and trypsin and that vary between types and between individual lots. This probably accounts for some of the variability in the preparation of hemopoietic cultures from the trout spleen.

The other important requirement for the development of hemopoietic cultures from the trout spleen was a high FBS concentration (20-30%). The high serum could be essential for the development of the stromal layer to a state that would promote myelopoiesis, and as well, supply the correct concentration of a specific cytokine or combination of cytokines to directly support myelopoiesis. One important cytokine could be granulocyte/macrophage colony-stimulating factor (GM-CSF), which was essential for the generation of large numbers of dendritic cells from mouse bone marrow cultures (Inaba et al, 1992). Previously, a high FBS concentration (20 %) has been noted to increase the duration of hemopoiesis in murine LTBM cultures (Eliason, 1983). However, unlike other culture systems, horse serum (HS) was completely ineffective in supporting the development of stromal islands and hemopoiesis in the trout spleen cell cultures. For murine LTBMC, HS was very effective but varied between serum batches (Dexter & Testa,1976). In a chicken bone marrow culture system, both HS and FBS

were effective for hemopoiesis but FBS was more favorable (Cormier & Dieter-Lievre, 1990). The failure of HS to support both the development of the stromal layer and hemopoiesis suggests that the stromal layer was important for the development of hemopoietic trout spleen cell cultures.

NON-HEMOPOIETIC CELL CULTURES

Although other methods of preparing trout spleen cell cultures did not lead to hemopoiesis, they did lead to the development of cell lines. A fibroblast-like line was developed from a primary culture that had been prepared by collagenase digestion and maintained in 10 % FBS; an epithelial-like cell line; from the outgrowth of explants in 10 % FBS. Previously, a cell line with reticular cell characteristics was developed from fragments of the rainbow trout spleen (Moritomo et al, 1990). Other fish spleen cell lines have been developed from the silver perch (Ellender et al, 1979), sturgeon (Hedrick et al, 1991) and black porgy (Tung et al, 1991). Whether any of these cell lines can support hemopoiesis is unknown, but several adherent cell lines from cultures of mammalian bone marrow (Itoh et al, 1989; Aizawa et al, 1994) and spleen (Piersma et al, 1984) can influence hemopoiesis in vitro, making them useful for investigating the interactions between stromal cells and hemopoietic stem cells.

Despite different dissociation protocols for the preparation of the spleen cell suspensions, horse serum (HS) favored the development of cultures in which macrophages predominated. Usually HS is considerably less effective than FBS in supporting proliferation of piscine (Ganassin & Bols, 1992) and mammalian cell lines (Jayne, 1991). For the trout spleen, macrophages might be less sensitive to detrimental factors or more responsive to maintenance factors in HS than other cell types in the spleen cell suspension. What these factors might be is unknown, but HS is known to differ from FBS in some significant ways. For example, adenosine deaminase activity is much less abundant in HS than FBS (Ullman et al, 1976). Regardless of the reasons for its effectiveness, HS possibly could be used to generally prepare relatively pure cultures of rainbow trout macrophages.

Although identifying the full developmental potential and all the different cell types of these cultures are beyond the scope of this paper, the general descriptions and

methods of obtaining these cultures should be helpful in fish immunology and virology. These cultures should lead to the development of in vitro models for fish hemopoiesis, similar to those developed over the last 20 years for mammals (Dexter et al, 1977; Spooncer et al, 1993), and allow the identification of factors regulating blood cell production. Secondly, they should allow studies of trout dendritic cells. Although antigen presenting cells have been assayed in catfish (Vallejo et al, 1992), and Peleteiro and Richards (1985) have speculated that cells similar to the Langerhans cell in mammals present antigen to the lymphocytes of the rainbow trout epidermis, dendritic cells have not been described previously in fish. The failure to detect them is likely due to their rarity. In mammalian tissues, the frequency of dendritic cells is very low, less than 1% (Steinman et al, 1986), and generating them in cell culture is one approach to the problem of obtaining enough cells for study. Inasmuch as primary cultures of human dendritic cells preferentially support the replication of human immunodeficiency virus type I, suggesting that dendritic cells act as a reservoir of virus infection (Langhoff et al, 1991), the cultures of the current study could be of value in fish virology.

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Chapter 3

An in vitro culture system that supports long-term production of functional myeloid cells from rainbow trout spleen and pronephros

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ABSTRACT

Long-term hemopoietic cultures, similar to those produced from mammalian bone marrow, have been generated from rainbow trout pronephros and spleen. The trout organs are dissociated, and placed in culture flasks, leading to the formation of a complex stromal layer on the culture surface. In time, small, round cells appear on top of the stromal layer, proliferate, and are released into the medium as mature progeny cells. Cultures that produce these progeny cells develop only after specific dissociation procedures and in medium supplemented with a specific amount and type of serum. Spleen cultures must be dissociated with collagenase and the medium must be supplemented with a high (30%) concentration of fetal bovine serum. Nonhemopoietic cultures resulted after explant outgrowth or dissociation with trypsin and in medium with low FBS concentrations or a horse serum supplement. These cultures contained non-adherent cells, including macrophages and fibroblasts, and could be passaged to form cell lines, but did not develop a non-adherent population. Kidney hemopoietic cultures resulted after dissociation by either mechanical means or collagenase digestion, and supplemented with 10, 20 or 30% FBS.

Spleen cultures produce macrophages and a unique cellular product with the characteristic morphology and motility of mammalian dendritic cells. The progeny cells produced by the pronephros include macrophages and neutrophils, and these progeny cells adhere differentially to extracellular matrix proteins. This adhesion is enhanced by exposure to the phorbol ester, phorbol -12-myristate 13-acetate (PMA).

1. INTRODUCTION

Long-term bone marrow cultures (LTBMC) (Allen et al, 1990; Dexter et al, 1977; Spooncer et al, 1993) have been valuable tools for studying the process of hemopoiesis in mammals. Recently, long-term cultures have been developed from the two major hemopoietic tissues of the rainbow trout, *Oncorhynchus mykiss*, the pronephros (Diago et al, 1993) and the spleen (Ganassin & Bols, 1996). In these cultures, a multi-layered stroma forms on the tissue culture surface. Cellular products develop in association with the stromal layer, and are released into the culture medium as mature cells. The cells produced by this culture system are difficult to identify definitively because of the scarcity of monoclonal antibodies to fish leukocyte surface markers. However, the major products of cultures of rainbow trout spleen include macrophages and cells with the characteristic morphology and motility of mammalian dendritic cells (Ganassin & Bols, 1996), and the major products of kidney cultures have been described as heterophils (Diago et al, 1993).

Both kidney and spleen cultures, like long term bone marrow cultures, are dependent upon supplementation of the culture medium with a high concentration of fetal bovine serum. The method of organ dissociation is also important to successful hemopoiesis, particularly from spleen tissue. Hemopoietic cultures from rainbow trout spleen are only established when the tissue is digested at 10°C for 12-18 hours with collagenase, and not when the tissue is disrupted using mechanical means, such as mincing, or forcing the tissue through a screen or a syringe. Digestion with other enzymes such as trypsin results in cultures that cannot sustain hemopoiesis. Rainbow trout kidney cultures, on the other hand, can be established successfully by either mechanical dissociation (Diago et al, 1993), or by enzymatic digestion.

Both kidney and spleen hemopoietic cultures will be useful in studies of the factors controlling production of immune system cells, and will provide a convenient source of cells for studies of their functions. For example, preliminary experiments indicate that the kidney culture products, which are primarily neutrophils, adhere differentially to various extracellular matrix (EM) proteins. This adhesion is enhanced by exposure to the phorbol ester, PMA.

2. MATERIALS AND METHODS

Preparation of kidney and spleen cell cultures

Cultures were initiated from rainbow trout spleen as previously described (Ganassin & Bols, 1996). Kidney cultures were prepared using the same protocol. Briefly, rainbow trout weighing 100- 250 grams were killed by cerebral fracture and their blood was removed by caudal puncture to reduce the blood volume in the kidney. A ventral incision was made, and the viscera were removed to expose the kidney. The connective tissue was removed, and the pronephric tissue was scraped out with a sterile scoop and placed in petri dish with 5 mL of sterile phosphate buffered saline supplemented with the antibiotics Gentamicin, (50 µg/mL) and Fungizone, (100 µg/mL) (Canadian Life Technologies, Burlington, ON). The tissue was teased apart with a sterile scalpel and scissors. At this point, two different dissociation methods were used. In the first instance, the tissue was forced through a 40-mesh screen of a tissue dissociation kit (Sigma). Alternately, an equal volume of collagenase A (Boehringer Mannheim, Dorval, Que.) was added to the kidney fragments, which were then incubated for 12 hours at 10 °C. After either dissociation step, the resulting suspension of cells and fragments was centrifuged in a tabletop centrifuge for 5 minutes at 1,000 rpm (HN-SII, International Equipment Co., Needham Heights, MA). The cell pellet was resuspended in the growth medium, Leibovitz's L-15, supplemented with 10, 20 or 30% fetal bovine serum (FBS) (catalog number 200-6140), or with 30% horse serum (HS) (catalog number 16050) from Canadian Life Technologies (Burlington, ON).

The cell suspensions in different growth media were added to 12.5 cm^2 flasks (Falcon, Oxnard, CA), and incubated at 22 °C. The tissue from each organ was distributed into three flasks. After two weeks, the medium was completely removed, the surface of the flask was rinsed with medium, and fresh medium containing penicillin (100 I.U. /mL) and streptomycin (100 µg/mL) was added. At this point the only cells that were present were those attached to the surface of the culture flask. Cultures were maintained at 22 °C, and fed by complete changes of medium until the appearance of hemopoietic foci. At this time, cells were harvested from the cultures weekly by removing half of the culture medium containing non-adherent cells, and replacing it with fresh medium.

Morphology and cytochemistry

A Nikon Diaphot inverted microscope with phase optics was used to observe and photograph living cultures in flasks. Non-adherent cells were harvested from a culture flask and a cytocentrifuge (Shandon Cytospin) was used at 500 rpm for 10 minutes to deposit the cells on to microscope slides. The slides were fixed for 5 minutes in methanol, and were then stained with Wright's and Giemsa stains (Sigma) in distilled water, 1:3:50 for 20 minutes, to distinguish general morphology. Positive staining for the leukocyte enzyme myeloperoxidase is a characteristic of rainbow trout neutrophils (Zelikoff & Enane, 1991). Myeloperoxidase activity was demonstrated using diaminobenzidine (DAB), with a modification of Sigma procedure 390. DAB was used as a substrate rather than p-Phenylenediamine diHCl and catechol, because staining results with the latter were unclear. Cytocentrifuged slides were fixed for 30 seconds at room temperature in fixative solution consisting of 5 ml of 37% formaldehyde mixed with 45 ml of 95% ethanol. Following fixation, they were washed in gently running tap water and air dried in the dark for 10 minutes. Fast DAB tablets (Sigma) were used to prepare the staining solution, and washed, fixed slides were incubated for 30 minutes in the dark in a 37°C waterbath. The slides were rinsed in tap water, allowed to air dry and counterstained with Acid Hematoxylin Solution (Sigma catalog No. 285-2) for 10 minutes.

Transmission electron microscopy

Non-adherent cells were harvested from kidney cultures and collected by centrifugation. Cells were fixed for 10 minutes in modified Karnovsky's fixative, pH 6.8, rinsed gently 3 times with 0.1 M sodium cacodylate, and post-fixed in osmium tetroxide. Cells were dehydrated in ascending ethanol concentrations, taken to acetone, then infiltrated and embedded in Epon:Araldite. Ultrathin sections were cut on a microtome, stained with lead citrate and uranyl acetate, and observed with a Phillips transmission electron microscope.

Cell Adhesion Assay

Wells of 48-well tissue culture plates (Costar, Cambridge, MA) were coated with laminin, fibronectin, collagen type I, or collagen type IV (Sigma). Collagen I and

collagen IV were dissolved in 0.1 M and 0.25% (v/v) acetic acid respectively. Both were sterilized by underlying with chloroform (Sigma, 1991), and diluted to working concentrations in Dulbecco's PBS. Laminin and fibronectin were purchased as sterile solutions and diluted further in Dulbecco's PBS. Wells were coated with 200 μL of each extracellular matrix protein, at final concentrations indicated in figure legends, by overnight incubation.

Following the overnight incubation, wells were washed twice with PBS, and l00 μL of L-15, with or without 100 ng/mL phorbol -12-myristate 13-acetate (Sigma) , was added to each well. Cultured kidney cells were prepared by collecting non-adherent cells by centrifugation, and resuspending in L-15 without serum to a concentration of 10^6 cells/ml. 100 μL of this suspension was added to each well, and to uncoated wells, for a final concentration of 10^5 cells per well, and 50 ng/mL of PMA in phorbol treated wells (Rüegg et al, 1992).

To quantify the degree of cell attachment, the fluorescent dye, 5-(6)-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) (Molecular Probes) was used. Although CFDA-AM has been shown to have a detrimental effect on cell attachment (De Clerk et al, 1994), in our assay it is not present during the period of cell attachment. After a 10 hour incubation, medium containing unattached cells was gently decanted from the culture vessels, and 150 μL of Dulbecco's PBS containing CFDA-AM at a concentration of 4μM was added to each well. The dye-containing buffer was aspirated out of each well after 30 minutes, and 250 μl of Dulbecco's PBS was added. The plate was scanned using the CytoFluor 2350 (Millipore), a cytofluorometer, with filters 485/530, using area scan at a sensitivity setting of 4.

3. RESULTS

Development of hemopoietic cultures

In both kidney and spleen cultures, collagenase dissociation led to hemopoietic cultures with a complex stroma that released cells into the medium. Hemopoietic spleen cultures could only be established with collagenase digestion, and not with trypsin or mechanical dissociation (Ganassin & Bols, 1996, summarized in fig. 3.1). Kidney tissue produced hemopoietic cultures when dissociated by either mechanical or enzymatic means. While spleen cultures absolutely required a supplement of 30% FBS to establish hemopoiesis, kidney cultures produced progeny cells when supplemented with 10 or 20% FBS, although the number of cells produced was greater with a 30% supplement (data not shown).

At the time of the first complete medium change, two weeks after culture initiation, only cells adherent to the tissue culture surface were present. In spleen cultures, the establishment of a complex stromal layer on the culture surface was followed by the appearance of small foci of round, phase bright cells (fig. 3.2b), which proliferated into large masses and produced non-adherent cells that were released into the culture medium. The time period from the initiation of the culture and the appearance of non-adherent cells ranged from 4 weeks to 6 months.

Kidney cultures developed in a different manner than those of the spleen (fig. 3.2). The stromal layer did not cover as much of the culture surface as the stromal layer of spleen cultures. The presence of numerous melanomacrophages gave a black appearance to the culture surface. Progeny cells did not develop in discrete foci, as was seen in spleen cultures, but rather over the entire surface of the stromal layer, and were much more numerous. The kidney cultures produced progeny cells as early as 2 weeks following culture initiation, and when supplemented with 30% FBS, maintained production for a minimum of 2 months. In cultures supplemented with a lower FBS concentration, hemopoiesis was not as prolonged, and the stromal layer declined and began to peel off of the surface after 2 months. In cultures supplemented with horse serum, a stromal layer did not develop, and non-adherent cells were not produced.

Generation of cell lines

As described previously (Ganassin & Bols, 1996), and summarized in figure 3.1, the techniques for producing hemopoietic cultures have led to the generation of numerous cell lines from rainbow trout spleen and kidney. These cell lines have been maintained and cryopreserved as described by Bols & Lee (1994), and are awaiting further characterization.

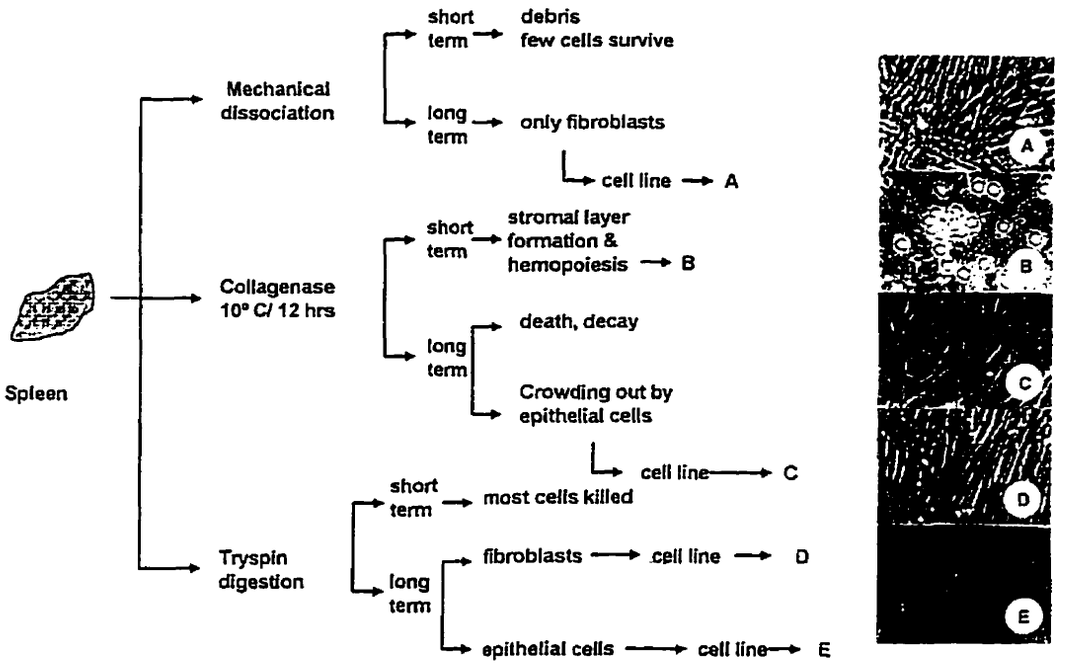

Figure 3.1: Effect of dissociation protocol on spleen culture development. Only specific dissociation protocols, as summarized in this chart, lead to hemopoietic cell cultures of rainbow trout spleen. Mechanical dissociation, including mincing tissue, or forcing it through a fine screen or a syringe, results in the death of most cell types. Only fibroblast-like cells survive, which can be passaged to form cell lines, **A.** Collagenase digestion, for 12 hrs at 10°C, leads to the formation of a stromal layer and the proliferation of hemopoietic cells, **B.** These cultures ultimately either die, or are crowded out by a cell type with epithelial morphology, **C**, which can also be passaged to form cell lines. Digestion of the tissue with trypsin results in the survival of either fibroblast-like, **D** ,or epithelial-like, **E** ,cells, and these will also form cell lines.

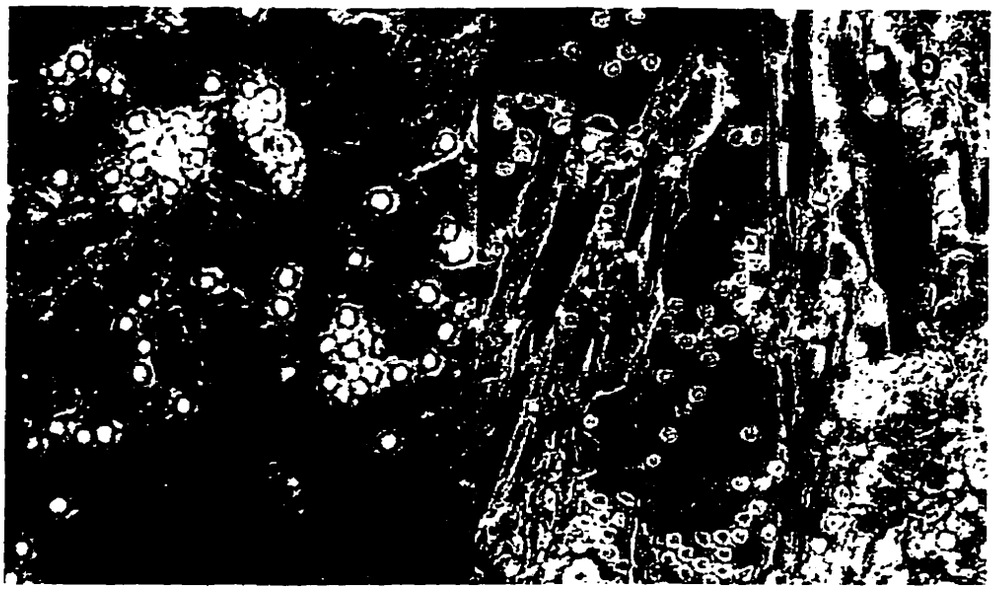

Figure 3.2: Spleen and kidney hemopoietic cultures differ in character. The production of progeny cells (the small, round, phase-bright cells evident in these micrographs), occurs in discrete foci associated with the complex stromal layer in spleen hemopoietic cultures, **a**. In kidney cultures, **b**, the stromal layer is not as extensive and contains numerous melanomacrophages (black pigmented cells). Progeny cells develop all over the stromal layer, and are not concentrated in foci.

Products of hemopoietic cultures

The progeny cells of spleen cultures have been previously described (Ganassin & Bols, 1996). The products of kidney cultures include neutrophils, which are myeloperoxidase positive cells (fig. 3.3c) , approximately 60 μm in diameter, often with lobed nuclei (fig. 3.3a). Another identifiable product is the monocyte or macrophage, with a diameter greater than 100 μm, an oval shaped nucleus, numerous lysosomes and residual bodies (fig. 3.3b), and negative staining for myeloperoxidase. Other cell types are not easily identifiable, and may be earlier developmental stages of neutrophils or macrophages.

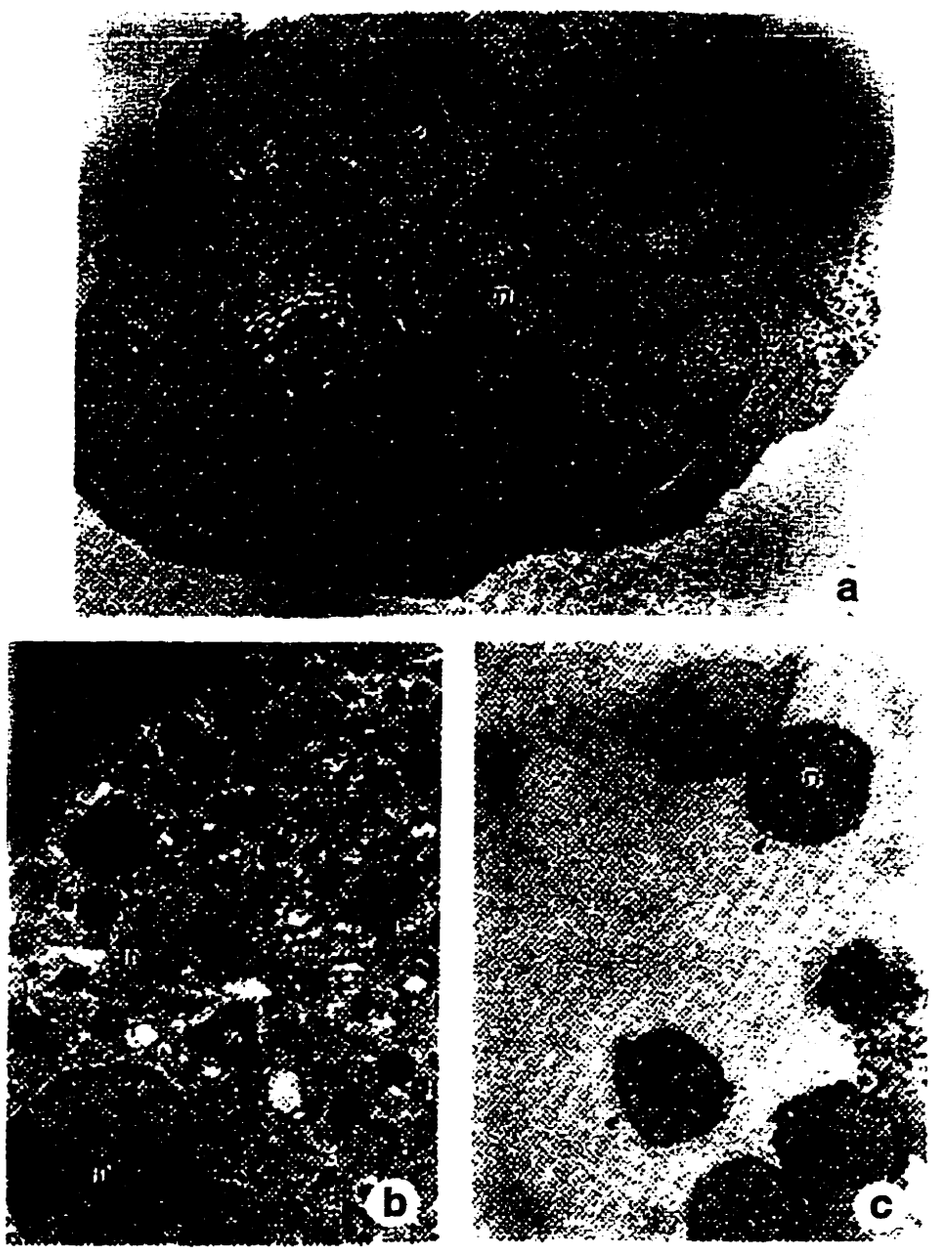

Figure 3.3: Characterization of the products of kidney hemopoietic cultures. The major products of these cultures are neutrophils (A), which have large multi-lobed nuclei (n) occupying approximately half of the cell area and a granular cytoplasm (TEM, Mag. 22,500 x , actual cell size 60 μm diameter). Fewer macrophages (B) are formed, and are easily distinguished by their larger size (TEM, Mag. 13,200 x, actual cell size, 110 μm diameter). The bean-shaped nucleus (n) occupies much less of the cell area, and there are numerous phagocytic vesicles (v) , residual bodies (r), and other inclusions. In cytocentrifuge preparations stained for the enzyme myeloperoxidase (C), most of the cells show dark granulation (arrowheads), indicating a positive reaction typical of rainbow trout neutrophils.

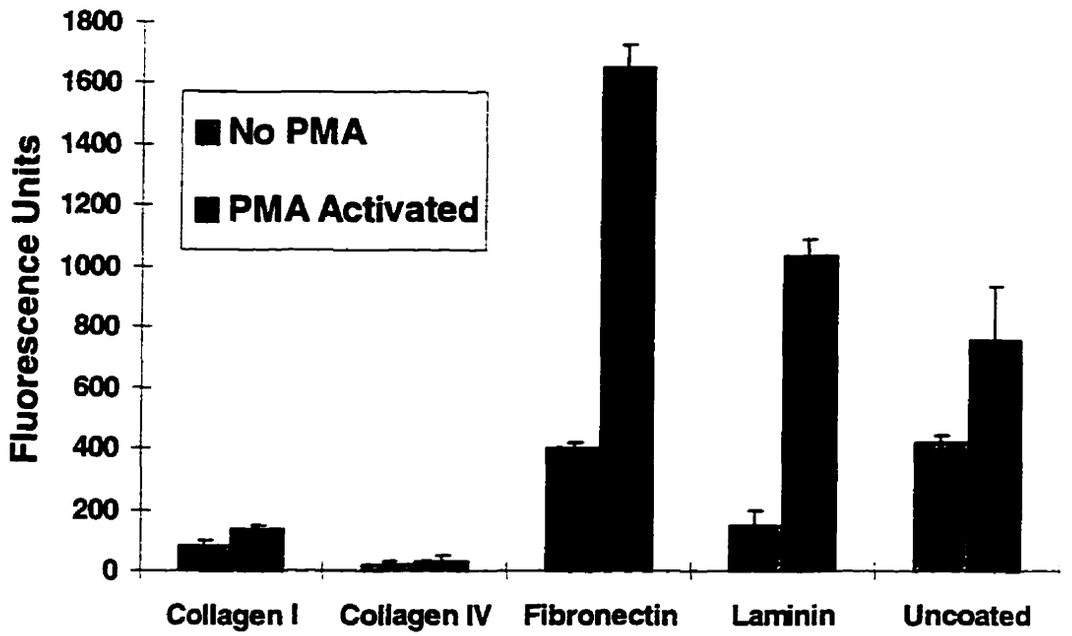

Figure 3.4: Adhesion assay of kidney culture products (primarily neutrophils). Wells of a 48 well plate (Costar) were coated with the extracellular matrix proteins collagen I or IV (10 µg/well), fibronectin (5 µg/well), or laminin (2 µg/well). Adhesion was measured after 10 hours. Cells were added in medium without a serum supplement, and with or without the phorbol ester PMA, and allowed to adhere for 10 hrs. After 10 hours, non-adherent cells were removed, and 150 µL of 4 µM CFDA-AM (carboxyfluorescein diacetate acetoxy methyl ester, Molecular Probes) was added to each well. After 30 minutes incubation, the solution was aspirated and replaced by Dulbecco's PBS, and the plate was scanned using a CytoFluor 2350 microfluorometer (Millipore), using filters 485/530, sensitivity 4. Results shown are of a single representative experiment, with error bars representing the standard deviation of triplicate wells.

Adhesion of kidney culture products

Unstimulated kidney culture products adhere to uncoated plastic and to surfaces coated with fibronectin to the same degree (fig. 3.4). Adhesion to laminin is approximately one half of adhesion to uncoated plastic, while adhesion to either collagen I or collagen IV is very limited.

When the phorbol ester PMA is included in the attachment medium, attachment to collagen I or collagen IV coated surfaces is relatively unaffected; however, the cells

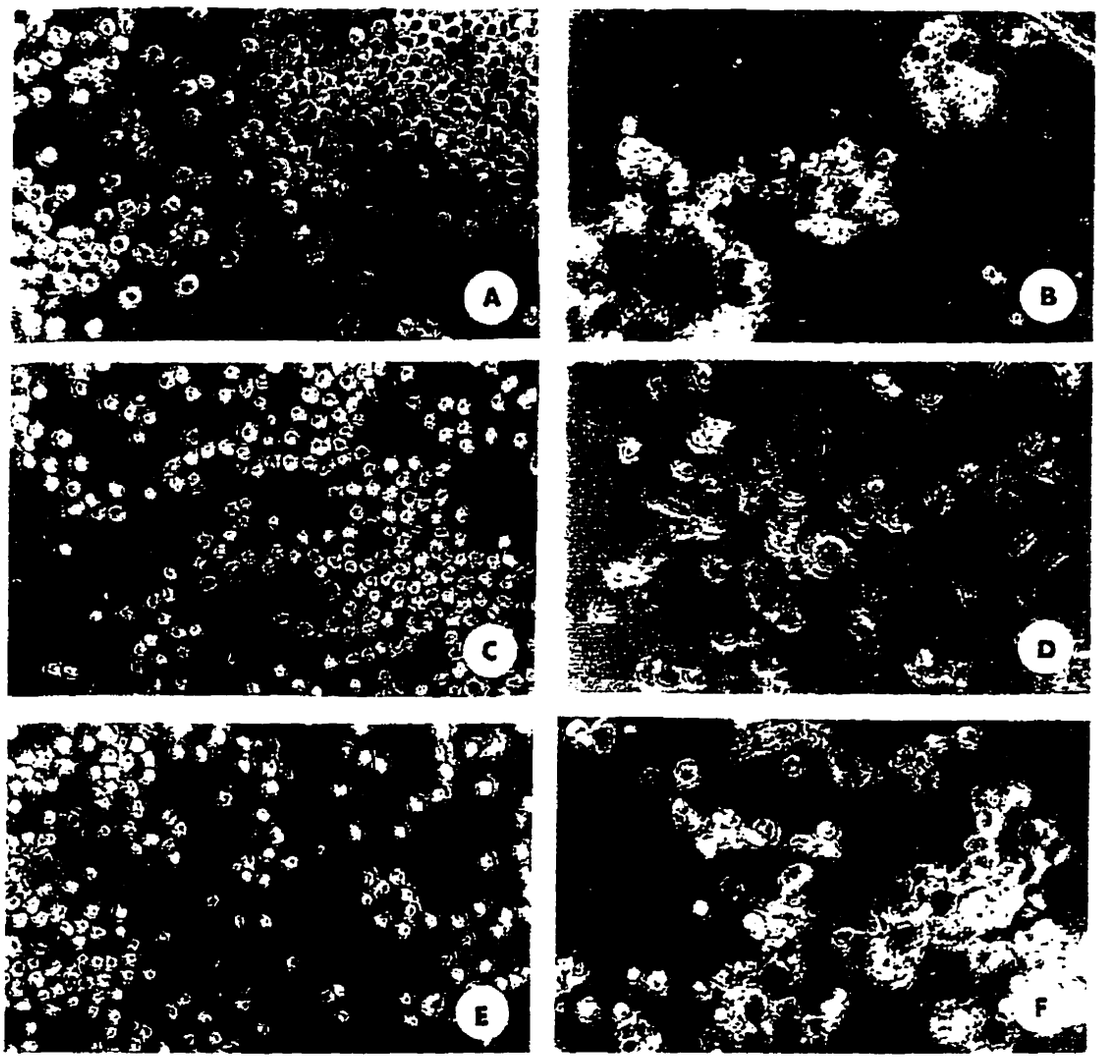

Figure 3.5: Appearance of cells adhering to extracellular matrix proteins. Without PMA in the attachment medium, cells did not attach appreciably to surfaces coated with collagen I (A), but attached to surfaces coated with fibronectin (C), or laminin (E). When PMA, 50 ng/mL, was added to the attachment medium, cells plated in wells coated with collagen I (shown) or IV (not shown, but similar) formed large aggregates, not attached to the culture surface (this photograph is taken in the plane of the floating cells). In wells coated with fibronectin (D), attachment was greatly enhanced and the cells rapidly spread. In laminin coated wells (F), attachment was much greater than in uncoated, but cells attached tenuously and did not spread appreciably. Phase contrast, original mag. 200x, photographed 1 hour after plating.

attach to one another and form large aggregates (fig. 3.5b). Attachment to uncoated surfaces is enhanced slightly by exposure to PMA. PMA dramatically enhances the attachment of kidney culture products to surfaces coated with fibronectin and laminin. The adherence of PMA stimulated kidney cells to fibronectin is four times that of the unstimulated controls, while adherence to laminin is increased five fold. Cells attach

strongly and spread in fibronectin coated wells (fig. 3.5d), but are weakly adherent and spread less in wells coated with laminin (fig. 3.5f).

4. DISCUSSION

Hemopoietic cell cultures have been established from the two major hemopoietic organs of the adult rainbow trout, the anterior kidney (pronephros) and the spleen. Cultures from these organs initiated and maintained under the same conditions differ in several respects, including the requirement for a specific dissociation protocol and serum supplement, the time course of culture development, the extent of stromal layer formation, and the progeny cells produced.

Kidney hemopoietic cultures can be established after dissociation of the tissue by mechanical means, or by enzymatic digestion, unlike those of spleen, which are only successful when initiated by collagenase digestion (Ganassin & Bols, 1996). In spleen cultures, digestion by collagenase is essential to the establishment of a complex stromal layer. In mammalian long-term bone marrow culture (LTBMC), the stromal layer provides cell-cell contacts and some of the diffusible regulatory molecules that stimulate blood cell formation and maturation. Other diffusible factors are provided by a high serum concentration supplementing the growth medium (Spooncer et al, 1993). The long period of time between culture initiation and the production of progeny cells in rainbow trout spleen cultures suggests that the stromal layer may produce some critical factor(s) that must accumulate to high enough levels to support hemopoiesis. Kidney cultures develop in a much shorter time than those of the spleen, and do not appear to require the establishment of an extensive stromal layer prior to cell production. Kidney cultures begin to produce non-adherent progeny cells very soon after culture initiation, which suggests that the factors required differ from those of the spleen, or are required in smaller amounts and are supplied adequately by the fetal bovine serum supplementing the culture medium.

Spleen progeny cells develop in discrete foci, and may require specific cell-cell contacts in addition to soluble factors, as has been observed in LTBMC (Allen et al, 1990). The associations between developing kidney cells and the stromal layer are less obvious.

The progeny cells produced by spleen cultures, as described previously (Ganassin & Bols, 1996), include monocytes, macrophages, and dendritic-like cells. The kidney cultures also produce monocytes and macrophages, but their major product appears to be neutrophils, as judged by their morphology, and positive myeloperoxidase staining. This division may reflect the *in vivo* situation.

Kidney cultures produce many more progeny cells than those of spleen. This is also true *in vivo*. The rainbow trout kidney is considered the primary hemopoietic organ, analogous to mammalian bone marrow. Histologically, far more blood cell production is observed there than in the spleen (Yasutake & Wales, 1983). The spleen has been considered analogous to the mammalian lymph node (Anderson et al, 1986), which functions primarily in the response to antigens and has a secondary role in blood cell production.

The culture conditions previously described for pronephric cultures (Diago et al, 1993), produced only small numbers of hemopoietic cells. When the pronephros is cultured using the same method and culture medium described for spleen cultures (Ganassin & Bols, 1996), the cultures reliably produce large numbers of neutrophils for a period of months. The cultures provide a convenient source of cells for further experimentation.

Preliminary experiments of the adhesive properties of the kidney culture products demonstrate the potential usefulness of these cultures. In the inflammatory response, the migration of leukocytes from the blood or tissues to the site of infection or tissue damage involves interactions with extracellular matrix proteins, such as laminin, fibronectin and collagen (Snyderman & Goetzl, 1981).

Our data shows that PMA enhances the attachment of the kidney culture products to laminin and fibronectin, and promotes the aggregation of the cells in the presence of collagen I or IV. The phorbol ester PMA is a potent activator of protein kinase C (PKC), its specific cellular receptor (Blumberg, 1988), and mimics the effects of physiological activators (Rosales & Juliano, 1995). Leukocytes of mammals respond to phorbol esters by increased cell-to-substratum or cell-cell adhesion, either due to increased expression of specific receptors on the cell surface, as has been reported in human

neutrophils (Yoon et al., 1987) or by the phosphorylation of proteins that are involved in adherence to extracellular matrix proteins (Pontremoli et al, 1987). The mechanism of the response of fish neutrophils to PMA requires further investigation.

Hemopoietic spleen and kidney cultures have many potential uses. First, they provide a model system for the study of the hemopoietic process similar to mammalian long-term bone marrow culture. LTBMC have allowed the study of numerous questions that are difficult or impossible to interpret in the more complex *in vivo* situation, including the identification and isolation of many of the known cytokines. The factors affecting rainbow trout blood cell production can now be examined and the identification and isolation of novel fish cytokines from these cultures is possible.

Secondly, these cultures provide a ready source of immune system cells for further studies. The dendritic-like cells produced by the spleen cultures will be useful in studies of antigen presentation, and of cellular motility. The kidney neutrophils can be used to help to elucidate the process of inflammation in fish.

Finally, the culture conditions result in the production of numerous cell lines, which are potentially useful in such areas as fish virology, toxicology, cell biology, and fish physiology (Bols & Lee, 1991,1994).

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Chapter 4

The development and characterization of a cell line, RTS11, from rainbow trout spleen, with characteristics of a myeloid precursor cell

ABSTRACT

A cell line arose spontaneously from a long-term hemopoietic culture of rainbow trout spleen and has been designated RTS11. For routine passaging, RTS11 requires 20-30 % fetal bovine serum, a high cell density, and medium from previous cultures. Through successive passages, RTS11 cultures maintain a balance between small, non-adherent cells that form the majority of cells and a few larger, granular cells that are either adherent or in suspension surrounded by clusters of the smaller non-adherent cells. The large granular cells appear to be macrophages because they are phagocytic and take up DiI-acetylated low density lipoprotein and acridine orange and stain for non-specific esterase, whereas the small round cells appear to be at an earlier stage of macrophage development. In proliferation assays, the non-adherent cells are stimulated strongly by lipopolysaccharide but weakly by the lectins Con A, PHA, and PWM, and are inhibited by adenosine. They are also stimulated by medium conditioned by the following rainbow trout cells : RTS 11, clone 1A (an another blood cell line), adherent spleen cells in primary culture, and PHA-treated peripheral blood leukocytes. By contrast, media conditioned by 5637, L929 and WEHI-3 cell lines, which are traditional sources of mammalian hemopoietic cytokines, inhibit proliferation. Rainbow trout head kidney leucocytes also show a positive proliferative response to RTS11 conditioned medium. Therefore, RTS-11 cells appear to both produce and respond to growth factors unique to rainbow trout.

1. INTRODUCTION

Numerous mammalian macrophage cell lines are available that differ in their degree of maturity, response to inducing agents, and functional characteristics (reviewed by Ralph, 1986). While they are not identical to macrophages, these variants have been a valuable resource in the study of macrophage biology, due to their convenience as a pure source of homogeneous cells, readily maintainable in culture (Ralph, 1986). Immature myeloid cell lines, such as HL-60 (Gallagher et al, 1979) and U-937 (Sundstrom & Nilsson, 1976) have become extremely useful models for the study of cellular development (reviewed by Harris & Ralph, 1985).

In addition, macrophage cell lines are often sources of cytokines and other factors influencing the growth and maturation of leukocytes. Some of these factors have autocrine effects, stimulating the growth of the cell that produces them. An example of this is the human myeloid cell line HL-60 (Heil et al, 1989).

Rainbow trout macrophages are morphologically and functionally very similar to those of mammals, and are presumed to develop in the same manner from stem cells originating in the spleen and pronephros. Little is known about the developmental stages of myeloid cells immediately following the stem cell, but they ultimately give rise to a progenitor cell known as the colony forming unit, granulocyte-macrophage (CFU-GM), which goes through several multiplication and differentiation stages before resulting in mature macrophages (van Furth, 1989). The morphologically distinguishable stages of macrophage maturation appear to be the same for fish as for mammals.

Most studies of fish macrophages have been undertaken using cells in primary culture. Although monocyte or macrophage cell lines have been reported recently from catfish (Vallejo et al, 1991), carp (Weyts et al, 1997), and goldfish (Wang et al, 1995), there are no examples of macrophage cell lines at any stage of maturity from rainbow trout.

This work describes a cell line with the characteristics of a promonocyte or earlier developmental stage of the myeloid lineage, which arose spontaneously from a long-term hemopoietic culture of rainbow trout spleen. These cells are predominately non-adherent, but contain a small proportion of more mature, adherent macrophage-like cells. They are stimulated to proliferate by lipopolysaccharide, and appear to secrete an autocrine growth factor that supports their growth. Their conditioned medium, in addition to stimulating their own growth, has mitogenic effects on rainbow trout leukocytes.

2. MATERIALS AND METHODS

A: CULTURE INITIATION

Establishment of long-term culture

The cell culture was initiated March 11, 1994, from the spleen of a sexually immature rainbow trout. The spleen was prepared for culture as previously described (Ganassin & Bols, 1996), and divided among three 25 cm^2 Primaria (Falcon) culture flasks, in L-15 medium supplemented with 20% FBS. The culture was maintained without disturbance other than periodic changes of medium for 10 months after the initiation of the culture. At this time, a complex stromal layer comprising fibroblast-like and epithelial-like cells had formed, with macrophage-like cells, and large mounds of phase-bright, small round cells adherent to the stromal surface (fig. 4.1a).

Subculture and establishment of the cell line

The culture was trypsinized to remove the adherent cell layer. Most cells detached easily and were transferred to new flasks: a portion of the stromal cells, however, remained attached and were left in the original flask. Fresh growth medium (GM), L-15 with 30% FBS was added to this heterogeneous stromal cell population. Formation of mounds of small, phase-bright cells continued to occur. This culture was subcultured routinely by removing the spent medium and non-adherent cells, then detaching the adherent cells with trypsin, and resuspending them in fresh culture medium. The production of non-adherent cells continued with repeated subculture. After the first 3 subcultures, the use of trypsin was discontinued, and cells were detached by incubation with Versene (Gibco).

Periodically, the non-adherent cell population was transferred to new flasks in an attempt to induce the cells to proliferate in the absence of the accompanying stromal cell layer.

B: ESTABLISHMENT OF OPTIMAL CULTURE CONDITIONS AND PROCEDURES FOR THE RTS11 CELL LINE

Selection of culture flasks

RTS11 were cultured in a variety of tissue culture flasks, dishes and plates of various sizes, in an effort to establish which culture vessel best promoted proliferation. These included 12.5 cm^2, 25 cm^2 and 75 cm^2 tissue culture treated flasks from Falcon, Corning and Nunc respectively, 25 cm^2 flasks not treated for tissue culture (Falcon), 60 cm^2 petri dishes (Nunc) and multiwell plates with 12, 24, 48, and 96 wells (Falcon or Corning).

Subculture procedure

Attempts to subculture RTS11 cells revealed several idiosyncrasies. The majority of RTS11 cells were non-adherent or semi-adherent and could be dislodged by sharply tapping the flasks against the palm of the hand. However, occasionally more of the cells adhered, and required scraping with a cell scraper (Falcon) to dislodge them from the surface. Initially, cells were collected by centrifuging at 1,000 rpm for 5 min in a table top centrifuge (IEC HN-SII, International Equipment Co., Needham Heights, MA). Cell viability was seriously compromised by centrifugation, and recovery of living cells was small. Centrifugation at lower speeds recovered few cells, which did not proliferate when fresh GM was added. Attempts were then made to subculture cells without centrifugation by dividing the cells into two new flasks along with their spent medium (hereafter referred to as conditioned medium), and adding an equivalent volume of fresh growth medium.

Determination of optimal seeding density

It was observed that cells added to flasks at higher cell density exhibited higher viability and proliferated faster than those added at lower density. The seeding density resulting in optimal growth was therefore determined by removing the cells from an actively growing flask of RTS11 and seeding duplicate 25cm^2 culture flasks with 5 mLs of cell suspension containing 16 x 10^5, 8 x 10^5, 4 x 10^5, or 2 x 10^5 cells per mL of conditioned medium. 5 mL of fresh GM was added to bring the total volume to 10 mL, resulting in seeding densities of 8 x 10^5, 4 x 10^5, 2 x 10^5, or 1 x 10^5 cells/mL. At intervals shown in the figure legend, a small sample (25μL) of cell suspension was removed from each flask and incubated with Trypan blue to stain non-viable cells. Viable cells were counted using a hemacytometer (Reichert Bright Line, Sigma).

Determination of optimal growth temperature and FBS concentratio:

The optimal growth temperature was determined by seeding duplicate flasks with 4 x 10^5 cells per mL as described above, and incubating the flasks at 5°C, 12°C, 18°C and room temperature (21°C +/- 1°C). To determine the optimai FBS concentrations, duplicate flasks were prepared in L-15 supplemented with 0, 10, 20 and 30% FBS, and incubated at 18°C. Aliquots were removed and counted as described above at the intervals shown in the figure legends.

Cryopreservation

RTS11 cells were frozen by collecting the cells by centrifugation, or by simply removing an aliquot, and resuspending them in growth medium containing a cryoprotectant. In attempts to improve viable cell yield after freezing, cells were resuspended in 90% FBS with 10% DMSO, in growth medium with 10% glycerol as cryoprotectant, and in 90% FBS with 10% glycerol. They were frozen by suspending the vial in the vapour phase over liquid nitrogen for 3 hours to allow slow freezing prior to being immersed in liquid nitrogen.

Effect of FBS on cell adhesion

As it was suspected that FBS inhibited the attachment of RTS11 to the tissue culture substrate, the ability of RTS11 cells to adhere to tissue-culture treated plastic in the presence of FBS concentrations ranging from 0-30% was assessed. Cells were incubated with the indicated FBS concentrations prior to their addition to culture wells, and attachment after 1-5 h was assessed using the assay described in chapter 3.

C: CHARACTERISTICS OF RTS11 CELLS

Morphology and cytochemistry

A Nikon Diaphot inverted microscope (Nikon Canada, Toronto, ON) equipped with phase optics was used to observe and photograph living cultures in flasks. To examine general morphology, non-adherent cells were deposited onto microscope slides using a cytocentrifuge (Johns Scientific Inc., Toronto, ON) and stained with Wright and Giemsa stain (Sigma), as previously described (Ganassin & Bols, 1996). Non-specific esterase, acid phosphatase and myeloperoxidase enzymes were demonstrated using Sigma kits. Sudan Black B, a marker of neutrophils, was used as described by Sheehan & Story (1947).

Incubation with acridine orange was used to indicate the presence of lysosomes characteristic of macrophages as described by Bayne (1986). Detection of mycoplasma contamination and observation of nuclear morphology was performed using Hoescht 33258 to stain DNA (Chen,1977). All of these procedures were performed on cells smeared onto microscope slides prior to staining.

DiI-acetylated low-density lipoprotein uptake assay

The fluorescent probe 1, 1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Molecular Probes, Eugene, OR) was used to indicate the presence of the scavenger receptor for lipoprotein. The "scavenger" receptor was named because it is found on cells of the macrophage/monocyte lineage, the "scavenger" cells (Goldstein et al, 1979), and these cells accumulate large amounts of DiI-Ac-LDL, resulting in bright focal fluorescence. Endothelial cells (Voyta et al, 1984) also accumulate this compound, resulting in a more diffuse, less intense staining. Cells were grown on 4 well chamber slides (Falcon) in normal growth medium. They were then incubated with 10 μg/mL DiI-Ac-LDL at room temperature in normal media for 4 h. The media was then removed and the cells were washed once with probe-free media for 10 min, rinsed with PBS and fixed with 10% buffered formalin for 5 min. Coverslips were mounted with 10% PBS in glycerol prior to viewing with a standard rhodamine filter.

Immunocytochemistry

Cells were examined for the presence of surface IgM, a marker for B cells (DeLuca et al, 1983), with the monoclonal antibody 1.14 (a kind gift of Dr. N. Miller). 10^6 cells were incubated for 30 minutes with the primary antibody, followed by incubation with the secondary antibody anti-mouse conjugated to FITC (Sigma). As a negative control, the primary antibody was omitted. Following staining, the cells were examined using a Coulter flow cytometer. As well as examining the fluorescence due to IgM positive staining, the flow cytometer was used to determine forward scatter (a measure of cell size) and side scatter (a measure of internal granularity).

D: EXAMINATION OF FACTORS AFFECTING RTS11 PROLIFERATION

Three types of assays were used to identify factors that affect the growth and differentiation of RTS11 cells. The first was an assay of cell number, by directly

counting cells in response to various treatments, as described above. The second type of assay involved treating the cells with a test substance, and scoring the effect on cell appearance and survival microscopically over time. The third was an assay of the ability of various treatments to stimulate the incorporation of thymidine into newly synthesized DNA. To demonstrate the utility of this assay in predicting cell growth, the response of RTS11 to the addition of 0, 10, 20, or 30% FBS was monitored by both assaying increase of cell number over time by directly counting cells, and by measuring ^{3}H-thymidine incorporation, and the results of the two assays were compared.

Growth of RTS11 in response to cell-line conditioned medium and to phytohemagglutinin conditioned medium

Preparation of cell-line conditioned medium

Conditioned medium was prepared from cell lines by plating in the usual growth medium and allowing the cells to attach and grow to confluence. The medium was then replaced with fresh growth medium and incubated for two weeks. The medium was then collected, and centrifuged in a table-top centrifuge at 3000 rpm to remove floating cells and debris. The supernatant was filtered through a 0.2 μm filter to ensure its sterility. Conditioned medium was used in concentrations ranging from 2.5-30%, as described for individual experiments. In all cases where conditioned medium was added as a supplement, non-conditioned medium, i.e., the normal growth medium for the cells that conditioned medium was collected from, was used as a control to ensure that any observed effects were not due to either the basal medium or residual FBS.

Conditioned medium from several cell lines of mammalian origin (ATCC, Rockville, MD) known to produce cytokines that stimulate hemopoiesis was prepared. These included 5637 cells (human bladder carcinoma; source of GM-SCF, G-CSF and IL-1) (Fogh et al, 1978), L929-cells (mouse fibroblasts; source of M-SCF) (Stanley & Heard, 1977), and WEHI-3 cells (mouse myelomonocytic leukemia cells; source of IL-3) (Lee et al, 1982).

Two cell lines and a primary culture of rainbow trout origin were also used to produce conditioned medium with potential modulating effects on hemopoiesis. These were RTS11 (as a source of putative autocrine factor), and clone 1A cells (peripheral

blood cell line; Bols, unpublished). In addition, conditioned medium was prepared from actively growing hemopoietic spleen cultures (see chapter 2).

Preparation of phytohemagglutinin leukocyte conditioned medium (PHA-LCM)

PHA-LCM was prepared from rainbow trout using the following procedure. Leukocytes were obtained from peripheral blood by centrifugation on Histopaque 1.077. The cells were washed twice in L-15 with 2% FBS by centrifuging at 400 g for 10 min, then resuspended at 10^6 cells/mL in L-15 with 5% dialyzed FBS and PHA at a final concentration of 20 µg/mL. 40 mL of the suspension was incubated in a 75 cm^2 tissue culture flask for 7 days. At the end of the incubation, the medium was centrifuged and the supernatant filter sterilized as described for conditioned medium. Aliquots were stored at -20°C and thawed immediately prior to use.

Growth assay - measurement of cell number

The ability of PHA-LCM and of conditioned medium from clone 1A cells and primary cultures of spleen cells (described in chapter 2) to promote proliferation of RTS11 was monitored using the cell number assay. Duplicate 12.5 cm^2 culture flasks were inoculated with 70,000 cells in 5 mL of L-15 with 30% FBS in the presence and absence of 20% v/v of each treatment. After 60 days of growth, non-adherent and adherent cells in each flask were removed and counted using a hemacytometer.

Effect of conditioned medium from various cell sources and to substances known to induce the differentiation of macrophages on the morphology and survival of RTS11 cells

RTS11 cells were collected by centrifugation and resuspended in either L-15 with no serum, or with 30% FBS. 50,000 cells were added to each well of a 48 well plate, and 20% conditioned medium from one of the following cell lines was added to triplicate wells; RTS11, clone 1A, 5637, and L929.

Other substances that are known to induce the differentiation of macrophages in other systems were obtained from Sigma (unless indicated otherwise) and tested at common concentration used to induce mammalian cell differentiation; 5 µg/mL calcium ionophore A23187 (Calbiochem), 10 nM phorbol ester PMA, PMA+A23187, 10 and 100 nM retinoic acid, 1.25% DMSO, 50 nM 1- α-25-dihydroxyvitamin D3 (Calbiochem), and 10 mM dibutyryl cAMP. The plates were incubated at 18°C and microscopic

observations were made weekly over a 2 month period to monitor changes in cell morphology and density.

Proliferation assay - ^{3}H-thymidine incorporation

The measurement of DNA synthesis was used as a quick measurement of the ability of a substance to influence cell growth. Assays were carried out in 96 well plates (Falcon), in a total volume of 200 µl per well. Because centrifuging these cells compromised their viability, RTS11 cells were counted and 50,000 cells were dispensed into each well of a 96 well plate with a very small volume of their spent medium. Treatments without FBS therefore may actually include a small amount of spent FBS from the medium transferred with the cells. Treatments were added, and the plates were incubated at 18°C for the period of time indicated in the figure legends. Twenty four hours prior to termination of the experiment, 1.0 µCi ^{3}H-thymidine (ICN) per well was added. The cells were then harvested onto glass fiber filters using a Skatron cell harvester (Skatron, Sterling, VA), and radioactive emmissions were counted in a Beckman liquid scintillation counter.

Chemicals tested were obtained from Sigma (unless indicated otherwise) and included lipopolysaccharide (LPS), phorbol -12-myristate 13-acetate (PMA), and the lectins concanavalin A (Con A) , pokeweed mitogen (PWM) and phytohemmaglutinin (PHA-P). Sera tested include FBS (Canadian Life Technologies), dialyzed FBS (dFBS), and rainbow trout serum, prepared as previously described (Ganassin & Bols, 1997). As the purine adenosine has been shown to have both stimulatory (Orrico et al, 1991) and inhibitory (Tanaka et al, 1994) effects on hemopoietic precursors, adenosine was also tested for its ability to modulate thymidine incorporation in RTS11, and in freshly isolated head kidney leukocytes.

All ^{3}H-thymidine incorporation experiments presented graphically were performed at least three times. For clarity, the results of a single representative experiment is shown in each case. For statistical analysis of individual experiments with different concentrations of a test compound, a single factor analysis of variance (Zar, 1974), was used to determine whether the compound had an effect. Two-tailed hypotheses (null hypothesis: treatment had no effect) were tested. If a difference was detected, Dunnett's test (Zar, 1974) was used to compare mean incorporation by control cultures

in order to determine the concentrations that had an effect. Two-tailed hypotheses (null hypothesis: treatment was equal to the control) were tested. The possibility of a type 1 error was set at 0.05 for all tests.

E: ASSAYS OF THE FUNCTIONAL ABILITIES OF RTS11 CELLS

RTS11 conditioned medium effect on rainbow trout leukocyte proliferation

The activity of RTS11 conditioned medium (CM) on leukocytes isolated from the head kidney of other rainbow trout was also tested. Head kidney tissue was aseptically removed from a rainbow trout, and placed in 10 mL L-15 supplemented with 10 IU/mL heparin (Sigma). The tissue was then forced through a 100 mesh/ inch metal screen using a pestle to dissociate the cells. The cell suspension was diluted by the addition of 20 mL of L-15 with heparin. Five mL of the resulting cell suspension was placed in a 10 mL centrifuge tube, and a syringe was used to underlay the cell suspension with 3 mL of Histopaque 1.077 (Sigma). The tubes were centrifuged for 30 minutes at 1300 rpm in an IEC centrifuge with a swinging bucket rotor, and the band of cells formed at the interface of the Histopaque and L-15 was collected. Cells were washed by centrifugation in L-15 without heparin prior to their use, and resuspended in L-15 without serum at a cell density of 10^6 cells/mL. The isolated head kidney leukocytes were then used as test cells in ^{3}H-thymidine incorporation experiments, performed as described for RTS11.

Conditioned medium from RTS11 was collected in the presence of 30% FBS, and filter sterilized before use. Leukocytes were also exposed to non-conditioned medium as a control.

Phagocytosis assays

Phagocytic ability was assessed in two ways; firstly, by incubation with latex beads, and secondly by incubation with yeast cells. The ability of RTS11 to phagocytose latex beads was assessed as previously described (Ganassin & Bols, 1996; see chapter 2). In addition, the ability of RTS11 to phagocytose yeast cells was tested by incubating the cells with Congo Red stained yeast cells, as described by Seeley et al (1990). Briefly, Congo Red stained yeast cells were prepared by adding 3 mL of Congo Red solution, 0.87% in PBS, to 1.5 g of yeast cells (*Saccharomyces cerevisceae*, Fleischmann). After 15 minutes at room temperature, 7 mL of distilled water was added, and the

mixture was autoclaved for 15 minutes to kill and fix the yeast. The cells were washed several times in HBSS to remove excess stain, and stored at 4°C until used in the assay. To test the ability of adherent RTS-11 cells to ingest yeast particles, the cells were allowed to attach to the slide surface of a Falcon multi-well chamber slide (VWR), and incubated with the yeast cell suspension for 60 minutes at room temperature at a yeast cell: test cell ratio of 40:1.

Respiratory burst assay

Neutrophils and macrophages of rainbow trout undergo a respiratory burst in response to stimulation by PMA. The ability of RTS11 to undergo this response was tested using the non-fluorescent dye 2',7'-dichlorofluorescin diacetate (DC-FHDA) (Molecular Probes, Eugene, OR). This dye forms the fluorescent product 2',7'-dichlorofluorescein when it is oxidized by cellular peroxidases and H_2O_2 generated during the respiratory burst (Bass et al, 1983). RTS11 cells were collected by centrifugation, resuspended in PBS with 500 mg/mL glucose and 10^5 cells/well were added to the wells of a 96 well plate. Freshly isolated rainbow trout head kidney leukocytes were dispensed to another set of wells for use as a positive control. DC-FHDA (50 μM in DMSO) was added to each well and allowed to equilibrate for 15 minutes prior to the addition of either DMSO or PMA to triplicate wells. Resulting fluorescence was measured using the CytoFluor Fluorescence Measurement System (Millipore), using the B/B filter set (λ=485/530) and a sensitivity of 3.

3. RESULTS

A: ESTABLISHMENT OF CELL LINE

Attempts to induce the non-adherent fraction of RTS11 cultures to proliferate in the absence of stromal cells were successful 3 years after the initiation of the original culture, when the cells spontaneously began to proliferate on their own. This proliferation only occurred when cultured using the conditions outlined below. To date, the non-adherent cell line has been maintained for over 8 months.

B: OPTIMAL CULTURE CONDITIONS AND PROCEDURES FOR THE RTS11 CELL LINE

Growth of RTS11 was greatly inhibited by culture in petri dishes or multiwell plates. In petri dishes, cells remained alive but became larger, with an increased cytoplasm:nucleus ratio (results not shown). Culture in 75cm^2 flasks also slowed proliferation drastically, even when the ratio of cell number to volume of medium was the same as that used in smaller flasks. The preferred culture vessels for RTS11 growth are either 12.5 cm^2 or 25 cm^2 tissue culture treated flasks, or 25 cm^2 non-treated flasks. 25 cm^2 non-treated flasks, containing a total medium volume of 10 mL, were adopted for routine culture.

The following procedure was adopted for routine maintenance of RTS11 cells. Saturation density was signaled by a change in colour of the L-15 medium from the original orange-red to yellow-orange, generally seen three to four weeks after seeding the culture flask. At that time, a cell scraper was used to remove any adherent and semi-adherent cells from the flask surface, and cells were resuspended evenly in the growth medium by pipetting up and down several times. Centrifugation was avoided, as it impaired cell survival. One half of the resulting cell suspension (5 mL) was transferred to a new culture flask, and 5 mL of fresh GM was added. This resulted in a cell density conducive to continuous proliferation as well as the transfer of enough conditioned medium to stimulate growth.

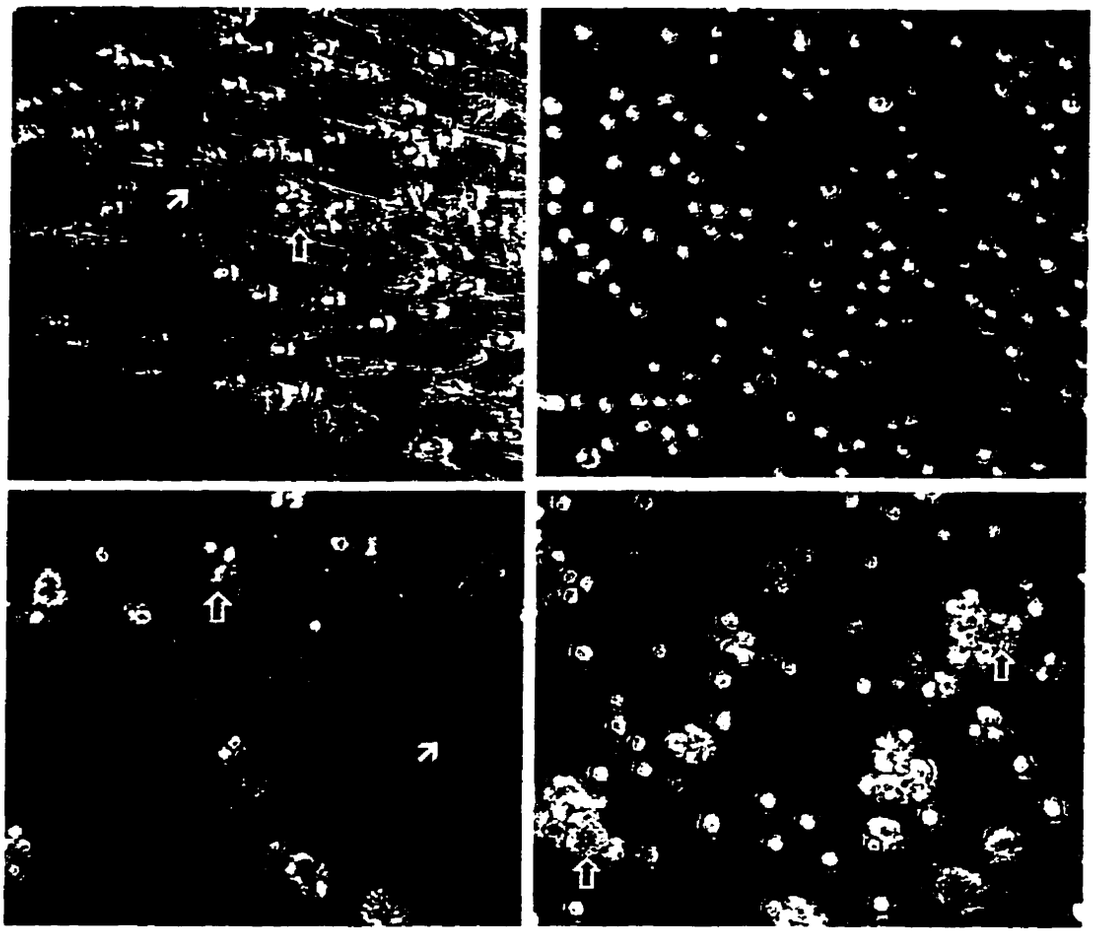

Figure 4.1: Appearance of RTS11 cells. In the original culture, **a**, (mag. 100x) both stromal elements and progeny cells are present. Progeny cells include flattened macrophage-like cells (↗) and round cells (⇧). After isolation of the RTS11 non-adherent cell line, **b**, the dominant cell type present is a small round, cell. A small percentage of cells in each culture have the typical morphology of macrophages. In **c**, the adherent form is shown (↗), with associated round cells (⇧). In **d**, the non-adherent form of macrophage-like cell (⇧) is usually found at the center of a cluster of round cells (mag. 200x for b, c, d).

To date, RTS11 cells have shown poor survival following cryopreservation. They do, however, recover and proliferate after storage at 5°C for periods of several months, and this is used as an alternate long-term storage method. Efforts to cryopreserve them successfully are ongoing.

C: CHARACTERISTICS OF RTS11 CELLS

Morphology and cytochemistry

Cells exhibiting three different morphologies were observed in RTS11 cultures. When examined by phase contrast microscopy, the dominant (>90%) cell type observed

was of uniform size, solitary, round, smooth-surfaced, phase-bright and non-adherent (fig. 4.1b). These will be referred to as "round cells". When the density was very high, many non-adherent cells settled to the bottom of the culture flask, but were not attached. Non-adherent clusters consisting of a central large, granular cell surrounded by round cells were also observed, but were present only in small numbers (fig. 4.1c). Well spread, adherent cells with a macrophage-like morphology were seen, sometimes with clusters of associated round cells (fig. 4.1d). If the cells were grown for long periods of time without a change of medium (8+ weeks), the percentage of these macrophage-like cells increased (fig. 4.1d). The central cells in clusters and those adherent to the culture surface both had characteristics of macrophages, and will be referred to collectively as "macrophage-like" cells.

Wright-Giemsa staining of cytospin preparations of RTS11 showed that the dominant cell type was round with smooth borders, and had a kidney shaped, eccentrically placed nucleus (fig 4.2a). The cytoplasm was scant, and devoid of obvious granules or vacuoles. The macrophage-like cells had an abundant cytoplasm, with many granules and vacuoles, and an oval nucleus (fig. 4.2b).

Table 4.1: Cytochemical staining characteristics of RTS11 cells

Stain	Description of results	
	Round cells	**Macrophage-like cells (adherent & non-adherent)**
myeloperoxidase	negative	negative
acid phosphatase	uniformly positive (turquoise) in a spot located next to the nucleus	positive
non-specific esterase	negative	mildly positive with small brown black cytoplasmic granulation
Sudan Black B	negative	negative
Acridine Orange	negative (nuclei are green, but no orange vacuoles are observed)	positive orange vacuoles
Hoescht 33258	• all observed fluorescence was associated with cell nuclei • the cell nuclei are eccentric, take up most of the cell, and have broad lobes, forming a cleft	• all observed fluorescence was associated with cell nuclei • the cell nuclei are eccentric, small and round-oval

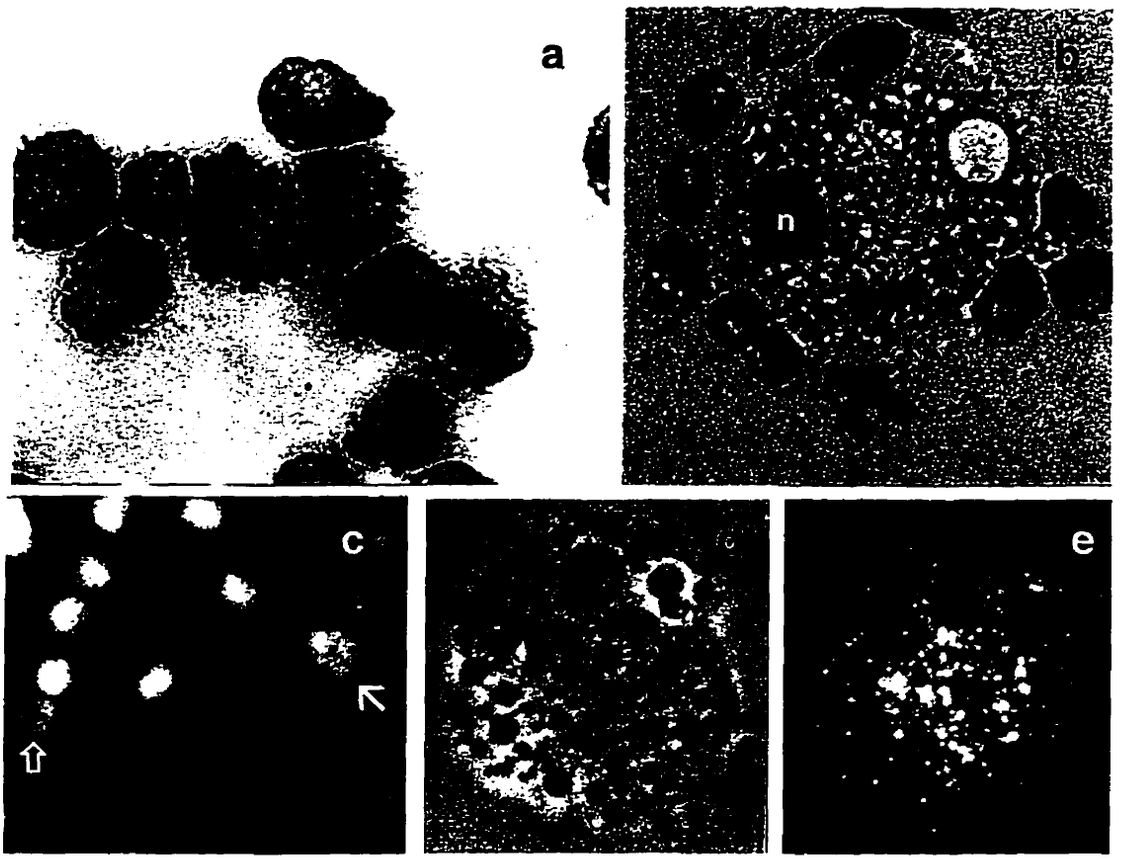

Figure 4.2: Staining characteristics of RTS11 (magnification 400x). Wright-Giemsa staining, **a & b**, of RTS11 round cells, **a**, shows the kidney shaped nuclei (n) and granular cytoplasm and a macrophage-like cell, **b**, with a small round nucleus and abundant vacuolar cytoplasm. The macrophage-like cell is from a non-adherent cluster and is surrounded by round cells. The morphology of RTS11 round cell nuclei (⇧) is clearly evident with Hoescht 33258 stain, **c**. Note the mitotic figure (↖). Adherent macrophage-like cells take up DiI-Ac-LDL, as shown in **d**, (phase contrast view), and **e**, (fluorescent view of same cell) .

DiI-acetylated low-density lipoprotein uptake assay

RTS11 round cells did not take up DiI-Ac-LdL. However, macrophage-like cells, both adherent and non-adherent, accumulated DiI-Ac-LdL in distinct vacuoles (fig. 4.2 d,e).

Flow cytometry

Flow cytometric analysis indicated that RTS11 cells did not express surface IgM. It also indicated that individual RTS11 cells were larger and contained more internal granules than lymphocytes (data not shown).

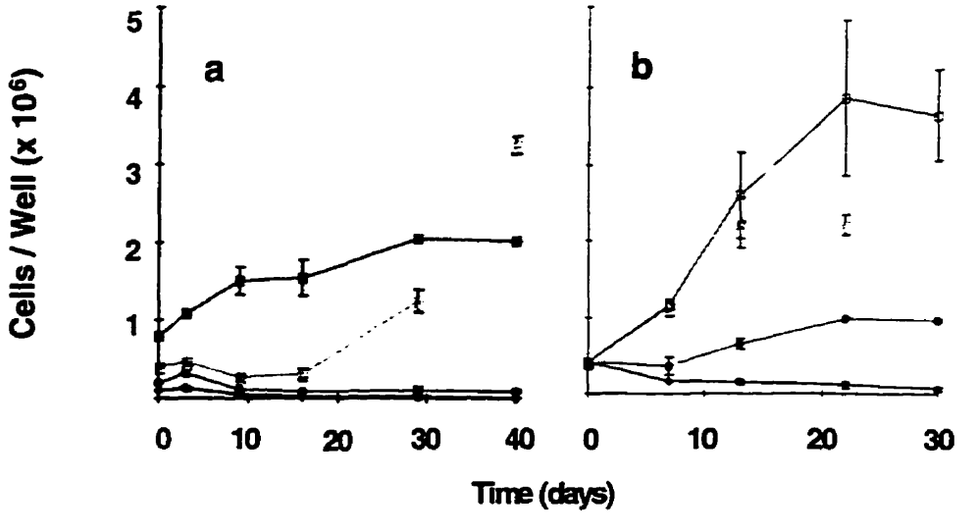

Figure 4. 3: Effect of seeding density, a , and temperature, b , on the proliferation of RTS11. In **a,** 25cm^2 culture flasks were inoculated with RTS11 cells at an initial density of 1 x 10^5 cells/mL (-◆-), 2x 10^5 cells/mL (-●-), 4 x 10^5 cells/mL (-■-), or 8 x 10^5 cells/mL (-□-). In **b** , 12.5 cm^2 flasks were inoculated with 4 x 10^5 RTS11 cells in L-15 with 30% FBS, and incubated at 5°C (-◆-), 12°C(-●-), 18°C(-■-), or 21°(-□-). All temperatures are ± 1°C. On the indicated days after culture initiation, an aliquot of cells was removed from each flask, incubated with Trypan Blue for 5 minutes and live cells counted in a hemacytometer. Values are the average of duplicate cultures, with error bars representing standard deviation.

Cytochemistry

The macrophage-like cells were positive for the enzyme non-specific esterase. They were also acid phosphatase positive, and had vacuoles that accumulated acridine orange stain (see table 4.1). The nucleus of these cells was generally round and small. No cells exhibited a positive staining reaction with Sudan Black B.

Round cells, in contrast, had a slightly clefted nucleus and a much smaller amount of cytoplasm. There were no apparent vacuoles that stained with acridine orange, and the only enzyme stain that showed reactivity was acid phosphatase, which was localized to a prominent spot near the nuclear cleft.

Mycoplasma and sterility testing

Hoechst 33258 staining was performed periodically to detect contamination by mycoplasma. The observed absence of fluorescence outside of the cell nuclei when stained indicated that the cells were not contaminated by mycoplasma.

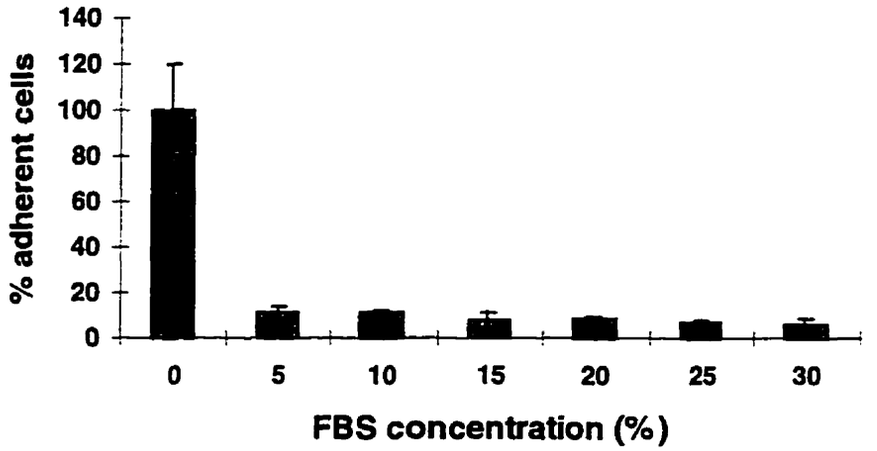

Figure 4.4: RTS11 adhesion to tissue culture plastic in the presence of various concentrations of FBS. RTS11 cells were preincubated with the indicated concentrations of FBS prior to the addition of 50,000 to each well of a 48 well plate. After incubation for 1.5 h, **a**, or 24 h, **b**, the wells were rinsed well with PBS, and 4μM CFDA-AM was added to each well for 45 m. Fluorescence was measured using the CytoFluor, with settings of B/B, sensitivity 5. All cells were adherent with no FBS, which was considered 100% adhesion. All percentage adhesion values were calculated by dividing the fluorescent units observed with the treatment by the fluorescent units observed when 100% of the cells adhered, and multiplying by 100.

Optimal cell density

Cells proliferated well when transferred at a relatively high cell density (fig. 4.3a). A cell inoculum of 8×10^5 cells/mL resulted in a doubling of the cell population in approximately 9 days, reaching a maximum density of 2×10^6 cells/mL after 29 days. With an inoculum of 4×10^5 cells/mL, growth was considerably slower. The population doubled in approximately 24 days, but then continued to grow, reaching a density of over 3×10^6 cells/mL after 40 days. With a smaller cell inoculum, there was little or no cell growth.

Optimal growth temperature

The effect of temperature of incubation on growth of RTS11 is shown in fig. 4.3b. Cells maintained at 5°C declined in number, while those maintained at 12°C increased slowly. While fastest growth occurred at room temperature (21°C ±1 °C), cells were routinely maintained at 18°C in a temperature-controlled incubator to avoid temperature fluctuations. Cells that were held at 5°C became adherent, but, when transferred back to an 18°C incubator, resumed growth and their non-adherent growth pattern after a lag period of several weeks.

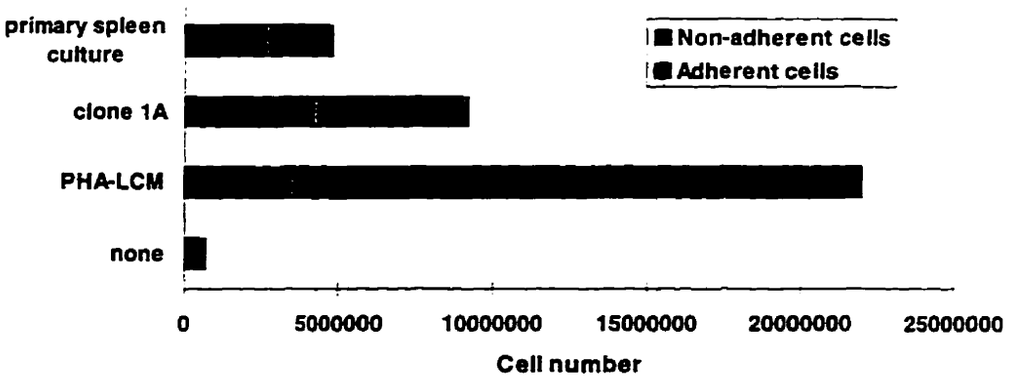

Figure 4.5: RTS11 response to rainbow trout crude extracts in a cell number assay. 70,000 RTS11 cells were incubated in 12.5 cm^2 culture flasks with 20% (v/v) of the indicated extracts in L-15 with 30% FBS. After 60 days, the non-adherent cells were removed and counted, and the adherent cells were removed with trypsin and counted.

Adhesion

FBS had a profound effect on the adhesion of RTS11 to the tissue culture surface (fig. 4.4). Without FBS, all cells were adherent. When 5-30% FBS was added to the culture medium, most cells did not adhere to the tissue culture surface.

D: FACTORS EFFECTING RTS11 PROLIFERATION

Growth assay - measurement of cell number

Treatment of RTS11 with various substances of rainbow trout origin greatly increased cell number over a 60 day period (fig. 4.5). Some treatments stimulated growth of the non-adherent, cell type, while others appeared to encourage adhesion and morphological changes, resulting in the adherent, macrophage-like cell type. PHA-LCM dramatically increased the number of both adherent and non-adherent RTS-11 cells present after 60 days of growth, but approximately 6 times more non-adherent cells than adherent cells were produced. Conditioned medium from primary cultures, and from the cell line clone 1A increased growth to a smaller degree. Clone 1A conditioned medium appeared to favour the proliferation of the adherent cell population, resulting in a confluent monolayer of adherent cells at the bottom of the flask at the termination of the experiment, with an approximately equal number of non-adherent cells present. Conditioned medium from spleen hemopoietic cultures also stimulated the production of

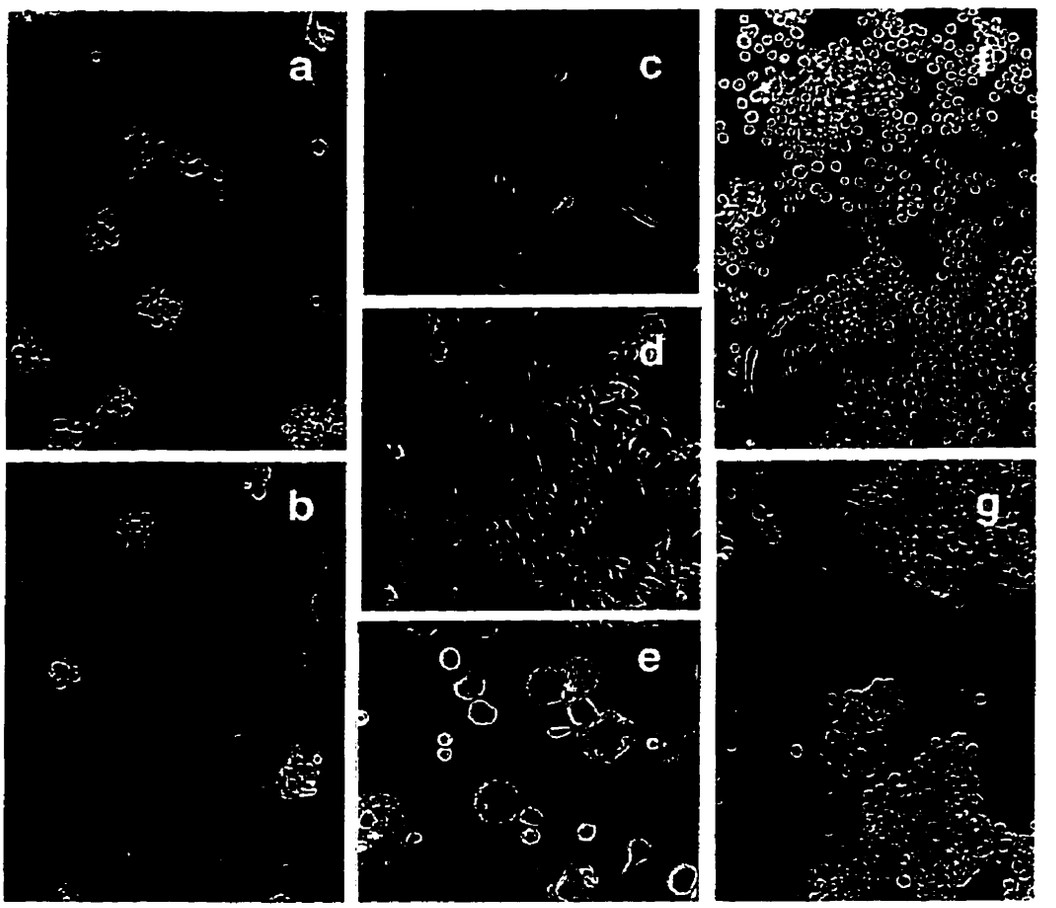

Figure 4.6: Morphology and density changes of RTS11 in response to PMA and to RTS11 conditioned medium in the absence of (a, c, f, &d) and in the presence of (b, e, g) 30% FBS. Cells are shown with no addition, **a**, and with the addition of only 30% FBS, **b**. Within 10 minutes of addition, PMA (10 nM) immediately promoted cell adhesion and spreading both without, **c**, and with, **e**, FBS. In the absence of FBS, culture for several weeks after exposure to PMA, without a change of medium, resulted in the formation of colonies of tightly packed, adherent cells, **d**. RTS11 CM, both without, **f**, and with, **g**, FBS, dramatically increased the survival and proliferation of RTS11, while retaining their usual morphology.

adherent and non-adherent cells to the same degree, but the overall cell numbers were not as great as seen with clone 1A conditioned medium.

Response to conditioned medium from various cell sources and to substances known to induce the differentiation of macrophages

Morphological and density changes were observed when RTS11 cells were incubated with various test substances (fig. 4.6). Only those inducing obvious changes are shown. Without serum (4.6a), RTS11 cells formed small clusters, with an overall decrease in cell number from the time of plating. With 30% FBS (4.6b), cell number was approximately the same, and clusters also formed, although individual cells appeared larger and more

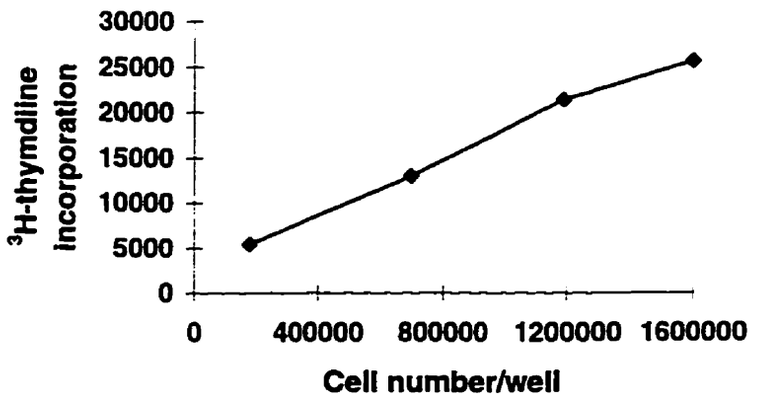

Figure 4.7: Correlation between ^{3}H-thymidine incorporation and growth of RTS11 in response to fetal bovine serum. For cell counting, 4 x 10^5 cells/mL were added to 12.5 cm^2 flasks in 10 mL of L-15 with 0, 10, 20 or 30% FBS. Flasks were incubated at 18°C for 14 days, at which time an aliquot of cells was counted using a hemacytometer. For thymidine incorporation, 50,000 cells were added to each well of a 96 well plate, and the indicated concentrations of FBS were added. Plates were incubated at 18°C for 4 days, at which time they received 1 μCi of ^{3}H-thymidine per well, and were incubated for an additional 24 hours prior to harvest with the Skatron cell harvester.

granular than without FBS. This lack of an increase in cell number with 30% FBS when the cells are cultured in multiwell plates contrasts with the situation when the cells are cultured in flasks (see fig 4.7). The phorbol ester PMA immediately caused cell attachment, both in the absence (4.6c) and presence (4.6e) of FBS. After prolonged culture (4+ weeks), colonies of small, tightly packed, adherent cells were observed in cultures with PMA and no FBS (4.6d). Conditioned medium from RTS11 cells, both in the absence (4.6f) and presence (4.6g) of FBS dramatically increased the number of RTS11 cells present. With FBS, they tended to clump together, while they remained well separated without FBS. The calcium ionophore A23187 caused immediate death of RTS11 cells both by itself and in combination with PMA, with or without FBS. The mammalian conditioned medium 5637 and L929, and the macrophage inducers retinoic acid, DMSO, dibutyryl cyclic AMP and 1- α-25-dihydroxyvitamin D3 did not enhance cell survival, growth or morphological change.

Response to fetal bovine serum

The response of RTS11 to fetal bovine serum was monitored using two different assays. In the cell number assay, the growth of RTS11 over time was best in L-15 supplemented with 30% FBS. Growth did not occur in the absence of FBS. The ^{3}H-thymidine incorporation assay demonstrated the same response, with 30% FBS promoting the

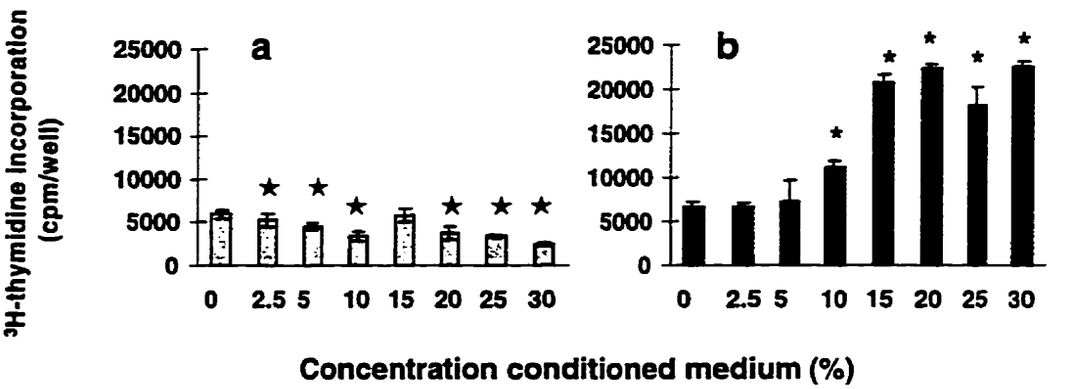

Figure 4.8: Response of RTS11 to its own conditioned medium. RTS11 cells were centrifuged and resuspended in L-15 without serum. 50,000 cells were added to each well of a 96 well plate, and various concentrations of RTS11 conditioned medium were added. In **a**, no FBS is present, while in **b**, 30% FBS was added. Plates were incubated at 18°C for 4 days, at which time they received 1 μCi of ^{3}H-thymidine per well, and were incubated for an additional 24 hours prior to harvest with the Skatron cell harvester. The means and standard deviations of triplicate cultures are shown. Values significantly ($p \leq 0.05$) lower than the control are indicated by a ★, while values significantly higher are indicated by an *.

highest level of DNA synthesis. The correlation between the two methods of measuring cell growth is shown in fig 4.7 (R^2= 0.9936). Subsequently, ^{3}H-thymidine incorporation assays, which can be performed much more quickly, were used to screen substances for their growth-promoting effects on RTS11 cells.

^{3}H-thymidine incorporation assays

Response to RTS11 conditioned medium

In the absence of FBS, conditioned medium from RTS11 did not stimulate proliferation (fig. 4.8a). However, in the presence of the usual supplement of 30% FBS, the addition of self-conditioned medium greatly enhanced thymidine incorporation by RTS11 (fig. 4.8b).

Response of RTS11 to conditioned medium from mammalian sources

The effect of conditioned medium from mammalian sources was dramatically different than that of CM from rainbow trout sources. In contrast to the stimulation by RTS11 conditioned medium, mammalian conditioned media did not stimulate thymidine

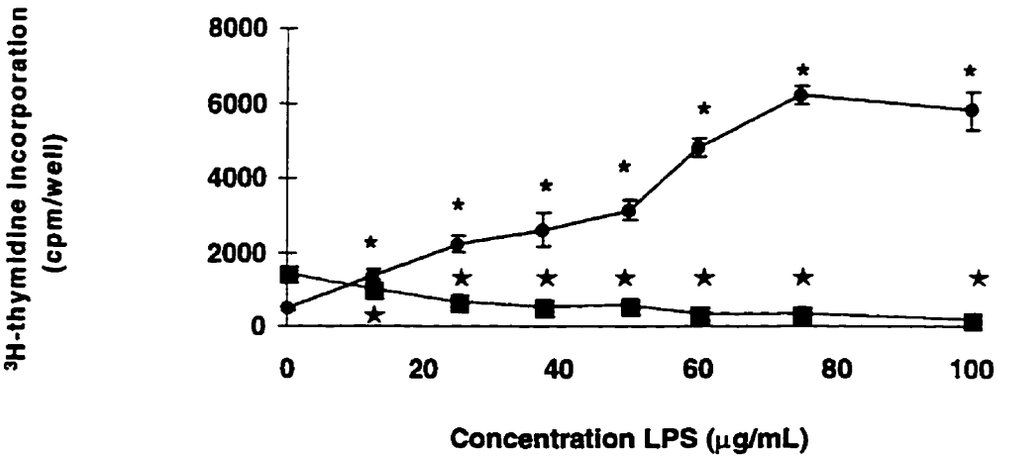

Figure 4.9: ^{3}H-thymidine incorporation by RTS11 in response to lipopolysaccharide with and without 30% FBS. 50,000 RTS11 cells were distributed to the wells of a 96 well plate, and the indicated concentrations of lipopolysaccharide were added in the absence (-I-) and presence (-n-) of 30% FBS. Plates were incubated at 18°C for 4 days, at which time they received 1 μCi of ^{3}H-thymidine per well, and were incubated for an additional 24 hours prior to harvest with the Skatron cell harvester. The means and standard deviations of triplicate cultures are shown. Values significantly ($p \leq 0.05$) lower than the control are indicated by a ★, while values significantly higher are indicated by an *.

incorporation by RTS11, either in the presence or absence of FBS (not shown). L929, 5637, and WEHI-3 conditioned medium inhibited thymidine incorporation by RTS11, while non-conditioned media of the same types were stimulatory. This confirms that the inhibition was in some way due to the mammalian cells and not their culture medium.

Response of RTS11 to leukocyte mitogens

Lipopolysaccharide:

In the absence of FBS (fig. 4.9), LPS increased thymidine incorporation by RTS11 in a dose dependent manner. Concentrations as low as 12.5 μg/mL were stimulatory, and this stimulation continued to a concentration of 75-80 μg/mL, then reached a plateau. In the presence of 30% FBS LPS inhibited the incorporation of ^{3}H-thymidine.

Lectins:

The effect of different concentrations of Con A on thymidine incorporation by RTS11 is shown in fig. 4.10 a & b. When there was no FBS included in the culture medium, thymidine incorporation by RTS11 was not stimulated by Con A. However, when the usual serum supplement of 30% FBS was included in the culture medium, low concentrations of Con A (1 and 5 μg/mL) significantly enhanced thymidine incorporation. The incorporation returned to basal levels at a Con A concentration of

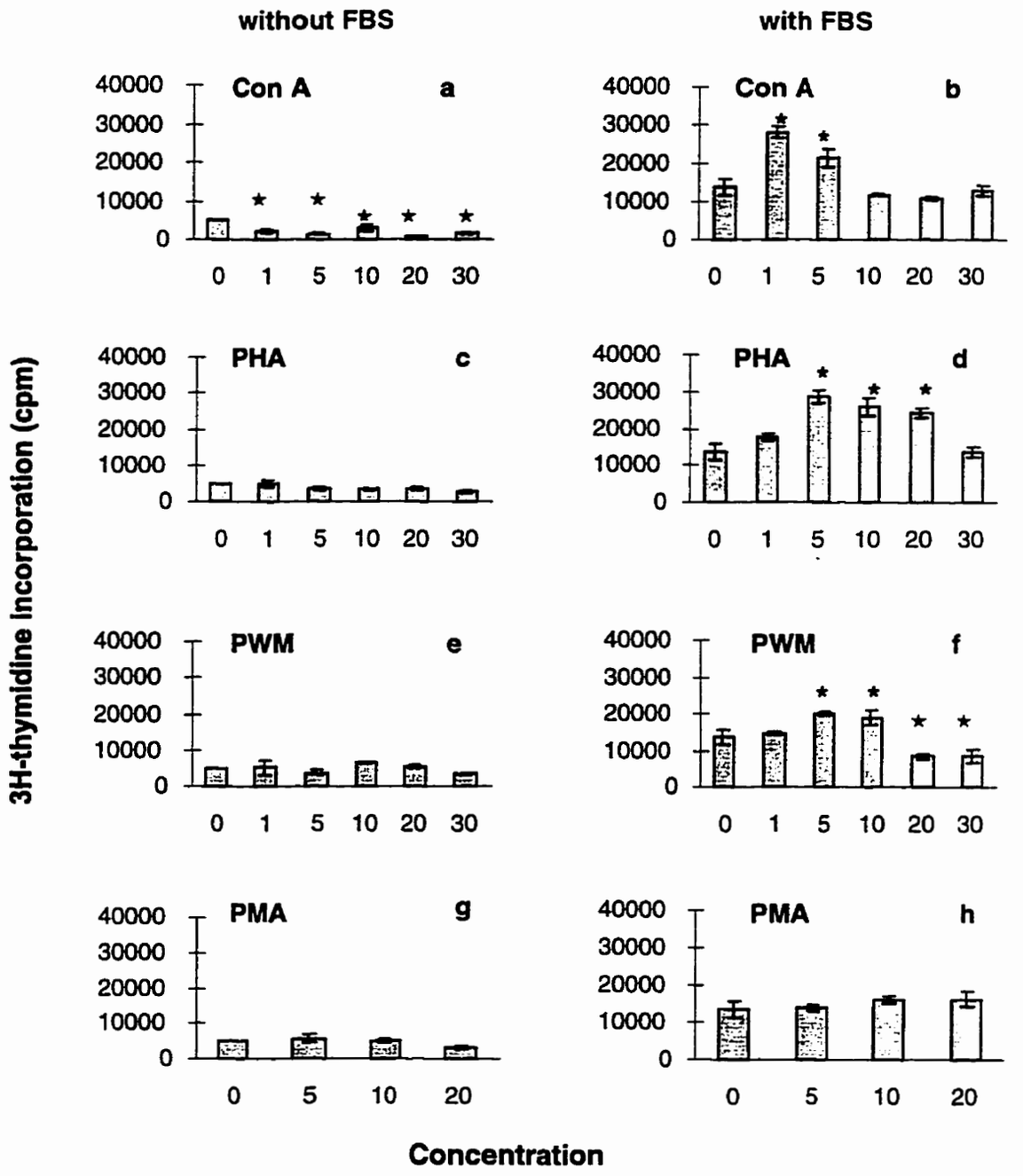

Figure 4.10: RTS11 ^{3}H-thymidine incorporation in response to lectins and the phorbol ester PMA in medium without serum (a, c, e, g) and with 30% FBS (b, d, f, h). Concentration of Con A , **a, b**, PHA , **c, d**, and PWM, **e, f**, are shown in µg/mL, while PMA , **g, h**, is in ng/mL. 50,000 cells were added to each well of a 96 well plate, mitogens were added, and incubated for 5 days at 18°C. 1 µCi per well of ^{3}H-thymidine was added for the last 24 hours of incubation. Values shown are the average of triplicate treatments, with error bars representing the standard deviation. Values significantly greater ($p \leq 0.05$) than the control are indicated by an *, while values significantly less ($p \leq 0.05$) are shown by a ⋆.

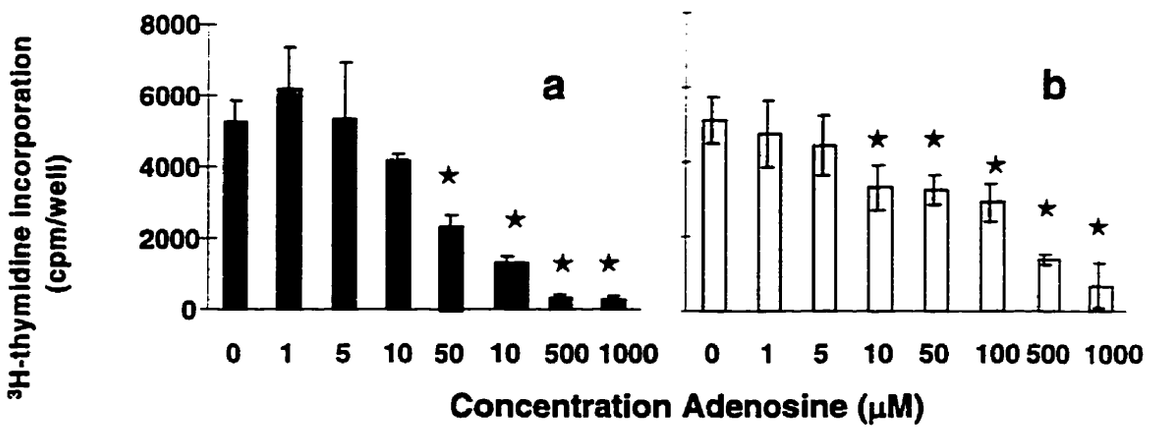

Figure 4.11: The effect of adenosine on ^{3}H-thymidine incorporation by RTS11. RTS11 cells were centrifuged and resuspended in L-15 without serum. 50,000 cells were added to each well of a 96 well plate, and the indicated concentrations adenosine were added, without, **a**, or with, **b**, 30% FBS. Plates were incubated at 18°C for 4 days, at which time they received 1 μCi of ^{3}H-thymidine per well, and were incubated for an additional 24 hours prior to harvest with the Skatron cell harvester. The means and standard deviations of triplicate cultures are shown. Values significantly ($p \leq 0.05$) less than the control are indicated by an ★.

10 μg/mL. The effect of PHA on thymidine incorporation by RTS11 is shown in fig. 4.10 c & d. In the absence of FBS, the response to PHA was similar to that seen with Con A. However, with the addition of 30% FBS to the culture medium, RTS11 were stimulated by PHA. This stimulation peaked at a concentration of 5-10 μg/mL, then declined and reached the level seen without PHA at a concentration of 30 μg/mL. PWM, shown in fig. 4.10 e & f, did not affect thymidine incorporation in the absence of serum, but in the presence of serum was stimulatory at intermediate concentrations (5-10 μg/mL), yet inhibitory at higher concentrations (20-30 μg/mL).

Phorbol ester.

PMA (fig. 4.10 g & h), a substance that is mitogenic by direct stimulation of protein kinase C, did not appear to enhance thymidine incorporation by RTS11 at any of the concentrations tested, either in the presence or absence of serum.

Adenosine.

Adenosine, both without FBS (4.11a) and in the presence of 30% FBS (4.11b), inhibited thymidine incorporation in a dose dependent manner. A noticeable inhibitory effect was present with10μM adenosine, and this inhibition increased with increasing dose. At 500

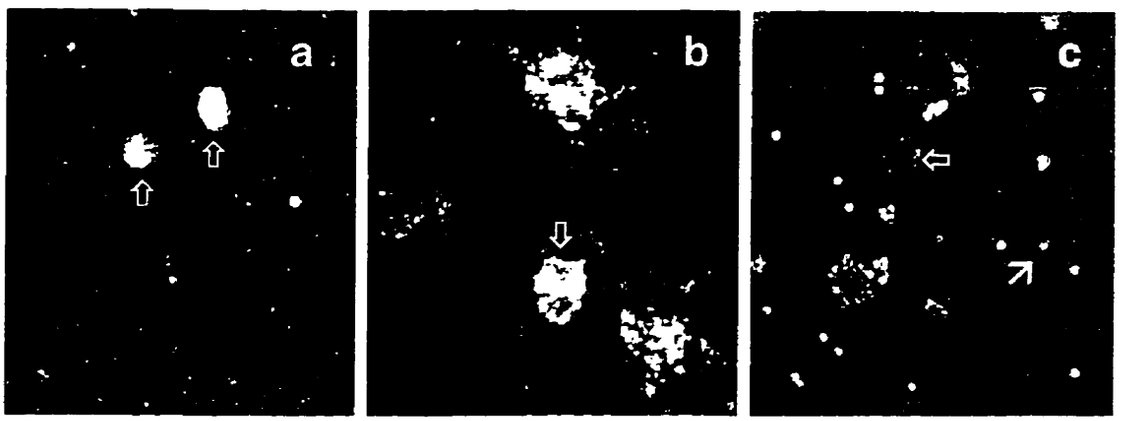

Figure 4.12: Phagocytosis by RTS11 cells. In **a**, the focal plane is on the non-adherent cells, which have not taken up latex beads, with the exception of the central macrophage-like cells in floating clusters (⇧). In **b**, the adherent macrophage-like cells (⇩) have take up large numbers of latex beads. In **c**, the adherent cells (⇦) have become engorged with yeast particles and have rounded up from the tissue culture surface. Most cells detached and became free floating in the medium. (↗) indicate free yeast particles. Phase contrast, original mag. 200x.

μM adenosine without FBS, or 1 mM in the presence of 30% FBS, thymidine incorporation by RTS11 was almost obliterated. With head kidney leukocytes, the same concentrations of adenosine neither inhibited nor enhanced thymidine incorporation (not shown).

E: RTS11 FUNCTIONS

Phagocytosis assays

RTS11 non-adherent round cells did not phagocytose either latex beads or yeast cells. The non-adherent macrophage-like cells ingested latex beads while the associated round cells did not (fig. 4.12 a). Adherent macrophage-like cells ingested so many beads that they rounded up and detached from the culture surface (fig. 4.12 b). Most of this type of cell were also able to phagocytose several yeast cells (fig. 4.12 c), which also resulted in them rounding up and detaching from the culture surface. Any RTS11 round cells that were associated with adherent cells did not phagocytize either latex or yeast particles.

Respiratory burst assay

RTS11 cells did not undergo a respiratory burst in response to stimulation with PMA (data not shown).

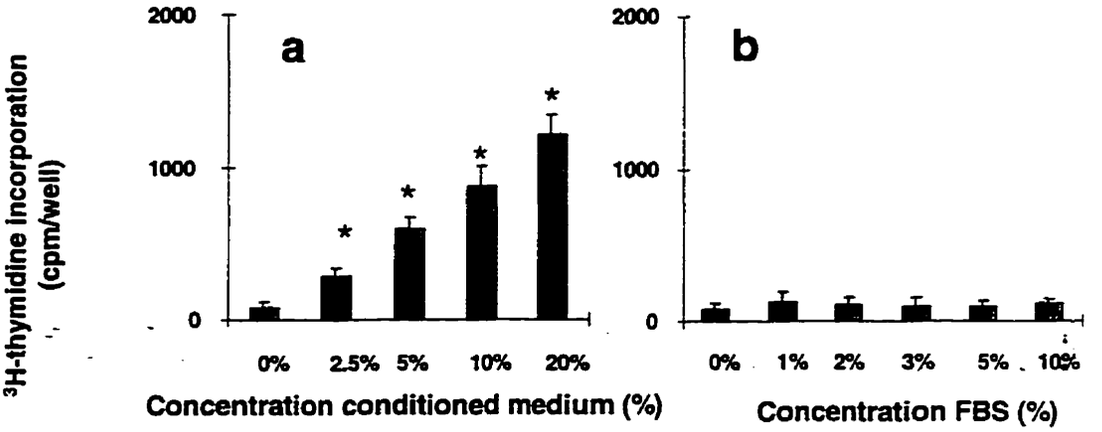

Figure 4.13: Effect of RTS11 conditioned medium on ^{3}H-thymidine incorporation by rainbow trout leukocytes. 50,000 head kidney leukocytes were added to each well of a 96 well plate, in L-15 medium without serum. Concentrations of conditioned medium, **a**, or FBS, **b**, as shown on the x axis were added. Plates were incubated for 5 days at 18°C, with 1 μCi per well of [^{3}H]-thymidine present for the last 24 hours of incubation. Values shown are the average of triplicate treatments, with error bars representing the standard deviation. Values significantly different from the control (p=0.05) are indicated by an *.

Leukocyte stimulation in response to RTS11 conditioned medium

^{3}H-thymidine incorporation by head kidney leukocytes from other fish was stimulated in a dose-dependent manner by the addition of RTS11 conditioned medium (fig. 4.13a). Concentrations of conditioned medium as low as 2.5% caused a significant increase in thymidine incorporation. Equivalent amounts of FBS did not demonstrate this effect (fig. 4.13b), confirming that the stimulation was not due to residual FBS left in the conditioned medium.

4. DISCUSSION

The RTS11 cell line is composed of two types of cells. The dominant cell type is a small, round, non-adherent cell, and the other cell type, present in small numbers, is large, very granular, and is present in both adherent and non-adherent forms. The number of larger, adherent cells is increased when cultures are held for prolonged periods of time without a change of medium.

Cell types in RTS11 cultures

These larger, granular cells appear to be macrophages. They have the typical morphology of macrophages, and possess several other macrophage characteristics. In both their adherent and non-adherent forms, they are actively phagocytic. As adherent cells, they have a well spread granular cytoplasm, with an oval to round nucleus surrounded by prominent vacuoles, typical of adherent macrophages. They are non-specific esterase positive, which is characteristic of fish macrophages (Zelikoff,1991), stain with acridine orange, also a feature of fish macrophages (Bayne, 1986), and take up DiI-Ac-LDL , which is a property of mammalian macrophages (Goldstein,1979).

RTS11 round cells appear to be a precursor cell, at an earlier stage of macrophage development than the adherent, granular cells. Monoclonal antibodies to leukocyte surface markers, which are used in mammalian systems to identify developmental stages of hemopoietic cells (Roitt, 1993), are scarce for fish (Coll & Dominguez, 1995), and cellular products of hemopoietic cultures are therefore identifiable only by their morphological and functional characteristics. RTS11 lack lymphoid markers, and lack the typical mitogenic responses of lymphocytes. They do not appear to be granulocytes, as their nuclei are not lobated and they do not stain for myeloperoxidase, a characteristic of rainbow trout granulocytes (Yasutake & Wales, 1983). Their morphology, round, non-adherent cells with a kidney shaped nucleus and slightly granular cytoplasm suggests a monocyte-like cell. They lack several characteristics of monocytes, which are phagocytic, undergo respiratory burst, and are non-specific esterase positive (Auger & Ross, 1992). The lack of these features could be due to their adaptation to long-term culture, as many monocytic and macrophage cell lines lack some of the characteristics of normal monocytes, or these features could be inducible

given the proper stimulus, as is seen with many immature macrophage cell lines including HL-60 and U-937 (Ralph, 1986). Alternately, they could be at an earlier developmental stage, and could be considered pre-monocytic, or myeloid precursor cells.

These less mature round cells are often seen clustered around the macrophage-like cells, either in suspension or on the culture surface. In mammalian hemopoietic tissue, macrophages are often found surrounded by developing hemopoietic precursor cells (Crocker & Gordon, 1985; Crocker & Milon, 1992) where they function as nurse cells, providing locally high concentrations of cytokines and growth factors to the developing cells. While this has not been observed in rainbow trout, there is a high concentration of macrophages present in the major hemopoietic tissues, the kidney and spleen (Tatner & Manning, 1984; Braun-Nesje, 1981; Rowley, 1990). The tendency of RTS11 round cells to associate with the mature macrophages in the cultures suggests the possibility that macrophages may perform a similar function in trout.

Comparison with other macrophage cell lines

In overall growth characteristics, RTS11 compares and contrasts with the other monocyte/macrophage cells from fish. Like the catfish monocyte cell lines (Vallejo et al., 1991; Miller & McKinney, 1994), RTS11 grows in an anchorage-independent manner and has several cell types. However, unlike the catfish cell line, RTS11 grows without the need for fish serum. The other two fish monocyte/macrophage cell lines are adherent. The goldfish line requires goldfish serum for growth (Wang et al., 1995), whereas the carp cell line (CLC), like RTS11, grows in FBS alone, although at much lower concentrations (Faisal and Ahne, 1990; Weyts et al., 1997). The goldfish cell line has a morphology similar to the adherent cells of RTS11 cultures, whereas CLC has an epitheloid morphology.

RTS11 has fewer of the functional characteristics of monocytes/macrophages than the other fish monocyte/macrophage cell lines and this probably reflects their different origin and stage of development. Like the catfish and carp cell lines, RTS11 produces and secretes growth factor(s). In the case of the catfish (Vallejo et al., 1991) and carp (Weyts et al., 1997), interleukin -1 is thought to be one of these factors. The adherent

cells in RTS11 cultures are like the cells of the other lines in being phagocytic and positive for non-specific esterase. However, the respiratory burst is undetectable in RTS11 cultures but present in the other fish lines. Additional properties, such as nitric oxide production, chemotaxis (Wang et al., 1995) and antigen presentation (Vallejo et al., 1991) have yet to be investigated with RTS11. The catfish and carp cell lines originated from peripheral blood, while the goldfish cell line arose from macrophages that had been isolated from the kidney. Therefore these cell lines are likely from more advanced stages in the monocyte/macrophage lineage than RTS11, which arose from long-term spleen cell cultures.

RTS11 round cells share several characteristics with macrophage-like cell lines that have been developed from mammals. Although macrophage-like cell lines vary widely in their characteristics, they are generally not as adherent as their normal counterparts (Ralph, 1986). Immature macrophage-like cell lines particularly are most often non-adherent (Ralph, 1986). This is not surprising, as, unlike other immature hemopoietic cells, macrophages begin as non-adherent cells and acquire adhesive ability as they mature. This is true for murine bone marrow precursors and for several human cell lines, such as HL60 and U-937 (Shima et al, 1995). All other hemopoietic cell types begin as adherent cells, and become non-adherent with maturity (Weinstein et al, 1989; Coulombel et al, 1992).

RTS11 cells share several other features with the human promyelocytic cell line HL-60. They are morphologically similar and both lack lymphoid morphology and surface markers, and have a spontaneously differentiated population of more mature cells. Both cell lines lack phagocytosis and respiratory burst activity (Harris & Ralph, 1985). In addition, both cell lines are non-adherent, and produce autocrine growth factors.

Mammalian leukocyte cell lines have provided excellent model systems of leukocyte functions, responses and interactions with other cells. RTS11 are potentially useful for a variety of studies. They could serve as models of the process of macrophage differentiation, the substances impacting hemopoiesis in rainbow trout, and the molecular mechanisms that are activated by these substances. As they appear to secrete a substance or substances that enhance their own growth and that of other

rainbow trout leukocytes, they are a potential source of what could be fish-specific cytokines.

Response of RTS11 cultures to serum

The interactions of RTS11 cells with serum are complex. RTS11 cells appear to require high concentrations of FBS (30%) to maintain proliferation. This could be due to a essential growth factor in FBS, available in trace amounts. Despite the requirement of high concentrations of FBS by RTS11, FBS by itself it is not sufficient to support cell growth. The cells proliferate most readily in the presence of their own conditioned medium and FBS, and when seeded at relatively high density. RTS11 conditioned medium also promotes thymidine incorporation by RTS11, and supports their long-term survival when cultured in multi-well plates, a condition which they do not normally tolerate. These observations suggest that an autocrine factor is produced by the cells that stimulates their continued proliferation. This is similar to the human promyelocytic cell line HL-60 that releases an activity into their culture medium that promotes their own proliferation, and maintains them in an undifferentiated state (Heil et al, 1989), and the mouse myelomonocytic leukemia cell line WEHI-3, which also requires its own growth factor (Broxmeyer & Ralph, 1977). RTS11, however, are able to respond to this putative autocrine factor only in the presence of FBS. This may be due either to the presence of a substance in FBS that potentiates the response to the autocrine factor, or to FBS stimulating the production of the autocrine factor. The promoter regions of hemopoietic growth factors contain serum-responsive elements, and cells such as macrophages and monocytes release growth factors more easily into the medium in the presence of FBS (Migliaccio et al, 1990).

FBS also influences the interactions of RTS11 cells with lectins. In the absence of serum, RTS11 are unresponsive to stimulation by Con A, PWM, or PHA, but in the presence of FBS, low concentrations of lectins are stimulatory. At higher concentrations, lectins again inhibit the incorporation of thymidine. Con A has been shown to interfere with the binding of the growth factor EGF on NIH 3T3 cells, by

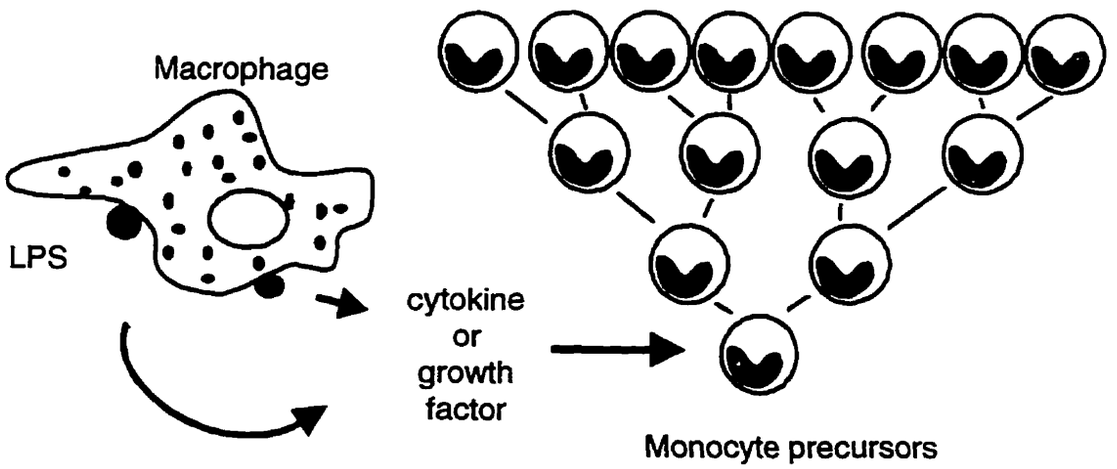

Figure 4.14: Postulated mechanism of LPS stimulation of growth of RTS11 cells. LPS may stimulate the growth of RTS11 by stimulating the production of a cytokine or growth factor by the small fraction of mature macrophage-like cells present. This substance may stimulate the division of RTS11 round cells, the monocyte precursors. This stimulation only occurs in the absence of serum.

sterically hindering its access to its receptor (Carpenter & Cohen, 1977). A similar mechanism could account for the inhibition by high concentrations of lectins observed in RTS11. Growth factors in serum presumably stimulate RTS11 growth by binding to their specific receptors. These effects may be physically blocked by large numbers of lectin molecules binding to their specific surface sugars. At lower concentrations of lectin, perhaps both lectin molecules and growth factor molecules in FBS are able to bind to their receptors and initiate their stimulatory effects.

Conversely, RTS11 growth is inhibited by lipopolysaccharide in the presence of serum. This could be due to LPS interacting with serum factors to induce RTS11 cells to differentiate rather than to proliferate. LPS interacts with surface receptors on macrophages and triggers a variety of signal transduction pathways, depending upon other signals that are received (Adams & Hamilton, 1992).

In general, exposure to LPS is an activating signal for macrophages, which encourages their transition to a more activated state, preparing them for bactericidal or tumoricidal activity, and inhibiting their proliferation (Adams & Hamilton, 1992). Its activity on less mature cells, however, may be slightly different. Immature mouse bone marrow cells were found to proliferate in response to LPS, but the presence of growth

factors such as IL-3 or GM-SCF inhibited this proliferation (Nardi et al, 1991). Perhaps growth factors in FBS are interfering with the response of RTS11 to LPS in the same manner.

In the absence of serum, lipopolysaccharide significantly stimulates DNA synthesis by RTS11. LPS is mitogenic for some macrophage-like cell lines (Ohki et al, 1992) and has been shown to increase the survival of human monocytes in culture (Mangan et al, 1991; Becker et al, 1987) by preventing apoptosis. Alternately, lipopolysaccharide could be acting by stimulating the small percentage of mature macrophages in cultures to release factors that enhance the proliferation of the less differentiated round cells (see fig. 4.14). Upon exposure to LPS macrophages from mouse (Aznar et al, 1990) and humans (Rinehart & Keville, 1997) release many factors, such as IL-1, IL-6 and G-CSF, which are able to induce the proliferation of early stage cells. In human cultures, endotoxin caused the release of these factors which then stimulated the proliferation of CFU-GM (Rinehart & Keville, 1997).

RTS11 are normally non-adherent, but adhere under several conditions. Their adhesion to the surface increases dramatically when serum is removed from the culture, when they are held for a prolonged period without fresh medium, or when they are treated with PMA. Many macrophage cell lines become adherent in the absence of serum (Muschel et al, 1977; Ralph, 1986), or when cultured in exhausted medium (Harris & Ralph, 1985). In addition, macrophage-like cell lines often adhere in response to an activating agent (Ralph, 1986). It seems that some factor or factors in FBS inhibits adhesion of RTS11, but that this factor is used up with prolonged culture. The cells then adhere, and become more macrophage-like. Adhesion may serve as a signal triggering genes involved in differentiation, leading to a more mature phenotype, as observed with human monocytes (Haskill et al, 1988). In addition, when RTS11 cells are treated with the phorbol ester PMA, they adhere and cease to proliferate. PMA induces several immature macrophage-like cell lines, including HL-60 (Harris & Ralph, 1985) and U-937 (Hass et al, 1989) to cease proliferation and to acquire some characteristics of mature macrophages. Further work is needed to study any further effects on RTS11, and whether PMA is a signal inducing a more mature phenotype.

Response of RTS11 cultures to other regulatory factors

Interestingly, RTS11 cells are inhibited by exposure to adenosine, a regulator of hemopoiesis in mammals. Cells exposed to adenosine at physiological concentrations show inhibition of thymidine incorporation, and morphological changes, both consistent with apoptosis. This effect is observed both in the presence and absence of serum. This effect is not noticed with head kidney leukocytes, which represent a mixed population of cells, indicating that the effect may be specific to cells of the macrophage lineage. The possibility that RTS11 are undergoing apoptosis is being investigated. Human thymocytes (Szondy, 1994), neutrophils (Walker et al, 1997), and the HL-60 cell line (Tanaka et al, 1994) are induced to undergo apoptosis by adenosine. Adenosine may be an important regulator of hemopoietic cell growth in fish, as it appears to be in humans (Orrico et al, 1991).

In spite of their similarities to mammalian cell lines and their responses, RTS11 do not respond to several mammalian stimuli but do respond to some fish-specific stimuli. Known sources of cytokines that promote the growth of mammalian hemopoietic precursors, including WEHI-3, L929 and 5637 conditioned medium, inhibit the growth of RTS11. On the other hand, RTS11 are stimulated to proliferate greatly upon exposure to crude extracts of rainbow trout origin. In addition to their own conditioned medium, RTS11 cells are stimulated to grow in the presence of PHA-LCM prepared from rainbow trout peripheral blood leukocytes strongly stimulated growth. The cells also respond to clone 1A conditioned medium, and to conditioned medium from primary spleen long-term cultures. These results support the possibility that RTS11 require factors unique to fish cells, and also suggests the presence of fish-specific cytokines in these crude extracts.

Production of growth factors by RTS11 cultures

In addition to its effects on RTS11 themselves, conditioned medium from RTS11 has growth promoting activity for rainbow trout leukocytes freshly isolated from fish. The putative autocrine factor may thus have activity of a more general nature, not specific to RTS11 cells alone. The nature of the growth factor or factors produced by RTS11 is currently unknown, but there are several possibilities. Many cytokines and hemopoietic growth factors are produced by mammalian leukocyte cultures. WEHI-3, for example, produces large amounts of IL-3 (Lee et al, 1982), which has a broad range of target cells,

while L929 produces mainly M-CSF (Stanley & Heard, 1977), with actions only on macrophages and their precursors. The activity produced by RTS11 could be analogous to M-CSF, since it stimulates RTS11 cells, which are of the macrophage lineage. Preliminary experiments show that this activity is much more mitogenic for cells isolated from the head kidney of trout than those isolated from the peripheral blood, or spleen. The population of leukocytes isolated from the head kidney contains many more immature cell types than that isolated from blood or spleen, and also contains many more macrophages (Braun-Nesje, 1981; Rowley, 1990). This suggests that the activity may be specific for immature leukocytes, or possibly macrophages. Its characteristics are currently under investigation.

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Chapter 5

A spleen stromal cell line, RTS34st, that supports the growth of rainbow trout macrophages and produces conditioned medium with mitogenic effects on leukocytes

ABSTRACT

A cell line, RTS34, similar in composition to long-term spleen cultures was developed. This cell line consists of a stromal cells layer with an associated cell population of macrophage-like cells that form proliferative foci and release non-adherent progeny cells into the culture medium. A stromal cell line, RTS34st was isolated from the RTS34 cell line. These cells are fibroblast-like and epithelial-like in morphology. RTS34st themselves incorporate ^{3}H-thymidine in response to serum and several lectins. In addition, RTS34st provide a hemopoietic inductive microenvironment for immature precursor cells. These stromal cells appear to selectively support the proliferation of macrophage-like cells from several sources, including autologous macrophages, head kidney macrophages and RTS11 myeloid cells. RTS34st appears to produce soluble factors capable of stimulating rainbow trout hemopoietic cells. Conditioned medium from RTS34st stimulates thymidine uptake in peripheral blood, spleen and head kidney leucocytes, as well as the cell line RTS11.

1. INTRODUCTION

The rainbow trout spleen is an active hemopoietic organ (Catton, 1951), composed of numerous cell types. These include red blood cells, leukocytes and their precursors, and the reticular or stromal cells, comprising endothelial cells, fibroblasts, and macrophages (Fangë & Nilsson, 1985). Developing blood cells require both direct physical contact with the cells of the microenvironment and the cytokines and other soluble factors produced by stromal cells (Dexter et al, 1977; Spooncer et al, 1993). Like mammalian leukocytes in bone marrow (Quesenberry, 1989), trout blood cells develop in the head kidney or spleen (Fänge, 1994) in close association with a delicate reticular network of stromal cells (Yasutake & Wales, 1983). These cells provide the microenvironment that supports hemopoietic stem cell self-renewal and the proliferation and development of mature, differentiated blood cells.

Stromal cell lines of mammalian origin have been invaluable tools for defining the interactions between hemopoietic cells and their environment (Deryugina & Müller-Sieburg, 1993; Zipori, 1989). These cell lines are heterogeneous and have a variety of applications. Some are useful in supporting specific populations of hemopoietic precursor cells (Collins & Dorshkind, 1987; Yanai et al, 1989), and others have been shown to maintain and expand primitive stem cells (Wineman et al, 1993; Rios & Williams, 1990). Several novel cytokines, such as IL-7 (Namen et al, 1988) and IL-11 (Paul et al, 1990) are produced by and have been isolated from stromal cell lines.

Despite their potential usefulness, few fish stromal cell lines have been reported. TPS, a rainbow trout pronephric stromal cell line, was described by Diago et al (1995). Other cell lines from fish hemopoietic organs, such as SP-2 from the silver perch spleen (Ellender et al, 1979) and BPS-1, BPS-4 from the spleen and BPK from the kidney of black porgy (Tung et al, 1991), or LTK from lake trout kidney (Ganassin, unpublished) could have stromal functions, but have not been tested for their ability to support the growth of hemopoietic progenitors.

Several cell lines have arisen spontaneously from long-term spleen hemopoietic cultures (chapter 2). One of these, RTS34st, has been maintained for over three years. This cell line consists of a heterogeneous population of epithelial-like and fibroblast-like

cells. These stromal cells support the survival and proliferation of rainbow trout macrophages from a variety of sources. In addition, conditioned medium from this cell line stimulates uptake of thymidine by leukocytes isolated from trout head kidney, and of RTS11 cells. Therefore, it appears that RTS34st cells provide both the microenvironment and the soluble factors necessary to promote rainbow trout macrophage growth *in vitro*.

2. MATERIALS AND METHODS

Establishment of long-term culture

RTS34 was initiated in September 1994, from a sexually immature rainbow trout weighing 98 grams. The fish was obtained from Rainbow Springs Trout Farms, Petersburg, ON, and maintained in 14°C flowing water and fed daily with commercial pellets. The 25.9 mg spleen was excised and prepared for long-term culture as previously described (chapter 2), and distributed into each of two 12.5cm^2 Falcon culture flasks with five mL of Leibovitz's L-15 medium supplemented with 30% fetal bovine serum (FBS). The medium was replaced after two weeks of culture, and replenished at monthly intervals thereafter. After 8 weeks of growth, a stromal cell layer consisting of an underlying layer of epithelial-like cells, with dendritic-like cells and macrophages dispersed over the surface, and foci of small, phase-bright round cells developed. As the number of cells in each focus increased, some became free floating in the culture medium.

Subculture and establishment of the cell line RTS34st

After 8 weeks of culture, one of the original flasks was treated with EDTA to dislodge the cells from the growth surface. The resulting cell suspension was divided into 2 new flasks, A and B, with fresh L-15 with 30% FBS. In both flasks, the cells proliferated quickly to cover the culture surface. Flask A had noticeably fewer macrophages, dendritic-like cells and hemopoietic foci than B. Both flasks were continuously passaged 1:2 at 4-6 week intervals; flask A and its descendants with trypsin, and flask B and its descendants by treatment for 20 minutes with EDTA.

In flask A, the number of cells other than stromal epithelial and fibroblastic cells declined with each passage. By the tenth subculture, only stromal cells remained. The resultant cell line was named RTS34st, and is routinely subcultured by trypsinization in the usual manner used for culture of fish cell lines (Ganassin & Bols, 1997a).

The descendants of flask B, on the other hand, comprised all of the cell types of the original population. After subculture 9, these cultures began to regularly produce hemopoietic foci and non-adherent progeny cells. The complex stromal layer, complete with foci, when detached with EDTA, covered the surface of the two new flasks, formed

new foci, and produced more non-adherent progeny. This cell line is referred to as RTS34.

CHARACTERIZATION OF THE NON-ADHERENT CELL POPULATION

Morphology and cytochemistry

Live cultures were regularly examined and photographed using a Nikon Diaphot inverted phase contrast microscope with fluorescence capability. Non-adherent cells were harvested from a culture flask and a cytocentrifuge (Shandon Cytospin) was used at 500 rpm for 10 minutes to deposit the cells on to microscope slides prior to staining. Wright-Giemsa and myeloperoxidase staining were carried out as previously described (chapter 2). Acid phosphatase and α-naphthyl-acetate esterase (non-specific esterase) activity were detected using a Sigma kit and protocol. Sudan Black B was performed as described by Sheehan & Story (1947) and acridine orange staining by the method described by Bayne et al (1986). Cultures were stained with Hoescht 33258, using the method described by Chen (1977), to distinguish nuclear morphology and to test for the presence of mycoplasma.

DiI-acetylated low-density lipoprotein uptake assay

The presence of the scavenger receptor for lipoprotein metabolism, a marker for endothelial cells (Voyta et al,1984) and cells of the monocyte/macrophage lineage (Goldstein et al, 1979), was determined as previously described (chapter 4).

Phagocytosis assay

The ability of both RTS34 non-adherent progeny and stromal cells to phagocytose foreign particles was assessed by incubating the cells with latex beads or with Congo Red stained yeast cells as previously described (see chapter 4).

CHARACTERIZATION OF RTS34 STROMAL CELLS, RTS34st

Morphology and cytochemistry

RTS34st cells were stained with the same array of cytochemical stains that were used for the non-adherent cell population, RTS34na.

Table 5.1: Antibodies used to screen RTS34st cells.

Primary antibody			Secondary antibody	
Specificity	**Type**	**Source**		**Source**
progeny cells from long-term spleen cultures (chap. 2)	polyclonal, raised in rabbit	Stevenson, University of Guelph	GAR-FITC	Sigma
E-51 - rainbow trout cellular fibronectin	polyclonal, raised in rabbit	Lee, University of Saskatchewan	GAR-FITC	Sigma
1.14 - rainbow trout IgM	monoclonal	Miller, University of Mississippi	RAM-FITC	Sigma

Rate of growth with various concentrations of FBS

The growth rate RTS34st in response to different concentrations of FBS was determined by dislodging the cells from the culture surface with trypsin in the usual manner. The cells were counted, and distributed equally into the wells of Costar 24-well culture plates, at an initial density of 2×10^5 cells per well in 1 mL of growth medium, supplemented with either 0, 5, 10, 20 or 30% FBS, in triplicate treatments. Growth was measured at three day intervals by using trypsin to dislodge the cells from the growth surface, and counting the cells using a Celloscope cell counter.

Optimum growth temperature

To determine the preferred growth temperature, cells were added to the wells of 24-well plates in one mL of medium supplemented with 30% FBS, and counted exactly as described above. One plate was incubated at each of 5°C, 12°C, 18°C and 21°C ± 1°C (room temperature).

Immunocytochemistry

RTS34st cells were stained with the antibodies described in table 5.1. For immunocytochemical staining, cells were allowed to adhere to 4-chamber slides in L-15 medium supplemented with 30% FBS. When cells were attached and spread, medium was removed, cells were rinsed twice with PBS, fixed with acetone, rinsed four times with PBS, and the primary antibody was added. As a negative control, the primary antibody was omitted in one chamber of the four chamber slide. After 1.5 h incubation in the dark, the primary antibody was removed, wells were rinsed three times with PBS, and the secondary antibody solution was added. After 1.5 hours, the secondary antibody solution was decanted, wells were rinsed four times with PBS, slide chambers were removed, and

coverslips were mounted with PBS/glycerol. Slides were observed using a Nikon inverted phase contrast microscope equipped with epifluorescence at a wavelength appropriate for the fluorochrome conjugated to the secondary antibody used in each case.

^{3}H-thymidine incorporation assay

Chemicals

Chemicals tested were obtained from Sigma. Cells were exposed to a panel of lectins including concanavalin A (Con A), pokeweed mitogen (PWM) and phytohemmaglutinin (PHA-P), castor bean lectin (CBL) and wheat germ lectin (WGL). Sera used include FBS (Canadian Life Technologies) and rainbow trout serum, prepared as previously described (Ganassin & Bols, 1997a). Sera from at least 10 fish was pooled and heated at 56°C for 30 minutes to inactivate complement prior to use.

Proliferation assay

Assays were carried out as described for RTS11 cells (chapter 4), with the following changes. RTS34st were dislodged with trypsin, centrifuged and resuspended in L-15. 15,000 cells were added to each well of a 96 well plate, and allowed to attach overnight. Treatments were added, and the plates were incubated at 18°C for 7 days, with ^{3}H-thymidine added for the last 24 hours. RTS34 na cells were not available in sufficient quantities to determine their responses in the absence of stromal cells.

Collection of conditioned medium

Conditioned medium was prepared from RTS34st cells by plating in the usual growth medium, L-15 with 30% FBS, and allowing the cells to attach. The cells were grown to confluence, and the medium was collected after two weeks of culture. At the time of harvest, medium was red-orange in colour and slightly cloudy in appearance. The medium was collected, and centrifuged in a table-top centrifuge at 3000 rpm to remove floating cells and debris. The supernatant was then filtered through a 0.2 μm filter to ensure its sterility. Experiments using conditioned medium that contained serum always contained L-15 with an equivalent amount of FBS (non-conditioned medium) as a control, to ensure that effects were not due to any remaining serum.

Effect of RTS34 CM on mitogenesis of freshly isolated leukocytes and RTS11 cells

The effect of conditioned medium from RTS34 was examined using four different sources of leukocytes. Leukocytes were isolated from the peripheral blood, spleen or head kidney of rainbow trout. RTS11 cells were used as an additional source. The ability of RTS34st CM (collected in the presence of serum) to stimulate ^{3}H-thymidine incorporation by leukocytes, in the presence or absence of 10% FBS was assessed using the assay as described for RTS11 in chapter 4. RTS11 cells were treated the same way.

Ability of RTS34 to function as a stromal layer and support the growth of immature hemopoietic cells

The immature myeloid cell line, RTS11, leukocytes isolated from head kidney, and non-adherent progeny cells from the parent cell line, RTS34, were used to demonstrate the ability of RTS34 to provide a hemopoietic inductive microenvironment. RTS34st were seeded into 75 cm^2 culture flasks (Nunc), and an inoculum of 1 x 10^6 head kidney leukocytes, RTS34na, or RTS11 cells, was added. As negative controls, identical flasks of RTS34st were maintained without the addition of cells, and an equal number of cells were incubated in the absence of a stromal layer. The development of RTS11 or leukocytes was monitored microscopically, and in cases where non-adherent cells were produced, by counting the production of these cells using a hemacytometer.

3. RESULTS

Morphology

Early cultures

The appearance of RTS34 cultures prior to separation of the stromal cell population is shown in fig. 5.1a. RTS34 stromal cells (fig. 5.1e) supported a population of other cells with the typical morphology of macrophages (fig. 5.1a). These macrophage-like cells were found both associated with the stromal layer cells, and alone in bare areas of the culture surface. As well, discreet foci of small, round, phase-bright cells were found scattered over the stromal layer soon after subculture of these parent cultures (fig. 5.1b).

RTS34na

After 2-3 weeks of culture, these foci become larger and non-adherent cells which were irregular in size and shape, with flaps of cytoplasm were released into the medium. When transferred to new flasks, these progeny cells adhered immediately and assumed a macrophage-like morphology (fig. 5.1d). Attachment to plastic or glass was rapid and occurred both in the presence and absence of FBS. When these non-adherent progeny cells were transferred to new culture flasks they attached to the surface, and could be maintained, occasionally showing limited proliferation, for a period of 1-2 months before they rounded up, detached from the surface, and died. Alternatively, non-adherent cells could be added to cultures of isolated stromal cells, RTS34st (fig 5.1e), where they adhered to the stromal layer and proliferated. When progeny cells were maintained in association with the adherent stromal layer, either in original or reconstituted (i. e. non-adherent cells added to isolated RTS34st cells) cultures, both cell populations remained, and could be routinely subcultured using EDTA to dislodge the cells from the culture surface.

RTS34st stromal cells

The sub-line of adherent stromal cells, RTS34st (fig 5.1e), were fibroblastic, with areas of cobblestone, epithelial-like morphology. Immediately following subculture, the

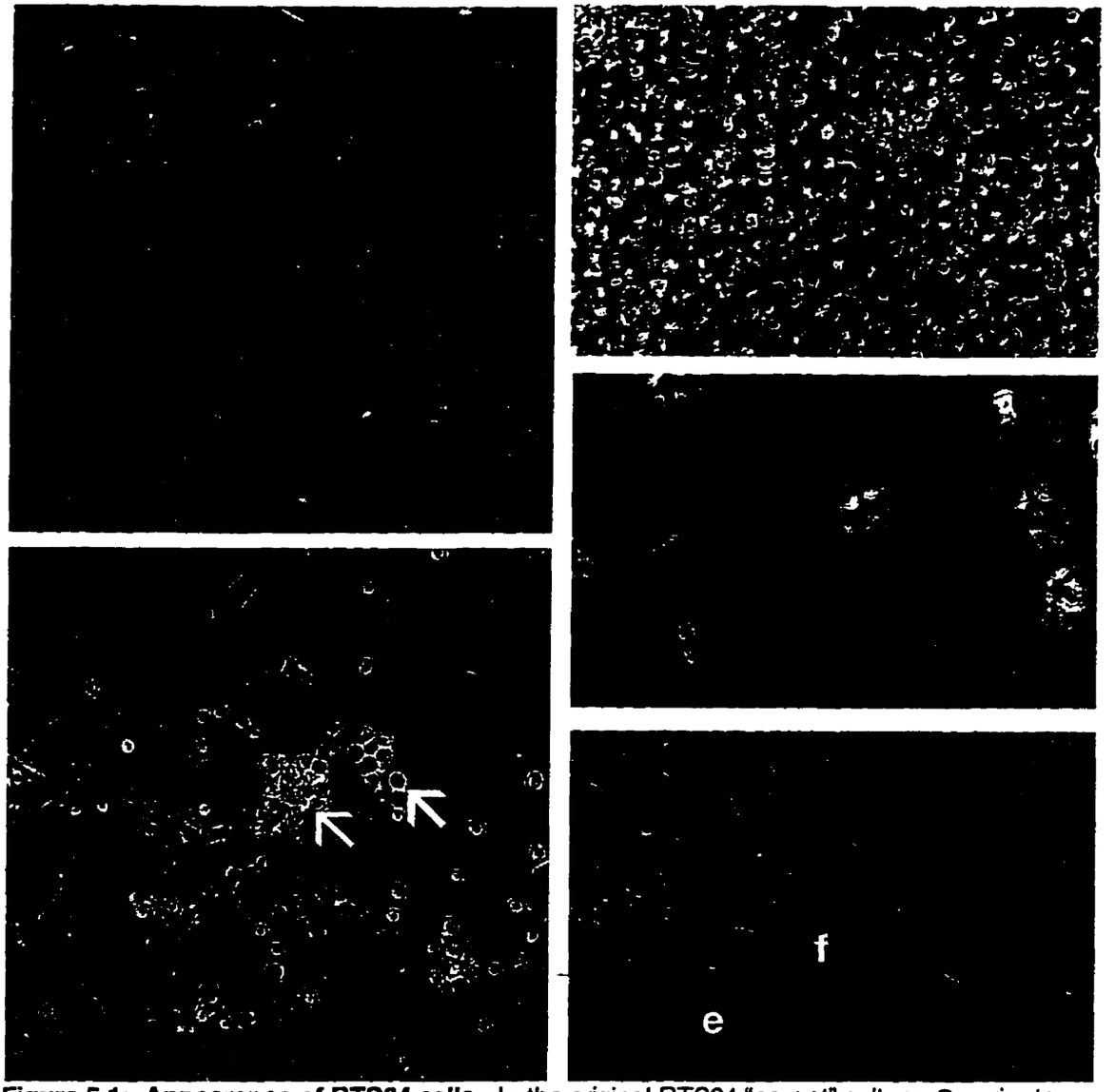

Figure 5.1: Appearance of RTS34 cells. In the original RTS34 "parent" culture, **a**, prior to isolation of the stromal cell line RTS34st, stromal cells with fibroblastic and epithelial morphologies are obscured by the macrophage-like cells (dark, spread, multi-polar cells) and non-adherent cells (round, phase bright cells) spread on top of the stromal cell layer. When the parent cell line was passaged, hemopoietic foci (↖) developed on the stromal layer, **b**. These cells proliferated and became non-adherent, **c**, and when seeded into new culture flasks, **d**, attached and assumed a macrophage-like morphology. RTS34st cells were isolated from the parent culture, and are shown in **e**. Fibroblastic (f) and epithelial-like (e) cell morphologies are evident.

cells are predominantly fibroblastic. As the culture grows, more and more epithelial-like cells appear. When maintained in culture past confluence, the fibroblastic cells are pushed aside as the epithelial-like cells grow and become the dominant cell type (not shown).

Table 5.2: Summary of characteristics of RTS34 non-adherent cells

Stain	Description of results
myeloperoxidase	most negative; a small fraction of cells positive
acid phosphatase	uniformly positive (turquoise) in a spot located next to the nucleus
non-specific esterase	mildly positive, with fine granules evident
Sudan Black B	negative
Acridine Orange	positive (nuclei are green, and bright orange vacuoles are observed)
Hoescht 33258	large, oval nuclei, no mycoplasma
DiI-Ac-LDL uptake	strongly positive, bright red fluorescence concentrated in large vacuoles
Phagocytosis	strongly positive

When maintained in L-15 supplemented with 30% FBS, the cells appeared to release substances into the medium, causing it to assume a slightly cloudy appearance, and a slightly more acidic pH. The cells and culture medium were tested for bacterial contamination in three ways. First, gram staining was performed, and revealed no bacteria. Secondly, samples of medium were inoculated into fresh tubes containing growth medium, and incubated at 18°C for 4 weeks, during which no growth was observed. Thirdly, the cells were stained with H33258, using the method of Chen, 1977, which showed no fluorescent staining outside of the RTS34cell nuclei. This test also confirmed that the cells were uncontaminated by mycoplasma.

CHARACTERIZATION

Cytochemistry

RTS34na:

Wright-Giemsa staining of cytocentrifuge preparations of RTS34na showed that these cells had a granular cytoplasm, with an eccentrically located, oval to kidney shaped nucleus. Most cells were mildly positive for α-naphthyl-acetate esterase, showing small black spots over the cytoplasm. All cells were acid phosphatase positive, a few over the entire cytoplasm, but approximately 99% of the cells in a large spot next to the nucleus. Only a few of the cells were myeloperoxidase positive, while the vast majority were negative. All cells were Sudan Black B negative. All cells had vacuoles in the cytoplasm that stained orange with Acridine orange stain, an indicator of macrophage lysosomal activity (Bayne, 1986).

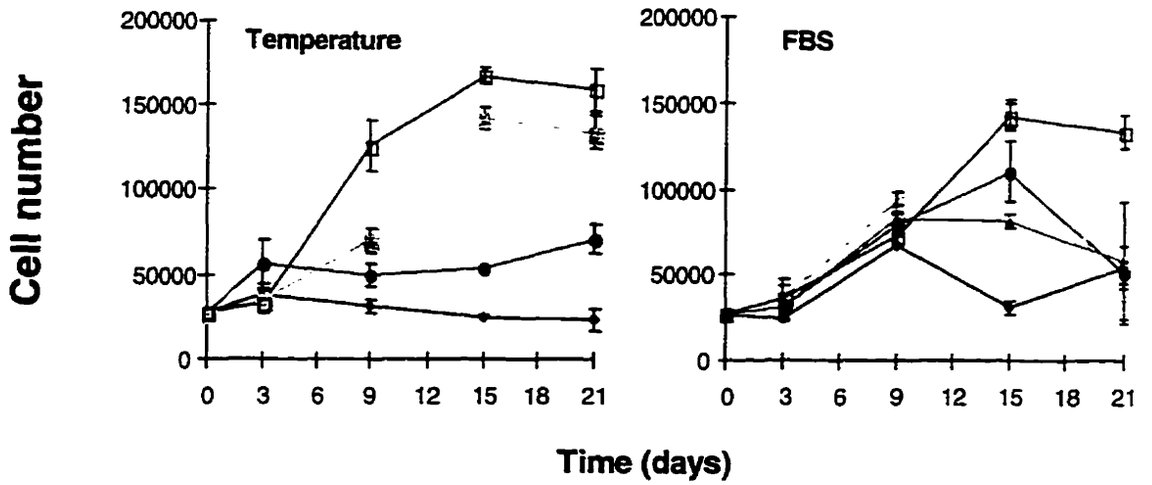

Figure 5.2: Growth of RTS34 in L-15 at different temperatures, a, and with different concentrations of FBS, b. In **a**, 6-well Falcon plates were inoculated with RTS11 cells at an initial density of 25,000 cells/well in L-15 with 30% FBS, and incubated at 5°C (-◆-), 12°C(-●-), 18°C( ), or 21°(-□-). All temperatures are ± 1°C. In **b**, 6-well Falcon plates were inoculated with RTS11 cells at an initial density of 25,000 cells/well in L-15 with 0% FBS (-◆-), 5% FBS (-▲-), 10% FBS (-●-), 20% FBS (-⧆-), or 30% FBS (-□-). On the indicated days after culture initiation, cells were removed from each well by trypsinization, and counted using a Celloscope cell counter. Values shown are the average of triplicate wells, with error bars representing standard deviation.

DiI-acetylated low-density lipoprotein uptake assay

RTS34na cells accumulated large amounts of DiI-Ac-LdL in vacuoles. Neither the fibroblastic nor the epithelial-like cells of the stromal cell layer took up DiI-Ac-LdL.

Phagocytosis

RTS34na cells were highly phagocytic, taking up many latex beads or yeast particles. The stromal cells comprising RTS34st, however, did not phagocytose significant numbers of either type of particle.

RTS34st

Cytochemistry

Wright-Giemsa staining clearly shows that RTS34st consist of two major cell types, fibroblastic cells and epithelial-like cells. Fibroblastic cells are bipolar in shape, while

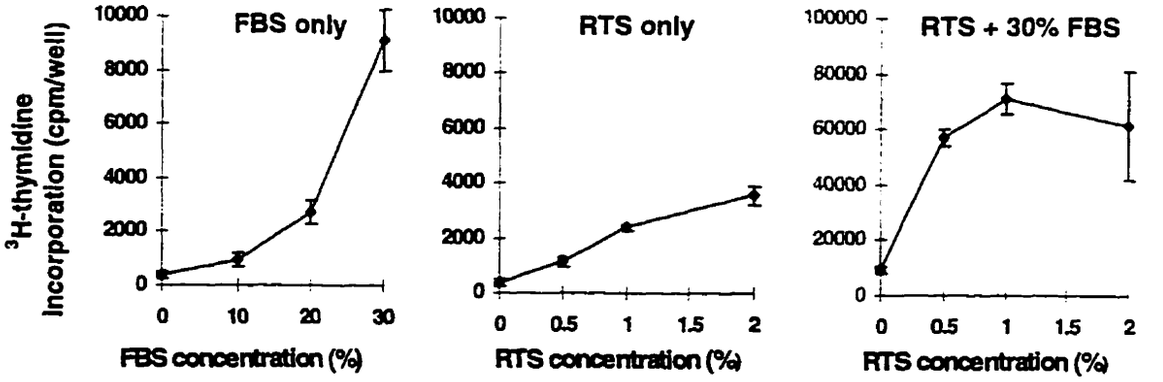

Figure 5.3: RTS34st response to serum as measured by ^{3}H-thymidine incorporation. 15,000 RTS34st cells were added to each well of a 96 well plate in L-15 without serum, and allowed to attach for 24 hours. (Note the different y axis values on the third graph.) The indicated concentrations of FBS without RTS, RTS without FBS, and RTS with 30% FBS were added to each well. ^{3}H-thymidine was added for the final 24 hours of incubation. After 7 days incubation, cells were trypsinized and harvested onto filter paper using the Skatron cell harvester. Values shown are the means of triplicate wells, with error bars indicating standard deviation. All treatments were significantly ($p \leq 0.05$) different from the control.

epithelial cells had varied shapes. No RTS34st cells demonstrated positive staining reactions for any of the cytochemical stains tested.

Immunocytochemistry

Of the antibodies tested, only the anti-fibronectin antibody showed positive staining. Fibronectin was localized within RTS34st fibroblastic cells as bright fibrils. The epithelial-like cells showed diffuse, all-over staining. A polyclonal antibody raised in rabbit against the non-adherent cell progeny of spleen cultures described previously (chapter 2) did not stain RTS34na cells.

Growth characteristics of RTS34st

RTS34st have been subcultured 25 times to date, using trypsin to detach the cells, and have maintained their mixed fibroblastic and epithelial-like morphologies. They can be frozen in liquid nitrogen, with 90% culture medium and10% DMSO, and retain their viability when thawed. RTS34st cells grow best with a serum supplement of 20% FBS

Table 5.3: Effect of lectins on mitogenesis of RTS34st in L-15 with 30% FBS

Lectin	Carbohydrate specificity[1]	Stimulation index[2]			
		1 µg/mL	5 µg/mL	10 µg/mL	20 µg/mL
PHA	N-acetyl-D-galactosamine	1.09	1.62 *	1.30 *	1.96 *
Con A	mannose glucose	1.02	1.20	1.05	1.48 *
PWM	poly-N-acetyllactosamine	0.73	0.88	0.95	0.35 ★
CBL	free terminal galactose	0.02 ★	0.05 ★	0.03 ★	0.01 ★
WGL	N-acetyl-D-glucosamine	0.83	1.21	1.79 *	0.95

[1] from Wu et al, 1988

[2] stimulation index is the ration of mean incorporation by the treated wells/incorporation to mean incorporation of control wells. Mean incorporation of control wells was 9122 ± 1158.
Values that are significantly greater than control values ($p \leq 0.05$) are indicated by an *. Values significantly less than control values ($p \leq 0.05$) are indicated by a ★.

(fig. 5.2 a) and at 22°C (fig. 5.2 b). They are, however, routinely maintained under the same conditions as the parent cell line, RTS34, with a serum concentration of 30% and at a temperature of 18°C. These conditions are optimal for the survival of the autologous macrophages that are associated with the stromal cells of RTS34.

^{3}H-thymidine incorporation in response to serum

The growth of RTS34st, as measured by ^{3}H-thymidine incorporation, showed 30% FBS was the most effective FBS supplement. Rainbow trout serum was stimulatory at very low concentrations, but not to the same extent as FBS. Rainbow trout serum and FBS acted synergistically: with both 1% RTS and 30% FBS, thymidine incorporation was elevated 6.2 fold over the incorporation caused by separate additions.

^{3}H-thymidine incorporation in response to lectins

Of the six lectins tested (table 5.3), CBL was toxic, causing agglutination of the cells and decreasing levels of thymidine incorporation to near zero values, even at the lowest concentration used, 1 µg/mL. PWM also inhibited DNA synthesis, but not to the same degree, except at the highest tested concentration of 20 µg/mL. Con A caused slight stimulation of DNA synthesis, with no apparent effect of concentration; all concentrations were equally effective. The greatest stimulation was seen with PHA at a concentration of 20 µg/mL. WGL had a positive effect at concentrations of 1-10 µg/mL, but the effect

Table 5.4: Effect of RTS34stCM on mitogenesis of freshly isolated leukocytes

Concentration RTS34st conditioned medium (%)[1]	Stimulation index[2] without serum				Stimulation index with 10% FBS			
	Blood	Spleen	Kidney	RTS11	Blood	Spleen	Kidney	RTS11
5	5.6 *	0.9	46.3 *	1.8 *	4.3 *	0.7	76.1 *	5.8 *
10	12.6 *	0.5	61.4 *	1.8 *	3.5 *	0.5	68.1 *	9.1 *
20	9.4 *	1.0	103.7 *	2.3 *	3.4 *	0.8	46.3 *	13.9 *
30	8.5 *	0.6	106 *	3.0 *	2.2 *	0.6	34.3 *	20.2 *

[1] conditioned medium was collected in the presence of FBS.
[2]stimulation index is the ratio of mean incorporation by the treated well/incorporation to mean incorporation by the control (wells containing an amount of FBS equivalent to that in the conditioned medium). Experiment was conducted with triplicate wells of each treatment. Standard deviation was ≤ 15% for all treatments.
[3]Values that are significantly greater than control values ($p \leq 0.05$) are indicated by an *.
[4]For RTS11 cells, experiments were conducted with 50,000 cells/well of a 96 well plate, for leukocytes, 100,000 cells were used. All cells were incubated with RTS34stCM for 5 days, with ^{3}H-thymidine added for the last 24 hours.

disappeared at a concentration of 20 µg/mL.

Effect of RTS34st conditioned medium on mitogenesis of leukocytes from different organs and on RTS11 cells

Leukocytes of different sources responded to conditioned medium from RTS34st with increased DNA synthesis. This stimulation of DNA synthesis (table 5.2) varied with the target cells used. In the absence of additional FBS, cells isolated from the spleen did not respond at all to conditioned medium, those of head kidney responded extremely strongly, and peripheral blood cells were intermediate in their response. RTS11 cells also responded strongly to RTS34stCM. With freshly isolated leukocytes, the inclusion of 10% FBS in the culture medium dampened the response. With the cell line RTS11, however, the presence of 10% FBS enhanced the response.

Ability of RTS34 to support the growth of immature hemopoietic cells

RTS34 na cells

Non-adherent cells from a flask of RTS34 (parent cultures), when added to the RTS34st monolayer, immediately attached and spread. They repopulated the stromal layer, and by 3 weeks after inoculation, the culture was indistinguishable from an original mixed culture. RTS34 na cells added to flasks without the stromal cells did not survive over the course of the experiment.

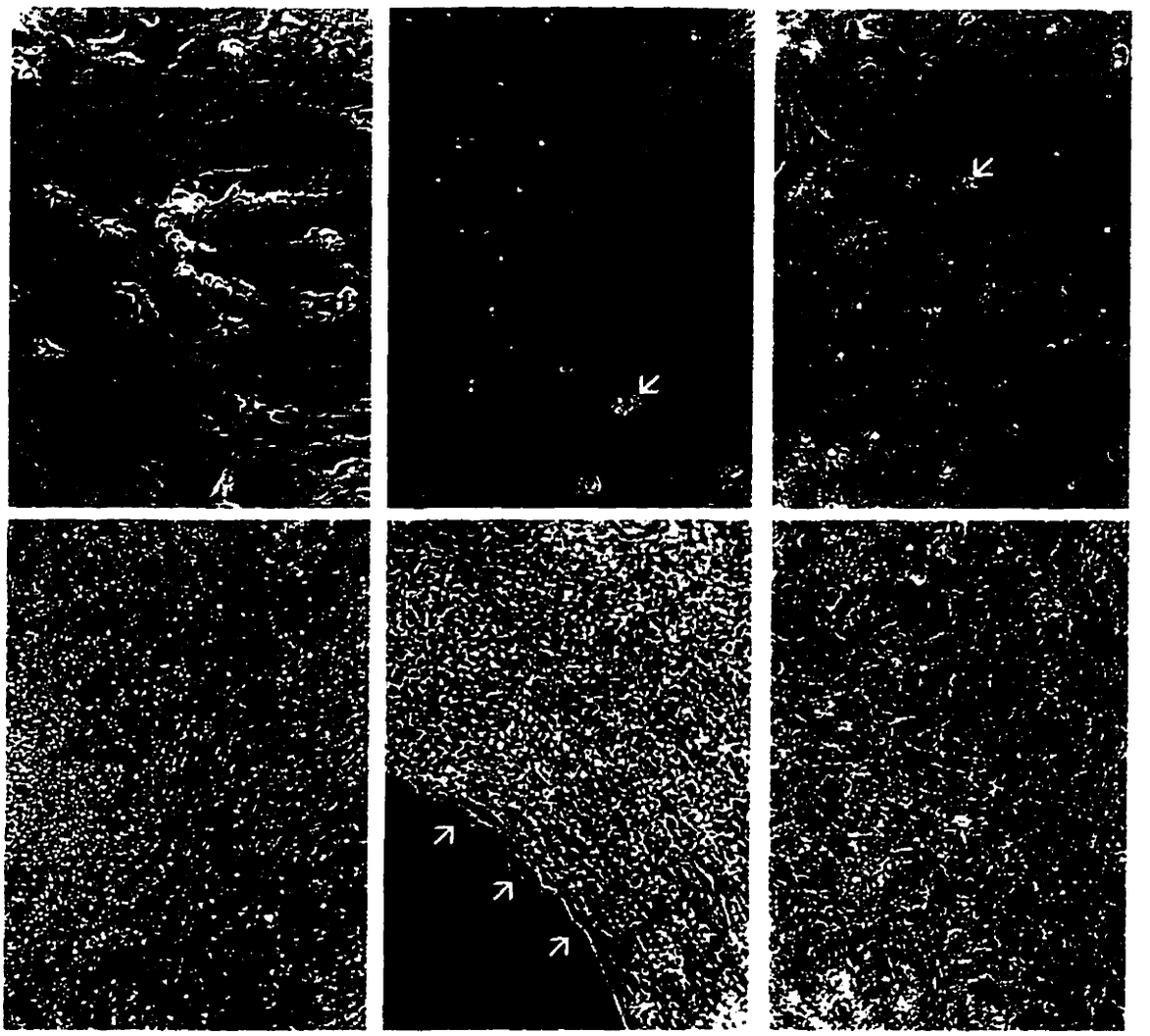

Figure 5.4: RTS34st cells provide a hemopoietic inductive microenvironment. RTS34st cells were seeded in 75cm^2 culture flasks and grown to form a growth-arrested monolayer. Non-adherent cells from , **a**, RTS34 cultures (↗) , **b**, RTS11 cultures, or **c**, head kidney leukocytes were added to each flask. Control flasks (not shown) contained RTS34st cells alone, or an equivalent number of non-adherent cells from each source. After 3 weeks of culture, cells from, **d**, RTS34, **e**, RTS11, and **f**, leukocytes had proliferated, while those in control cultures were dead. Note **e**, where proliferation of RTS11 cells took place only in association with the fibroblastic portions of the cell layer. Arrows indicate the boundary between the epithelial-like cells and the fibroblastic-like cells with attached, proliferating cells. In **f**, the adherent, proliferating cells had the morphology of macrophages. Original magnification 200x, except **d**, which was 100x.

RTS11 myeloid cells

When normally non-adherent RTS11 cells were added to a confluent layer of RTS34, they quickly adhered and flattened (fig. 5.4 b). Approximately 3 days after seeding, small foci of RTS11 cells were observed. These gradually increased in size and by 2 weeks, began to produce non-adherent progeny cells. After 8 weeks of culture, 3.17×10^6 non-adherent, live, round cells were in suspension in the culture, and the fibroblastic cell surface was almost obscured by attached RTS11 cells and foci (fig. 5.4 e), and did not appear to adhere to epithelial morphology cells. No non-adherent cells were produced by a parallel culture of RTS34 alone, and RTS11 maintained in a culture flask at the same density without the stromal layer cells died.

Head kidney leukocytes

Similarly, cells from the head kidney leukocyte preparation rapidly adhered to the fibroblastic RTS34 cells, flattened (fig. 5.4 c) and proliferated (fig. 5.4 f). These cells had the morphology of macrophages. Proliferation was not as extensive as that seen with RTS11, and did not result in non-adherent progeny cells. Head kidney leukocytes added to a parallel culture flask without stromal cells survived but did not proliferate.
When the culture was examined using phase contrast and UV light, autofluorescence of the macrophages on top of the stromal layer was pronounced. This autofluorescence is a characteristic of rainbow trout macrophages, even those freshly isolated from tissue (personal observation).

4. DISCUSSION

RTS34st is a stromal cell line that supports the growth of macrophage-like cells from various rainbow trout sources and produces conditioned medium that stimulates ^{3}H-thymidine incorporation into DNA of trout leukocytes. The successful establishment of RTS34st with the maintenance of these functions is probably due to the conditions under which it was established. As has been described for successful establishment of human stromal cell lines (Zipori, 1989), RTS34 was only subcultured when all of the cell populations originally present had ample time to increase in number which would enable them to survive passage. Subsequent subcultures were done at long intervals of up to two months. This allowed the stroma to maintain some cell-cell associations, and encouraged the survival of cells that may have required contact with their neighbors.

RTS34st cells are of at least two distinct morphologies, a fibroblastic-like cell and an epithelial-like cell type. Neither of these RTS34st cell types take up DiI-Ac-LDL, and so are unlikely to be endothelial in origin (Voyta et al, 1984). Morphologies of mammalian stromal cell lines are also highly variable: they may be fibroblastic, endothelial, epithelial or adipocytic, and these morphological features do not correlate with their production of cytokines or with their abilities to support hemopoietic development (Deryugina & Müller-Sieburg, 1993). It is unclear whether this heterogeneity reflects phenotypic plasticity of a single cell population or multiple cell populations of different origin and function (Zipori et al, 1985).

Histochemically, RTS34st cells are unremarkable, not staining strongly for any of the assayed enzymes. This is typical of stromal cell lines, which are generally myeloperoxidase negative and may or may not express alkaline phosphatase, acid phosphatase or α-naphthyl-acetate esterase. Acid phosphatase activity was detected in mouse bone marrow cell lines of the MBA series developed by Zipori et al, (1985), and these cell lines actively supported hemopoiesis. In general, however, there seems to be little correlation of enzyme markers with morphology or function in stromal cell lines (Deryugina & Müller-Sieburg, 1993).

The RTS34 stromal layer supports the growth of macrophages and macrophage-like cells from three sources. The first of these are autologous macrophage-like cells, RTS34 progeny cells, which originate as non-adherent round cells and become adherent as they mature. This pattern of development is typical of macrophages, as all other hemopoietic cells start as adherent precursors, and become non-adherent with maturity (Shima et al, 1995). Their identification as macrophages is confirmed by their morphology and their accumulation of high levels of DiI-Ac-LDL, which is taken up by macrophages and monocytes via the scavenger receptor (Goldstein et al, 1979). They are also non-specific esterase, acridine orange and acid phosphatase positive, and highly phagocytic, other rainbow trout macrophage characteristics (Zelikoff et al, 1991).

Secondly, the myeloid cell line RTS11 (chapter 5) proliferates in response to co-culture on RTS34st. These cells are immature cells of the monocyte-macrophage lineage, and proliferate but do not differentiate when co-cultured with RTS34st. Finally, the addition of preparations of rainbow trout leukocytes from head kidney to a confluent contact-arrested monolayer of RTS34st results in the selective adhesion and proliferation of macrophages from the originally mixed population of cells. Co-culture of hemopoietic precursor cells with a growth arrested fibroblast feeder layer is a technique that has proven successful with a variety of cell lines, such as M-MOK, a human immature megakaryoblastic cell line (Itano et al, 1995), and with primary cultures of macrophages from a variety of species (Talbot et al, 1996).

In these systems, as well as in RTS34, the mechanism by which the stromal cells support the developing hemopoietic cells is unknown. The growth supportive ability of RTS34 could be due solely to their production of a soluble factor. In the absence of the stromal cell themselves, conditioned medium from RTS34st enhances the uptake of ^{3}H-thymdine by RTS11 cells and freshly isolated rainbow trout leukocytes. This conditioned medium does not, however, support the proliferation of the autologous macrophage population, RTS34 na. These cells, which appear to be relatively mature macrophages, proliferate in methylcellulose culture in response to LPS (see chapter 6), and proliferate in association with the stromal cell layer, which demonstrates that they do have some capacity to proliferate. They do not, however, proliferate in culture flasks on their own, even with conditioned medium. This suggests that they have an additional requirement not met solely by soluble factors, and that physical contact with stromal cells is

necessary. It is possible that direct physical contact between a stromal cell and a macrophage sends a signal leading to proliferation of the macrophage or that binding of the precursor cell to the fibroblast feeder layer allows the local cytokine concentration to reach sufficient levels to allow each precursor cell to respond fully (Deryugina & Müller-Sieburg, 1993).

The selective association of macrophages with RTS34st fibroblastic-like cells could be related to the production of fibronectin by these cells. Fibronectin has been shown to promote the adhesion and activation of macrophages from various sources (Weinstein et al, 1989; Bohnsack et al, 1985: others). Fibronectin and other extracellular matrix proteins can bind cytokines, which in this form deliver effective signals to developing hemopoietic cells. Granulocyte-macrophage colony stimulating factor (GM-CSF), for example, binds to heparin sulphate, a glycosaminoglycan of the bone marrow extracellular matrix (Gordon et al, 1987; Roberts et al, 1988). This binding is necessary for biological activity. The growth pattern of RTS11 cells could indicate that the fibroblastic cells are producing a localized, possibly matrix bound factor.

The adhesion of RTS11 and head kidney leukocytes to RTS34st could also be mediated by lectins. Primitive hemopoietic progenitors with specific lectins on their surfaces home to specific stromal niches that support or inhibit their development and proliferation (Hardy & Megason, 1996; Tavassoli & Minguell, 1991). Binding of lectins to stromal cells stimulates growth factor production. Addition of the lectin PWM to irradiated bone marrow stroma caused an increase in the production of GM-CSF and IL-3 (Alberico et al, 1987). These observations demonstrate that surface sugars on various stromal cell types are of great importance in hemopoiesis.

RTS34st cells are stimulated to incorporate ^{3}H-thymidine by a variety of lectins, indirectly indicating the presence of particular glycosylation on their surfaces. Lectin binding to cell surface sugars can trigger a signal transduction pathway leading to DNA synthesis, as is commonly demonstrated in lymphocyte mitogenesis assays. Depending on the tissue targeted and on the lectin tested, lectins act as stimulators or inhibitors of cell proliferation. In several human colorectal cancer cell lines (Ryder et al, 1994), peanut agglutinin (PNA), WGA and ConA stimulate proliferation, and PNA, mushroom and coral tree lectins have been shown to be mitogenic to normal vascular cells (Sanford & Harris-Hooker, 1990). WGA

and Con A inhibit the mitogenic actions of several peptide growth factors in human fibroblasts. On the other hand, Con A stimulated thymidine incorporation in mouse lung cells, but inhibited it in cancerous lung cells (Vilarem et al, 1978).

Lectin binding has been examined histochemically in rainbow trout intestinal epithelium (Pajak & Danguy, 1993) and sacular macula (Khan et al, 1991), and has been used to separate out particular cell populations in rainbow trout liver (Blair et al, 1995). There do not, however, appear to be any published reports of lectin stimulation of DNA synthesis by fish cells, other than lymphocytes. Another rainbow trout cell line, clone 1A from peripheral blood (unpublished) also responds to a panel of lectins, but with a different pattern of responses than RTS34st (unpublished results). This suggests that rainbow trout, or possibly fish cells in general, can be stimulated to proliferate by binding of lectins to their surface sugars. The lectin responsiveness of RTS34st could also be peculiar to the particular fish used to initiate the cell line. There are differences in glycosylation patterns between individuals of the same species, for example, the human ABO blood groups are due to differences in cell surface glycosylation, and historically, lectins were used to distinguish between them (Sharon & Lis, 1989). If differences are evident across cell lines, perhaps the responses to a panel of lectins could be used as part of a cell line "signature". This could be useful in the identification of cross-contamination between cell lines, a potential problem in any cell culture lab where a variety of cell lines are used (Kozak, 1992; Hay, 1992).

RTS34st cells secrete a factor or factors into their culture medium that causes increased uptake of ^{3}H-thymidine into DNA in several cell types: freshly isolated peripheral blood and head kidney leukocytes, and RTS11 promonocytic cells. This factor is most active on head kidney leukocytes, and appears not to stimulate spleen leukocytes. This could be due to the cell composition of leukocytes isolated from each tissue source. Isolation of spleen leukocytes with a Histopaque gradient results in a relatively greater number of lymphocytes, and fewer other cells than when peripheral blood or kidney is treated in the same manner. The kidney is a particularly rich source of macrophages (Braun-Nesje, 1981; Rowley, 1990), and it appears that the putative factor(s) has activity for cells of the monocyte-macrophage lineage. Its characteristics and specificity are being investigated further, and compared to the activity of RTS11 conditioned medium described in the previous chapter. While the range of action of the two conditioned media appears to be similar, they elicit different levels of response in

freshly isolated leukocytes, and interact differently with FBS. RTS11 conditioned medium's stimulation of ^{3}H-thymidine incorporation is enhanced by the presence of FBS, while that of RTS34 is inhibited (Ganassin & Bols, 1997b).

Stromal cell lines in general have been valuable sources for the identification and isolation of cytokines, and the availability of a large variety of stromal cell lines differing in their characteristics has been an asset for these purposes (Deryugina & Müller-Sieburg, 1993). RTS34st could well prove to be a source of novel fish cytokines and growth factors, enhancing our understanding of factors controlling the production and activity of the cells of the fish immune system. RTS34st cells also provide a valuable alternate system for culturing rainbow trout macrophages for other studies.

Finally, the growth stimulation of RTS11 cells in co-culture with RTS34st provides a potential model system for the study of macrophage development. The effects of exogenous substances on hemopoietic cell production could easily be assayed using this co-culture system.

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Chapter 6

Development of methods for culturing rainbow trout hemopoietic cells in semi-solid media and for quantifying their growth in response to sera and mitogens

ABSTRACT

As a first step in the identification of growth factors and substances affecting hemopoiesis in fish, a clonal culture system for rainbow trout hemopoietic cells was developed. Of four semi-solid matrices tested, only methylcellulose supported colony formation by rainbow trout head kidney cells. Serum was essential for this, and rainbow trout serum (RTS) gave better results than fetal bovine serum (FBS). Most of the colonies formed in RTS consisted of macrophage-like cells, which suggests the presence of a macrophage colony stimulating factor, analogous to the M-CSF found in mammalian sera. As an alternative measure of proliferation, ^{3}H-thymidine incorporation by cells in methylcellulose was measured successfully. This allowed a comparison of mitogenic responses by head kidney cells in methylcellulose and in liquid culture. With serum or serum plus lipopolysaccharide (LPS), ^{3}H-thymidine incorporation was always higher in methylcellulose cultures. In addition to rainbow trout head kidney cells, non-adherent cells from cultures of rainbow trout spleen stroma were able to form colonies in methylcellulose. Therefore, as well as being useful in identifying hemopoietic growth factors, this clonal culture system should be useful in identifying and quantifying the cellular products of hemopoietic cultures.

1. INTRODUCTION

Clonal culture systems have been invaluable in elucidating the roles of cytokines and growth factors active with mammalian leukocytes, because upon stimulation with the appropriate factors, leukocytes respond by forming colonies. However, for fish hemopoietic cells, such culture methods have been used infrequently and have yet to be optimized. Culture in soft agar has been used and shown to support the growth of carp (Caspi et al, 1980a, 1980b) and rainbow trout leukocytes (Finegan & Mulcahy, 1987). Low melting temperature agarose, commercially known as SeaKem, was used by Moritomo et al, (1993) to demonstrate the response of carp granulocytes to carp serum. Fibrin clot cultures have been successfully applied by a single group to culture hemopoietic cells from rainbow trout. They have examined the effect of the common leukocyte mitogens, PHA, Con A, and LPS on the colony forming ability of head kidney leukocytes (Estepa & Coll, 1992;1993). While methylcellulose is perhaps the most popular choice for clonal culture of mammalian hemopoietic cells, there has been only one report of its use with fish cells (Kodama et al, 1994).

Usually clonal growth in semi-solid medium is determined by counting colonies, a tedious, time-consuming and subjective process. An alternative screening method, that would allow rapid screening of very small samples is therefore desirable. One possible alternative is the use of incorporation of ^{3}H-thymidine to detect increases in cell number. The ability of mesenchymal cells to incorporation thymidine in a methylcellulose and agar culture system has been demonstrated (Assoian et al, 1989), but this sort of assay does not appear to have been tried with hemopoietic cells.

The goals of this chapter are to compare the utility of four semi-solid culture systems for the growth of rainbow trout head kidney leucocytes and to study the proliferative response of rainbow trout head kidney leucocytes to piscine and mammalian sera in the optimal semi-solid medium, methylcellulose. An alternative method of monitoring growth in methylcellulose was also assessed, and the ability of non-adherent cells from rainbow trout spleen stromal cultures (RTS34) to proliferate in methylcellulose was tested.

2. MATERIALS AND METHODS

Evaluation of semi-solid matrices

The cost of each of four commonly used semi-solid matrices was assessed, and initial screening of the four chosen semi-solid matrices was performed to evaluate the relative ease of the procedure, and to demonstrate the successful growth of head kidney leukocytes in that medium. The four matrices were low-melting point agarose (Sea-Kem), as used by Moritomo (1993) soft agar (Difco) as described by Finegan & Mulcahy (1987), fibrin clots formed by the addition of fibrinogen to thrombin (Sigma), as outlined by Coll (1994), and methylcellulose, 4000 cp, (Sigma M-0512), using the method of Hatzfeld et al (1994).

Preparation of methylcellulose

Methylcellulose was prepared using the method described by Hatzfield et al (1994). Briefly, mc was added to a sterile 2 L flask, and autoclaved. Sterile tissue culture grade water was boiled and gradually added to the mc while mixing vigorously on a magnetic stirrer. The mixture was cooled in a laminar flow hood with constant stirring, and sterilized double strength L-15, prepared from powder, was added, to give a final concentration of methylcellulose of 2.1%. The mc mixture was stirred overnight at 4°C, aliquotted into sterile plastic tubes, and frozen at -20°C to break up the fibers. One day prior to use, the frozen mc was thawed at 4°C.

Isolation of head kidney leukocytes

Head kidney leukocytes were chosen for these assays as the yield of macrophages (Secombes, 1990) and a variety of other cells, particularly immature cells, is much greater from kidney tissue than from spleen or peripheral blood.

Head kidney tissue was aseptically removed from a rainbow trout, and placed in 10 mL L-15 supplemented with 10 IU/mL heparin (Sigma). The tissue was then forced through a 100 mesh/ inch metal screen using a pestle to dissociate the cells. The cell suspension was diluted by the addition of 20 mL of L-15 with heparin. Five mL of the resulting cell suspension was placed in a 10 mL centrifuge tube, and a syringe was

used to underlay the cell suspension with 3 mL of Histopaque 1.077 (Sigma). The tubes were centrifuged for 30 minutes at 1300 rpm in an IEC centrifuge with a swinging bucket rotor, and the band of cells formed at the interface of the Histopaque and L-15 was collected. Cells were washed by centrifugation in L-15 without heparin prior to their use, and resuspended in L-15 without serum at a cell density of 10^6 cells/mL.

Preparation of RTS34na cells

Non-adherent cells from a culture of RTS34 were harvested by removing the culture medium and centrifuging at 1000 g for 5 minutes. The cell pellet was resuspended in L-15 without serum, and cells were diluted to a concentration of 10^6 cells/mL.

Serum

FBS was purchased from Canadian Life Technologies. Rainbow trout serum was prepared as previously described (Ganassin & Bols, 1997). Serum was pooled from at least 10 fish, and heat-inactivated by incubation at 56°C for 30 minutes.

Other test substances

In addition to FBS and rainbow trout serum, the common T cell mitogens *Phaseolus vulgaris* lectin (phytohemmaglutinin or PHA), and *Canavalia ensiformis* lectin, (Concanavalin A, or Con A), and the B cell mitogen lipopolysaccharide from *E. coli* (LPS) were tested for their ability to stimulate colony formation.

Initiation of CFC assays

Methylcellulose, 1.5 mL of 2.1%, was placed into plastic vials (sterilized 5 mL scintillation vials). β-mercaptoethanol (5×10^{-5} M) and BSA (1mg/mL, Boehringer Mannheim #1081 489) were added. These additives are commonly used in mammalian CFC assays, and improve colony formation. Other additions were made as described in individual figure legends. The final concentration of methylcellulose used was 1.05% (v/v), determined in preliminary experiments to be firm enough to keep the cells well separated, but still allow colony formation. The methylcellulose and other components were mixed thoroughly using a vortex, and left overnight at 5°C to allow all components to diffuse together and form a homogeneous mixture. The mixture was brought to room temperature the following morning and one volume of cell suspension of either head kidney leukocytes, or RTS34na cells was added to 5 volumes of the mixture. A vortex was used to distribute the cells evenly throughout, and 1 mL syringe was used to

dispense the viscous mixture into the wells of multi-well plates (Falcon) using the following volumes: 100 µL/ well in 96 well plates, 200 µL/well in 48 well plates, and 500 µL/well in 24 well plates.

Scoring of colony formation

Colony counting and identification

Plates were examined using a Nikon Diaphot inverted microscope (Nikon Canada, Toronto, ON) with phase optics, and the number of colonies (clusters of 10 or more cells) were counted. Colonies were photographed, and individual colonies of each type were carefully removed from the methylcellulose using a 25 µL volume glass capillary tube, and either smeared on to microscope slides or deposited onto slides using the Shandon cytocentrifuge, then stained with Wright-Giemsa stain to distinguish their morphology.

Using ^{3}H-thymidine incorporation to quantify colony formation

The ability of cells cultured in methylcellulose to incorporate ^{3}H-thymidine was assessed in 96 well plates by adding 1 µCi/well of thymidine in 100 µL of L-15 to each well, which already contained 100 µL of methylcellulose-containing medium and cells. The methylcellulose dissolved in the additional liquid, which allowed the cells to take up the radiolabel. The assay was otherwise the same as described for RTS11 cells and leukocytes (chapter 4).

3. RESULTS

Evaluation of semi-solid matrices

Rainbow trout head kidney cells consistently formed colonies in methylcellulose, but not in other semi-solid matrices. Upon initiation in methylcellulose, cultures contained isolated, well-spaced cells (fig. 6.1a). Approximately two weeks later, colonies became evident in methylcellulose that had been prepared in L-15 with the appropriate supplement. Depending upon the supplement, the colonies were seen clearly after a 3-4 week incubation (fig. 6.1 b)and were counted easily with the aid of a Nikon inverted phase contrast microscope. By contrast, in fibrin clots, agarose and agar, colonies of head kidney cells formed infrequently or not at all. Methylcellulose was viscous but not solid, and during the course of culture, most cells and developing colonies settled near the bottom of the culture wells. The cells could easily be removed from the matrix for staining or further propagation. A summary of the relative merits of the four semi-solid matrices are presented in table 6.1. Although each method has potential advantages and disadvantages, methylcellulose was selected as the matrix for further study because it consistently yielded colonies, was relatively easy to use, and was inexpensive.

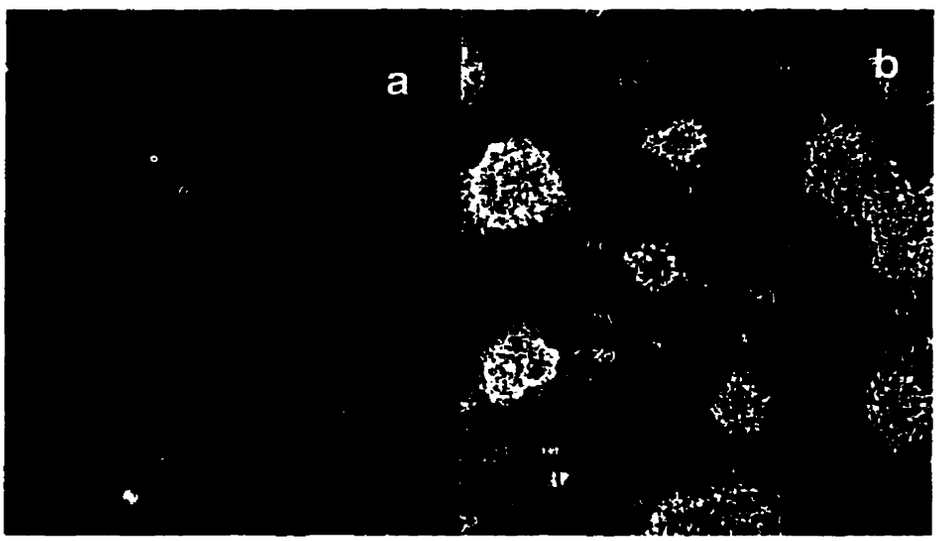

Figure 6.1: Formation of colonies by rainbow trout kidney cells in methylcellulose. Photograph **a** illustrates the appearance of cells in methylcellulose at the time of culture initiation in 1.05% methylcellulose with 10% FBS and PHA (20µg/mL); photograph **b** shows the culture 4 weeks later. At initiation, individual cells were well dispersed within the methylcellulose so that the spaces between cells were sufficient for the growth of colonies. Colonies were clearly evident 3-4 weeks later.

Table 6.1: Comparison of agarose, agar, methylcellulose and fibrin clots as semi-solid matrices for the culture of hemopoietic cells

Component (concentration/mL)	Cost [e]	Advantages	Disadvantages
low-melting point agarose[a] (10mg/mL)	$608.00/ 100g $.061/ mL		• requires exposure of cells to potentially lethal temperatures • viscous, requires practice to dispense
agar[b] (3 mg/mL)	$34.90/ 100 g $.001 / mL	• inexpensive • easy to handle	• requires exposure of cells to potentially lethal temperatures • removal of cells for fixation or propagation is difficult
fibrin clot[c] (fibrinogen: 0.4 mg/mL) (thrombin: 4 NIH U/mL)	$ 18.00/100 mg $.072/ mL $ 122.20/ 100 U $4.89/ mL	• fixation of cells is easy	• high cost of components • technically difficult • fibrinogen must be extensively dialyzed to remove salts • variability between batches • thrombin and fibrinogen are themselves mitogenic and may effect results
methylcellulose[d] (10 mg/mL)	$22.40/ 100 g $.002/ mL	• inexpensive • easy to prepare • does not dehydrate quickly • cells are not exposed to temperatures beyond their thermotolerance • proliferation can be measured in situ • cells can easily be removed from mc for staining and propagation	• viscous, requires practice to dispense • methods for staining for permanent record are time-consuming [f]

[a] concentration from Moritomo et al, 1993
[b] concentration from Metcalfe ***
[c] concentration from Coll,1994
[d] concentration from this study
[e] Prices from 1996 Sigma Chemical catalogue, in Canadian dollars
[f] Ozawa et al, 1982; McMahon & Hankins, 1980

Influence of sera on colony formation

Serum was an essential supplement for the formation of colonies in methylcellulose

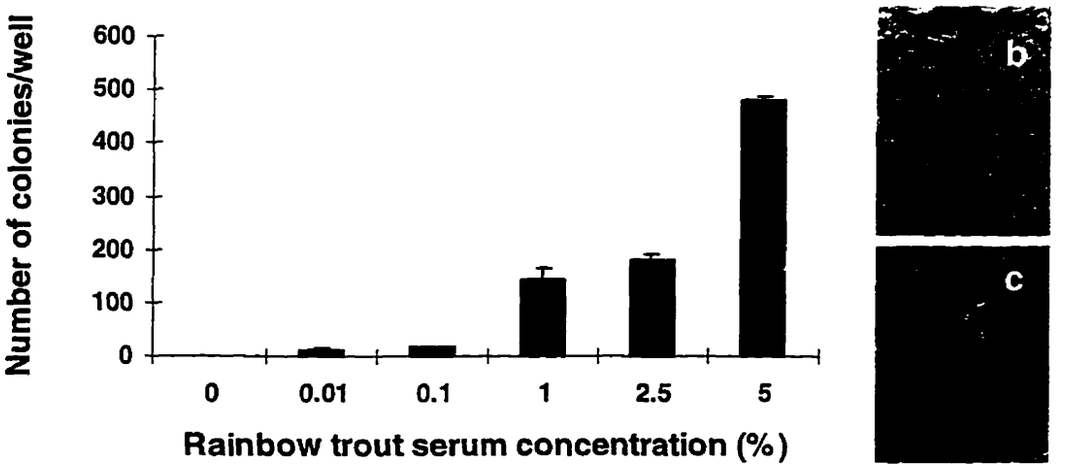

Figure 6.2: Rainbow trout serum promotes colony formation by head kidney leukocytes, and the proliferation of adherent macrophage-like cells. In **a**, 30,000 head kidney leukocytes were distributed into the wells of a 48 well tissue culture plate in 200 µL of L-15 medium with 1.05% methylcellulose with the indicated concentrations of rainbow trout serum. Colonies were counted 20 days after culture initiation. Values represent the mean of triplicate wells, with error bars indicated the standard deviation. Adherent macrophage-like cells of rounded, **b**, or stellate, **c**, morphologies also appeared in cultures supplemented with RTS.

by rainbow trout head kidney cells, and rainbow trout serum(RTS) gave more consistent results than fetal bovine serum (FBS). RTS consistently promoted the development of colonies, with the number of colonies increasing with increasing concentrations of RTS (fig 6.2). On the other hand, with 10, 20 or 30% FBS as the culture supplement, colonies appeared sporadically, with no obvious correlation with FBS concentration. However, FBS plus RTS consistently supported colony development. Colonies became evident after 10 days of culture.

The colonies that formed in methylcellulose varied in overall appearance and in cellular composition. Three distinct colony types were distinguished (fig. 6.3). A type A colony consisted of thirty or more small, uniform cells that were tightly packed together (fig 6.3 a). A type B colony (fig. 6.3 b) was smaller (10-20 cells) and composed of much larger, granular cells that were identified in cytospin preparations as macrophages (fig. 6.3 d). With RTS as a culture supplement, the colonies that formed were almost always type B. A type C colony contained small round to tear-drop shaped cells that were dispersed rather than tightly packed (fig. 6.3 c). With FBS, the colonies that formed were usually type C. These colonies were particularly difficult to count precisely, as they tended to be very large and the boundaries of individual colonies overlapped and

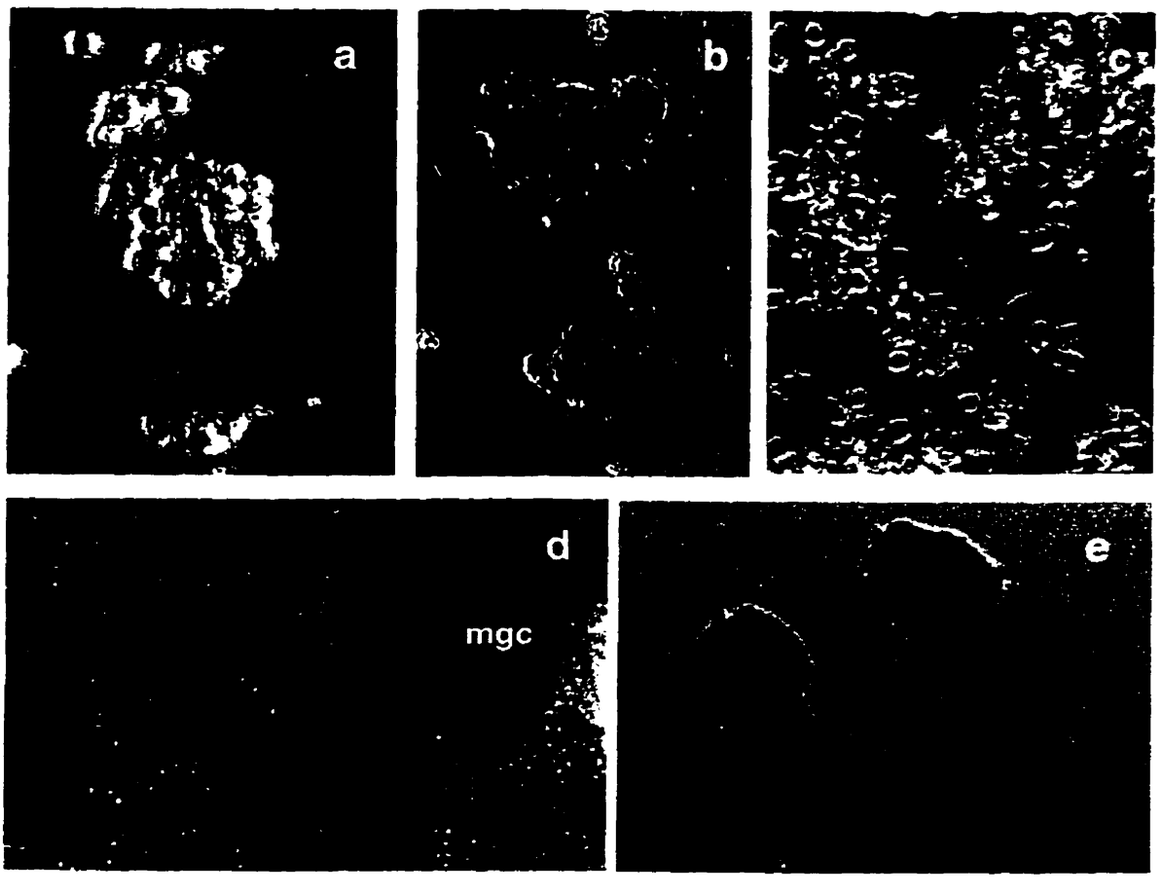

Figure 6.3: Three distinct colony types formed by rainbow trout kidney cells in methylcellulose. A type A colony is shown in **a**, a type B colony in **b**, and a type C colony in **c**, as viewed with a phase contrast microscope (original mag. 200x). In a and c, the mitogenic supplement was 10% FBS, in b, 10% FBS and 5% RTS. Photographs d and e are of cells taken from colony types B and C, collected onto slides by cytocentrifugation, and stained with Wright-Giemsa (mag 400x). In **d**, cells from a type B colony show macrophage-like characteristics, including a multinucleated-giant cell (**mgc**).

were hard to distinguish. All three of the different colony types were seen in small numbers in cultures with the mitogen PHA. The cells from type A and type C colonies were similar in appearance in cytospin preparations (fig. 6.3 e). While the cell type was not identifiable, both had oval to round nuclei and were smaller than macrophages, but larger than lymphocytes.

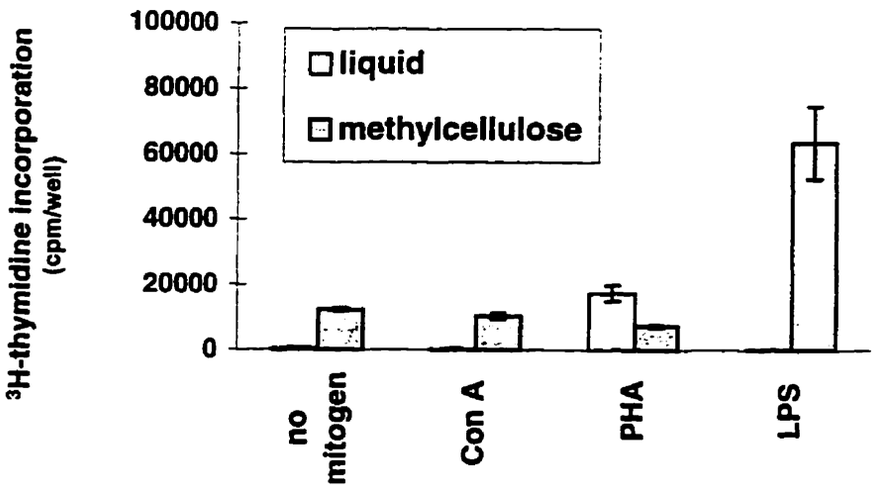

Figure 6.4: Comparison of mitogenic responses by head kidney leukocytes in methylcellulose and in liquid culture. For methylcellulose cultures, 15,000 leukocytes in 100 µL of 1.05% methylcellulose in L-15 with 10% FBS and the indicated treatment were dispensed into each well of a 96 well plate. For liquid cultures, treatments were identical except that methylcellulose was not included in the medium. In both culture types, the dose of Con A and PHA were 20 µg/mL, while LPS was present at 100 µg/mL. After 10 days of incubation, incorporation of ^{3}H-thymidine was measured as described in the Materials and Methods. Values shown are the mean of triplicate treatments, with error bars representing the standard deviation.

Monitoring proliferation in methylcellulose

Several problems were encountered with counting colonies formed in methylcellulose. Firstly, the large scattered type C colonies were difficult to enumerate. Secondly, macrophage colonies often adhered to the tissue culture plate and spread, making the colony boundaries indistinct and the colonies difficult to count. This was particularly true with cultures more than 20 days old. With the addition of LPS to serum-containing medium, an additional problem became evident. Single cells and small cell clusters (2-5 cells) which were too small to be considered colonies, appeared between the larger colonies which were also seen with serum alone. The cells in these clusters were generally larger and rounder than the leukocytes present at the time of culture initiation. Thus, potential growth-promoting activity could be missed by direct colony counting, because the numbers of larger colonies remained unchanged by LPS. Therefore, an possible alternative method of measuring growth, ^{3}H-thymidine incorporation, was investigated with cells in methylcellulose.

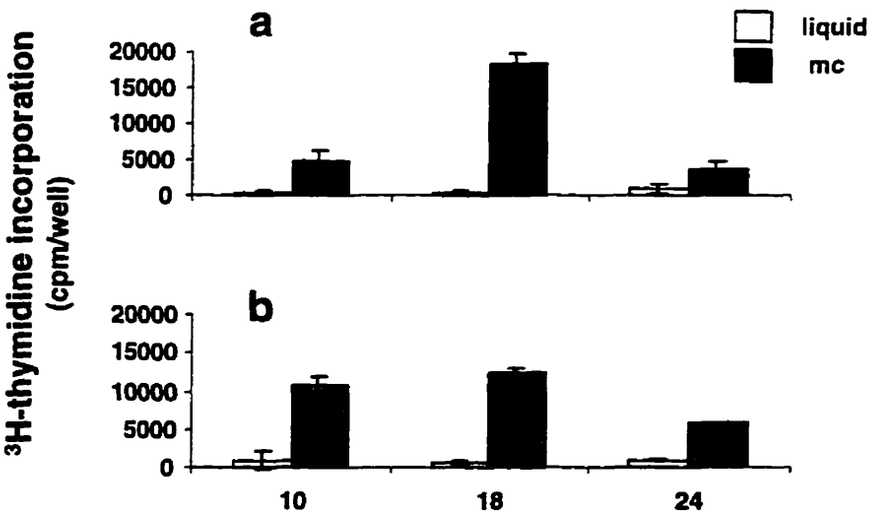

Figure 6.5: Response of head kidney leukocytes to different sources of sera. (a), FBS, and (b), FBS + RTS, in liquid and methylcellulose culture. For methylcellulose cultures, 15,000 leukocytes in 100 µL of 1.05% methylcellulose in L-15 with 10% FBS were dispensed into each well of a 96 well plate. For liquid cultures treatments were identical except that methylcellulose was not included in the medium. Incorporation of 3H-thymidine was measured after 10, 18, and 24 days as described in the Materials and Methods. Values shown are the mean of triplicate treatments, with error bars representing the standard deviation.

The enhancement of ^{3}H-thymidine incorporation by head kidney cells in response to mitogenic supplements was detectable in 1.05% methylcellulose, using the Skatron cell harvester. This is shown in fig 6.4. Head kidney leukocytes responded strongly to LPS. By contrast, with the addition of Con A or PHA to cultures with either FBS (not shown) or RTS (fig 6.4), ^{3}H-thymidine incorporation was not enhanced. Similarly, in colony counting assays, neither of these treatments increased colony formation above their serum controls, nor increased the number of single cells and clusters. Therefore, ^{3}H-thymidine incorporation is an additional, and perhaps, more encompassing measure of the proliferative or mitogenic response in methylcellulose.

Influence of methylcellulose on mitogenic response of head kidney leukocytes

The proliferative response to sera by rainbow trout leukocytes was better in methylcellulose than in liquid culture (fob 6.5). In liquid cultures with either 10% FBS or 10% FBS + 2% RTS, ^{3}H-thymidine incorporation by head kidney leukocytes was low for at least 24 days. In methylcellulose with the same serum supplements, ^{3}H-thymidine incorporation remained high for at least 24 days. As measured by ^{3}H-thymidine incorporation, the proliferative response to serum was always at least four and as much as fifty fold higher in methylcellulose cultures (fig. 6.5).

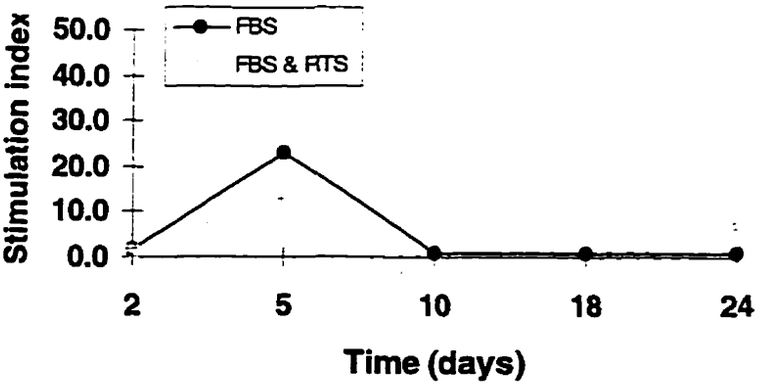

Figure 6.6: Time course of PHA stimulation in liquid culture with sera supplements from different sources. 50,000 leukocytes in 100 µL of L-15 with 10% FBS, or 10% FBS + 2% RTS were dispensed into each well of a 96 well plate. PHA (20 µg/mL) was added, and incorporation of ^{3}H-thymidine into triplicate was measured at the indicated times after addition, as described. Standard deviation was ≤ 15%. Stimulation index is the mean of the treatment/mean of the control, which contained serum but no PHA.

As well, the proliferative response to specific mitogens was profoundly dependent on whether the cultures were in liquid or methylcellulose medium, and whether RTS was present or not. In liquid culture with FBS, PHA elicited a strong mitogenic response within the first 3-7 days of culture but not for longer culture periods (fig 6.6). However,

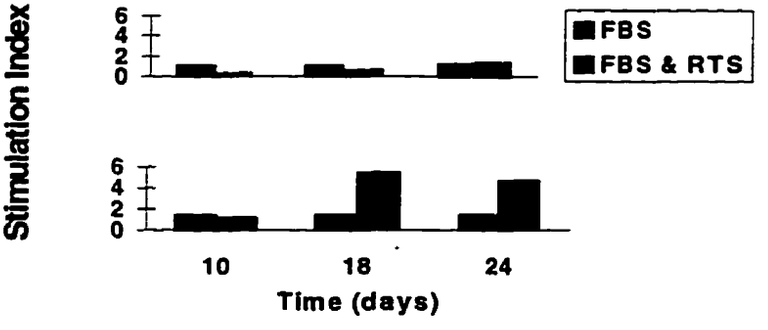

Figure 6.7: Stimulation by LPS in liquid (*top*) or methylcellulose (*bottom*) culture. For methylcellulose cultures, 15,000 leukocytes in 100 µL of 1.05% methylcellulose in L-15 with 10% FBS were dispensed into each well of a 96 well plate. For liquid cultures treatments were identical except that methylcellulose was not included in the medium. LPS (100 µg/mL) was added to each well, and incorporation of ^{3}H-thymidine was measured 10, 18, and 24 days after addition, as described in the Materials and Methods. Standard deviation of all treatments was ≤ 15%. Stimulation index is the mean of the treatment/mean of the control, which contained serum but no LPS.

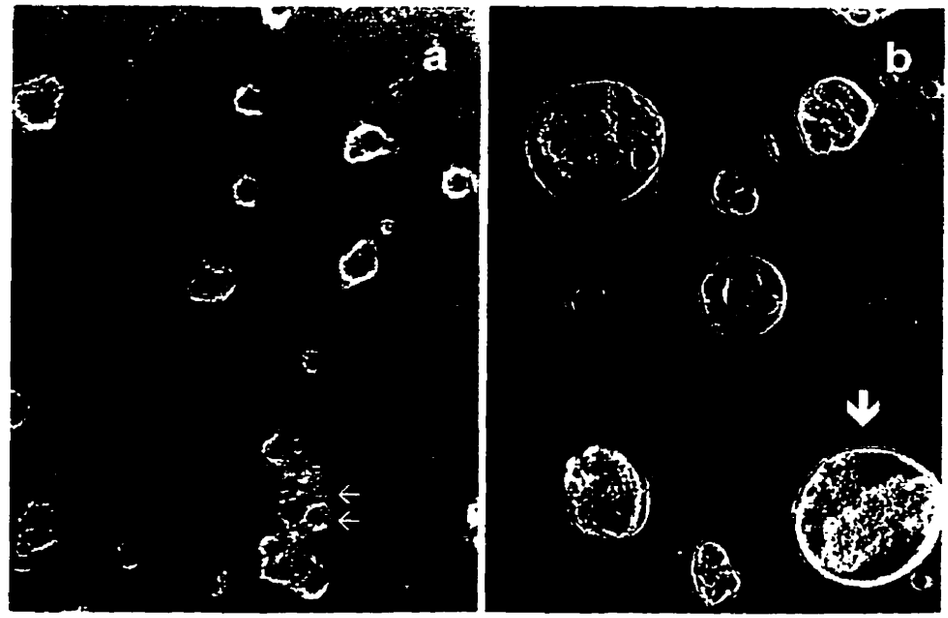

Figure 6.8: RTS34st form colonies in methylcellulose. RTS34n/a colonies formed in response to PHA, **a** , are small and consist of large, granular cells (←). Colonies formed in response to LPS, **b** , are larger and each appears to be surrounded by a gas bubble (⬇).

in liquid cultures with FBS plus RTS, PHA enhanced ^{3}H-thymidine incorporation for at least 18 days. In methylcellulose with FBS alone or with FBS plus RTS, PHA failed to enhance 3H-thymidine incorporation during a 24 day culture period. These results contrast and. completely with the results for another mitogen, LPS. In liquid culture with FBS alone or FBS with RTS,LPS was not mitogenic. However, in methylcellulose with FBS plus RTS, but not with FBS alone, LPS strongly stimulated ^{3}H-thymidine incorporation at 18 and 24 days (fig. 6.7).

Growth in methylcellulose of non-adherent cells from RTS34 cultures

Growth of the non-adherent cells (RTS34na) from RTS34 cultures was examined in methylcellulose with 30% FBS alone, or with 30% FBS plus either PHA (10 µg/mL) or LPA (100 µg/mL). Only a few colonies formed with 30% FBS alone, but the number was enhanced with LPS. Both mitogens changed the appearance of the colonies. The colonies formed with PHA (fig. 6.8) were larger than with FBS alone and consisted of large, granular cells. With LPS, even larger colonies formed, and curiously, the colonies were surrounded by what appeared to be gas bubbles (fig. 6.8).

4. DISCUSSION

Evaluation of semi-solid matrices

Methylcellulose has many advantages over other methods used in CFC assays, and was chosen as the matrix for the culture of rainbow trout hemopoietic cells. Methylcellulose is inexpensive, easy to prepare, does not dry out for long periods, and allows the cells to be removed easily for staining and propagation. Unlike the use of either agar or agarose, the use of methylcellulose does not expose rainbow trout cells, which survive only short periods at temperatures higher than 28°C, to temperatures beyond their thermotolerance (Mosser et al, 1986).

Rainbow trout serum and colony formation

Rainbow trout serum appears to be an important supplement for the formation of substantial numbers of colonies by fish head kidney leukocytes in semi-solid matrices. In the current study colony formation was absent without serum, sporadic with FBS, and enhanced by increasing concentrations of rainbow trout serum (RTS). In another study using rainbow trout kidney leucocytes in methylcellulose, Kodama et al (1994) observed few colonies in the absence of serum and an increasing number with increasing RTS concentrations. In both studies the cells of these colonies appeared to be macrophages. Moritomo et al, 1993, showed similar colony development with carp kidney granulocytes grown in agarose. This suggests that RTS and possibly other fish sera contain macrophage-colony stimulating factor (M-CSF) or granulocyte/ macrophage-colony stimulating factor (GM-CSF), or the fish equivalent.

Monitoring proliferation in methylcellulose

The use of a ^{3}H-thymidine incorporation assay in methylcellulose cultures has at least two advantages over colony counting as a method of scoring growth promoting activity. Firstly, ^{3}H-thymidine incorporation is simple to perform and quick, and could be used as a rapid screening method, identifying test substances that have effects on leukocyte proliferation, in 96 well plates. This has the advantage of using only small amounts of extracts, and could be used to screen cell culture supernatants and crude extracts from organs of rainbow trout for their production of colony stimulating factors. Promising additives could then be used in larger scale assays, with colony counting as

an end point. The initial screening step could save both time and resources. Scoring colony formation by ^{3}H-thymidine incorporation is simple, rapid and permits a quantitative and objective analysis of colony formation. It is not, however a complete substitute for colony counting. This is because the observation and staining of colonies is necessary to determine exactly what types of cells are responding.

A second advantage is that ^{3}H-thymidine incorporation allows the detection of mitogenic responses that do not result in the production of enough cells to be scored as colonies. This was the situation seen with LPS and rainbow trout head kidney cells, where the formation of colonies did not adequately reflect the growth-promoting effects of LPS. This was also noted by Estepa & Coll (1992), in fibrin clot cultures. They found that no stimulation of colony formation was apparent when LPS was used, but that LPS promoted cell survival of an adherent type of cell.

Several interrelated phenomena likely contributed to the greater ^{3}H-thymidine incorporation by head kidney cells in methylcellulose culture than in liquid culture. Overall, methylcellulose seems to provide a superior physical environment for the proliferation of these cells, as cell survival is greatly increased. Additionally, methylcellulose may keep polypeptide growth factors released by cells concentrated near the cells and thus aid the autocrine stimulation of growth. Indeed, this could also be extended to paracrine growth stimulation. For example, the increase in ^{3}H-thymidine incorporation by the head kidney cells in response to LPS was observed only after 18 days of growth in methylcellulose. In this time colonies of macrophage-like cells have appeared independent of LPS. These colonies could release a paracrine growth factor(s) that stimulates ^{3}H-thymidine incorporation by a different cell type. LPS would cause the increase in ^{3}H-thymidine incorporation by increasing the release of the growth factor(s) from the colonies of macrophage-like cells (see Figure 4.14) and/or by increasing the responsiveness of the target cell to the growth factor(s).

The modulation of the proliferative response by the semi-solid matrix has been seen with other matrices. Both fibrinogen and thrombin, the two components required for fibrin clot culture, have been shown to modify the mitogenic response of various hemopoietic cell types. Fibrinogen increased the effect of IL-3 on early human progenitor cells (Zhou et al, 1993), and was mitogenic to T and B cell lines (Levesque et

al, 1986). Thrombin enhanced human T-cell (Naldini et al, 1993) and mouse lymphocyte (Scher, 1987) proliferation. Their use in CFC assays could thus be a confounding factor.

Effect of lectins on colony formation by fish leukocytes

The response of fish blood cells to PHA in semi solid medium is complex. Rainbow trout cells formed several types of colonies in methylcellulose in response to culture with PHA, confirming the results reported by Estepa & Coll (1992), with rainbow trout head kidney leukocytes cultured in fibrin clots. They found that four morphologically distinct types of colonies formed with PHA treatment. Finegan & Mulcahy (1987) showed 2 types of colonies forming from peripheral blood lymphocytes cultured in agar, after an initial liquid culture step to promote blast formation. One of these consisted of lymphocytes and lymphoblasts, while the second type was not identifiable. Caspi et (1990, 1992) found PHA necessary to stimulate colony formation by carp peripheral blood lymphocytes. Although in mammals it is classically considered a T cell mitogen, PHA seems to stimulate the growth of numerous cell types in fish.

No colony formation was observed with Con A. Estepa & Coll (1992) found that a dose of 100 µg/mL was needed to demonstrate the same level of colony formation seen with 2 µg/mL PHA. Perhaps a higher concentration of Con A than that used in this assay is necessary to stimulate colony formation.

Effect of LPS on colony formation by fish leukocytes

Colony formation in response to LPS appears difficult to demonstrate with fish leukocytes. Although rainbow trout peripheral blood lymphocytes formed colonies in agar in response to LPS (Finegan and Mulcahy, 1987), LPS did not enhance colony formation by head kidney leukocytes in methylcellulose in this study or in fibrin clots in a study by Estepa and Coll (1992). Estepa and Coll also tried LPS from a variety of aquatic bacteria (*Vibrio anguillarium, Aeromonas salmonicida, Yersinia ruckeri, Aeromonas hydrofila, Aeromonas florescens, and Aeromonas sobria*) but still colony formation was not increased. The use of ^{3}H-thymidine incorporation in methylcellulose should aid further studies on the action of LPS on fish leucocytes.

Colony formation by non-adherent cells from RTS34 cultures

The non-adherent cells from the rainbow trout spleen stromal cell cultures (RTS34) were able to form colonies in methylcellulose, demonstrating that methylcellulose cultures could be used in the future to monitor the cellular products of hemopoietic cultures. This particular example had several complexities. The non-adherent cells from the rainbow trout spleen stromal cell cultures (RTS34) were identified previously as belonging to monocyte/macrophage lineage (Chapter 5). Colony formation by these non-adherent cells was enhanced in 30% FBS by LPS, but the proliferation of RTS11 cells was inhibited in the presence of FBS by LPS. The different results could indicate that the response to LPS changes in the macrophage lineage at different stages and/or in different organs.

Surprisingly, the colonies that formed in LPS-containing medium appeared to have a bubble of gas around them. LPS stimulates the release of several oxygen species, including H_2O_2 and O_2 (Auger & Ross, 1992), and this apparent gas bubble could contain one of these or a product formed by their reaction with chemicals found in the growth medium, L-15.

Other potential applications of this method

In addition to screening substances for hemopoietic growth modulating activities, the semi-solid nature of methylcellulose allows colonies to be easily removed from the matrix . This method therefore holds promise for the development of clonal cell lines of fish leukocytes and other cells. When substances necessary for their survival are identified in these cultures, clones could be picked from methylcellulose and transferred to medium containing these substances, and propagated as clonal cell lines.

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Chapter 7

The cell culture approach to fish hemopoiesis: variations, problems and the future

Three general types of cell cultures have been developed as tools to study hemopoiesis in rainbow trout. These culture types differ in the amount of cell separation that is done prior to culture initiation, the time for the culture to yield results, and the life of the culture. One type is long-term hemopoietic cultures. These were developed from the spleen (Chapter 2) and head kidney (Chapter 3) and initiated from the almost complete organ with little or no attempt to separate specific cell types before hand. The result is the growth of a complex mixture of cells that can support the development of differentiated products. These cultures are slow to develop and persist for many months but not indefinitely. From these cultures, cell lines (Chapters 4 and 5) can be developed. The development of these can take months to years but they have the potential to be grown indefinitely. Cultures in semi-solid media are initiated with isolated leukocytes (Chapter 6) and are maintained only for 3-4 weeks. Each culture approach yields valuable information but has problems to be overcome to maximize their usefulness in the future.

Long term hemopoietic cultures: The problem of identifying dendritic cells

One of the biggest problems with long-term hemopoietic cultures at this time is identifying their cellular products. This is due mainly to the paucity of antibodies specific to rainbow trout blood cell types. The problem is most complex with dendritic cells and this is discussed below.

In many ways, dendritic cells are very similar to macrophages (see fig. 7.1 for a summary of similarities and differences). Macrophages are found in the same tissues and organs, and some types can morphologically resemble dendritic cells, although there are subtle differences. Macrophages show remarkable phenotypic plasticity, and display a wide variation in morphology depending upon their location and state of activation. For example, in mammals, resident bone marrow macrophages, which serve as nurse cells for developing hemopoietic cells (Crocker & Milon, 1992) have many ramifying processes or "dendrites" and look very much like dendritic cells.

Another complicating factor is that dendritic cells and macrophages may both develop from a hemopoietic cell stage as late as the monocyte. Peters et al (1993) have shown

that monocytes isolated from human peripheral blood will mature in culture into macrophages under one set of culture conditions, or dendritic cells under another. The precise signals that trigger differentiation into one state or another are unclear. These findings suggest that dendritic cells are intimately related to macrophages, and arise from the same precursor cells. The relationship between dendritic cells, monocytes, and macrophages requires some clarification. Perhaps dendritic cells are a class of macrophage with slightly different characteristics.

The dendritic cells arising from rainbow trout spleen in long-term culture differed morphologically from macrophages, although the morphological versatility of macrophages casts some question upon this as a criterion. Dendritic-like cells were smooth surfaced, and less well-spread on the tissue-culture plastic than macrophages. They possessed veils and many dendrites of various sizes emerging from a relatively small cell body. They had irregularly shaped nuclei, and few cytoplasmic vacuoles, and showed little ability to phagocytose foreign particles. They did not exhibit positive staining for any of the leukocyte enzymes shown in table 7.1. The most interesting evidence was their dramatic motility, particularly as shown in time-lapse video. The rainbow trout dendritic-like cells constantly extended and retracted their processes, veils or dendrites, while macrophages seen in the same field gently flowed over the culture surface in amoeboid fashion. Movement of vesicles in the macrophages, and ruffling of their edges was also evident.

The presence of dendritic cells (DC) in the tissues of rainbow trout, or indeed, in any fish, was controversial but is becoming commonly accepted. The possibility that fishes may possess Langerhans cells (LC) was first proposed by Mittal et al (1980), in the swamp eel, *Monopterus cuchia*. Cells with the unusual morphology of dendritic cells have been isolated from the skin of rainbow trout (Peleteiro & Richards,1985; Davidson et al, 1993). These may be analogous to human LC, which are antigen presenting cells (APCs) present in the skin that carry antigen via the afferent lymphatics into the paracortex of the draining lymph nodes, where the antigen is presented to lymphocytes (Roitt, 1993).

Table 7.1: Characteristics used to distinguish dendritic cells from macrophages

	Dendritic cells	Macrophages
FUNCTIONS		
phagocytosis	+/-[1,2]	+++[1,2]
T cell stimulation	+++[1,2]	+[1,2]
pinocytosis	+/-[1]	++[1]
ENZYMES		
myeloperoxidase	+/-[1,2]	+++[1,2]
a-napthylbutyrate esterase (NSE)	+/-[1,2]	+++[1,2]
ATPase	+/-[1]	+++[1]
acid phosphatase	+/-[1]	+++[1]
ANTIGENS[A]		
MHC class II	+++[2]	+[2]
Lamin A/C, lamin B	+++[2]	+++[2]
INDUCERS OF DIFFERENTIATION		
IL-3 + M-CSF	+[2]	+[2]
tocopherols & choecalciferol	+/-[2]	?
unknown serum factors	-[2]	+[2]
MORPHOLOGICAL CRITERIA		
veils	++[1,2]	-[1,2]
dendrites	+++[2]	+/-[2]
phagolysosomes, vacuoles	-[1,2]	+++[1,2]
stretched, irregular nucleus	+++[2]	-[2]
round, regular nucleus	-[2]	+++[2]
ruffled surface when adherent	no[1]	yes[1]
smooth surface when adherent	yes[1]	no[1]
BEHAVIOUR IN CULTURE		
glass adherence	transient[1]	strong[1]
increase in size and number of organelles	no[1]	++[1]
increase in number of processes	yes[1]	no[1]
endocytosis increases	no[1]	yes[1]
MOVEMENT		
constantly extend and retract processes	+++[1]	-[1]
membrane ruffling, formation & centripetal migration of peripheral pinocytic vesicles	-[1]	+++[1]

Parameters are - (negative) to +++ (strongly positive)
Numbers refer to publications listed below
[A] Only antigens of a general, i.e. not species specific nature are included
compiled from [1] Steinman et al, 1980 & [2] Peters et al, 1993

After intramuscular injection of *Aeromonas hydrophilla* in carp, antigen was found associated with cells of the melanomacrophage centres at least one year following

immunization, and Ig secretion in these structures was increased. This suggests the presence of immune complexes such as those which are retained on follicular dendritic cells in mammals (Secombes & Manning, 1980). Ellis et al (1976) found that intraperitoneally injected particles in the plaice, *Pleuronectes platessa* L., associated with cells with a dendritic morphology.

Several authors have speculated on the occurrence of dendritic cells in fish, although they have yet to be isolated. In mammals, dendritic cells are a trace cell type, and a difficult, multi-step isolation procedure is necessary for their purification. The absence of reports of isolation of dendritic cells from fish may be due more to the lack of attempts to find them, rather than their absence.

The long-term spleen culture system described in chapter 2, while allowing the production of dendritic-like cells, did not produce them in sufficient quantities to allow extensive further experimentation. The time from culture establishment until cells could be harvested was long. Most importantly, functional hemopoietic cultures did not develop from every fish, but randomly arose from a small percentage of them. All cultures require routine care and observation, and, finally, time constraints made the use of this culture system impractical. Efforts were thus turned to the development of cell lines from spleen hemopoietic tissues, which had the potential to ensure a more reliable supply of cells, in larger numbers.

Cell lines

The general procedure for initiating cell lines from fish is presented in the appendix (Ganassin & Bols, 1997). The most important consideration, generally, in successful establishment of cell lines from rainbow trout is not to rush the time of the first subculture. This is often true with cell cultures from other species. Zipori (1989), who started many functional stromal cell lines from mouse bone marrow, pointed out the importance of not applying strong selective pressures when trying to establish cell lines of rare cell types. He recommended maintaining cultures in their original flasks for many months, and avoiding the use of trypsin, which can damage sensitive cells. Jörgen Fogh (1978), who established many hundreds of human tumor cell lines, points out that, for successful cell

line establishment "Patience is pertinent, and we have become even more patient as the years have passed".

A major problem with developing hemopoietic cell lines from rainbow trout and other fish is that the cell lines arise spontaneously and the mechanism behind their immortalization is unknown. There is little or no control over what types of cell lines to expect. Direct immortalization procedures, such as the use of oncogenic viruses and genes as has been done with mammalian cells, would allow for greater control. However, these have been used only rarely with fish (Tamai et al, 1993; Burns et al, 1993) but hold promise for the future.

Short-term primary cultures in semi-solid medium

Proliferation in semi-solid medium was optimized for head kidney leukocytes. The colonies that form in these cultures can be used to characterize hemopoietic precursor cells and to identify factors that regulate hemopoietic cell proliferation and differentiation. The major problem to be overcome with semi solid medium is the development of a more rapid and convenient method of monitoring proliferation.

Future directions

Despite the problems to be overcome, in the future, the three cell culture approaches can be used in conjunction to help unravel the network of controls involved in regulating hemopoiesis and the activities of blood cells in fish. For example, indications are that two spleen cell lines, RTS11 and RTS34st are producing factors that have specific activity on fish leukocytes. The next step will be to screen crude organ extracts and the two described cell line and several other rainbow trout cell line supernatants for growth promoting activity. One method will be using ^{3}H-thymidine incorporation by head kidney leukocytes to measure growth both with and without methylcellulose. This is necessary because it is obvious that cells respond differently in the two types of medium. Promising extracts will be identified, and used in a larger scale methylcellulose colony forming assay to determine what types of cells are being produced.

Using these assays as a screening method to monitor activity, factors from culture supernatants and crude extracts can then be purified. This is an approach that does not

appear to have been applied in fish cytokine research, but has resulted in the identification of many mammalian cytokines and growth factors.

The cell lines RTS11 allows many interesting types of research. By varying culture conditions and additives, it may be possible to induce maturation of these cells. There are indication that PMA induces a macrophage-like phenotype, and it should be determined whether or not this is accompanied by measurable functional changes. If so, these cells will provide a model system of macrophage development in rainbow tour, similar to that supplied by the HL-60 human cell lines. It will be interested to determine whether RTS11 be induced to form granulocytic cells, or if are they committed to the macrophage lineage, and what factors will influence their development.

In addition, co-culture of RTS11 with RTS34st is a potential model system for hemopoiesis in fish. Approaches commonly using to assay for growth factors affecting hemopoietic precursor cells, such as ^{3}H-thymidine incorporation or growth in semi-solid media is necessary to allow a quantitative approach, but do not address several basic facts. Hemopoiesis is an extremely complex process. Cells develop *in vivo* in close contact with one another, with complex interactions taking place between stromal and developing hemopoietic cells. The local "cocktail" of cytokines and growth factors that each cell may encounter in hemopoietic tissue may be unique (Zipori, 1989). Long-term spleen and kidney hemopoietic cultures imitate the *in vivo* condition, but are themselves complex. Being able to reconstruct *in vitro* systems from basic, well-characterized cellular components, in an attempt to mimic the *in vivo* environment, provides an additional method for investigating hemopoiesis and studying cell-cell interactions. This is an important application of stromal and leukocyte cell lines.

In more general terms, the availability of new cell lines of hemopoietic tissues provide a source of cells for various purposes. An interesting use may be for the propagation of intracellular parasites, or bacteria such as the causative agent of bacterial kidney disease, *Renibacterium*, which is refractile to culture.

Monocyte/macrophage cell lines are also useful in studies of antigen processing and presentation. In addition, a homogeneous source of macrophage-like cells may have applications in immunotoxicology studies. The complexity of the immune system makes

the examination of immunotoxic substances difficult, particularly *in vivo.* Toxicants can subtly affect the immune system by interfering with the development of a particular lineage of hemopoietic stem cell, and assays of effects as subtle as this have yet to be developed (Burrell et al, 1992). A simplified hemopoietic cell culture system may help to directly identify this sort of effect.

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Appendix

Development and growth of cell lines from rainbow trout, Oncorhynchus mykiss

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INTRODUCTION

The applications of fish cell lines are numerous. They continue to be used for detecting and studying fish viruses, which was the original motivation for their development. A rapidly expanding application is in the field of toxicology (Babich & Borenfreund 1991). In this discipline, cell lines can be used in *in vitro* assays that are simpler, more rapid, and less expensive than tests with whole fish. Other subjects making use of cell lines are the molecular biology, physiology, endocrinology and immunology of fish (Bols & Lee, 1991).

The development and growth of fish cell lines (figure 1) uses the same basic methodology as that developed for mammalian cells. The growth media consist of basal media that are commonly used for mammalian cells supplemented with fetal bovine serum (FBS). One difference is a reduced requirement for glutamine (Bols *et al.* 1994). Leibovitz's L-15 is the basal medium most commonly used to culture fish cells. Its unique amino acid composition allows buffering by free gas exchange with the air, and does not require the use of added buffers or a CO_2 incubator (Leibovitz, 1963).

Another important difference is that proliferation takes place over a wide temperature range but usually occurs at temperatures much lower than 37 °C (Bols et al., 1992). Fish cell lines may conveniently be grown at room temperature.

Many fish cell lines have been described. A recent review of the literature counts 159 fish cell lines from 74 fish species representing 34 families of fish (Fryer & Lannan, 1994). Nearly all these cell lines grow in an anchorage-dependent manner. The cell lines have been generally derived from a limited number of organs, with the embryo being perhaps the most common source. What follows is a reproducible method for developing and growing cell lines from nearly all organs of rainbow trout.

REAGENTS AND SOLUTIONS

Initiation solution

Initiation solution is buffered saline supplemented with high concentrations of antibiotics. To prepare enough initiation solution to start 5 separate cultures, add 50 µl of Gentamicin (Sigma G1397) and 500 µl of Fungizone (Gibco 15295-017) to 25 mL of sterile buffered saline solution, such as Hank's Buffered Saline Solution or Phosphate Buffered Saline. The saline used must contain magnesium, which is a cofactor for the activity of collagenase.

Collagenase A solution

To prepare 500 mL of 1 mg/mL collagenase A (Boehringer Mannheim, Laval, Que), add the contents of a 50 mg bottle to 500 mL of buffered saline solution. To sterilize, first prefilter the solution through a glass fiber filter, then filter with a 0.2 µm filter, such as a Gelman VacuCap. If the solution is too viscous to pass easily through the 0.2 µm filter, it may be necessary to first pass it through a filter of 0.8 µm. Aliquot in 5 mL volumes into 15 mL sterile centrifuge tubes, label and store at -20 °C, where it is stable for 1 year. Thaw the enzyme just before use. Collagenase should not be refrozen, or stored at refrigeration temperatures (2-8 °C).

Growth medium

Prepare 100 mL of L-15 (Gibco) with 30% FBS, by aseptically dispensing 30 mL of FBS into a sterile 100 mL bottle. Add 2 mL of penicillin-streptomycin solution (Gibco 15140-015), and 68 mL of L-15, with or without phenol red.

BASIC PROCEDURE - DISSOCIATION OF ORGANS INTO SUSPENSION OF SINGLE CELLS AND CLUMPS OF CELLS

Materials

- ☐ rainbow trout (~100 g each). This is a convenient size to work with, but cultures can be initiated from fish of any size, from embryos to large adults.
- ☐ tricaine methanesulfonate (MS222) (Syndel Laboratories, Vancouver, BC)
- ☐ initiation solution, 5 mL for each culture to be initiated
- ☐ Collagenase A solution, 1 mg/mL (5 mL for each culture to be initiated, freshly thawed)
- ☐ Growth medium

MS222 (Tricaine methanesulfonate) is irritating to eyes and mucous membranes. Wash it off immediately if it is splashed onto exposed skin.

1. Prepare 60 mm petri dishes (one for each culture) to receive the dissected tissue by dispensing 5 mL of initiation medium into each. The dishes may be weighed at this point if it is necessary to keep track of the amount of tissue used.

2. Sedate rainbow trout by immersing in water containing MS-222 (1:10,000), or, alternatively, stun with a blow to the head.

3. If the spleen or kidney is to be cultured it is useful to remove excess blood cells, which can interfere with the attachment of other cell types. Bleed fish as thoroughly as possible, either by severing the caudal peduncle, or, with larger fish, by collecting the blood via caudal puncture. Blood collected this way can be used to prepare serum or plasma for later experiments (see supplementary procedure). This step is not necessary for the culture of other organs.

4. Rinse the outside of the fish with 70 % ethanol. To remove organs other than the brain or pituitary make a ventral incision from the operculum to the dorsal fins. This step allows access to the spleen, liver, Corpuscles of Stannius and gonads.

5. Sever the organ from blood vessels and ligaments with a scalpel, and place it in a petri dish containing 5 mL of initiation solution. Reweigh the dish at this point to determine the weight of the dissected tissue.

6. Small organs such as the spleen can be used as is to initiate cultures. For larger organs such as the liver, which are in excess of 1 gram in weight, process approximately 1 g of material as outlined below.

7. Carefully dissect away any adherent tissue from the desired organ or gland. A dissecting microscope may be necessary to ensure the absence of other types of tissue.

8. Mince the tissue with a scalpel and small scissors into pieces approximately 1 mm^3 in size.

9. Add 5 mL of collagenase A solution to the dish, cover the opening with Parafilm and place at 10-12 °C for 12-18 h.

10. Remove the dishes from the incubator, and aseptically transfer the resulting cells and tissue fragments to a 15 mL centrifuge tube.

11. Centrifuge for 5 minutes at 1,000 rpm to pellet.

12. Discard the supernatant, and add 6 mL of L-15 containing 30 % FBS. Pipet up and down several times to dissociate the tissue, and distribute evenly into three 12.5 cm^2, two 25 cm^2, or one 75 cm^2 culture flask. The use of Falcon 12.5 cm^2 flasks allows many replicate cultures to be produced, which can subsequently be subjected to a variety of treatments. Bring medium volume to 10 mL in 75 cm^2 flasks, or to 5 mL for smaller sizes. Alternatively, if you wish to experiment with various other serum supplements, resuspend is 6 mL of L-15 without serum, distribute evenly to flasks, and then add desired serum supplement to each culture individually.

INITIATION AND MAINTENANCE OF PRIMARY CULTURES

Materials

- growth medium

1. Distribute cell suspension into either one 75 cm^2 , two 25 cm^2, or three 12.5 cm^2 flasks.

2. If possible, incubate flasks at 18 or 19°C. The cells grow better if maintained at a stable temperature, but, depending upon the desired application, cultures can be incubated at room temperature if incubators are unavailable.

3. To use as a primary culture, observe the flasks periodically under a phase contrast microscope and allow the cultures to remain undisturbed until a confluent monolayer of cells is produced. The time required for this to occur varies from 24 hours or less with gonadal tissue, to a period of several months to a year for other tissues such as the brain or gill.

4. Remove all non-adherent material by aspiration, then rinse the monolayer gently with room temperature culture medium. Add a volume of fresh L-15 with 30 % FBS, 5 mL for 12.5 or 25 cm^2 flasks, and 10 mL for 75 cm^2 flasks.

If the culture is maintained for a long time period, renew the growth medium periodically by the same method. Generally, the medium does not become noticeable acidic as it will with cultured mammalian cells. Renewal of the medium is only necessary when the culture begins to look less healthy, i.e. When numerous vacuoles are evident and/or the cells begin to detach slightly.

SUBCULTIVATION ("PASSAGING") AND EARLY DEVELOPMENT OF A CELL LINE

Materials

- Trypsin solution, 0.1%
- Growth medium

1. To successfully initiate cell lines from rainbow trout, maintain the primary culture at confluence for a period of several weeks to months before the first attempted subcultivation.

2. To passage, remove fresh growth medium from the refrigerator, and allow it to warm to room temperature before proceeding.

3. Examine the flask to be passaged under the phase contrast microscope. Note the general health of the culture, and check for contamination before proceeding. Cells to be passaged should be healthy, contamination free, and confluent.

4. You may wish to photograph the culture prior to passaging for your records, as subcultivation may dramatically change the cell types present.

5. Under the tissue culture cabinet, aspirate off the old medium, and add 1.5 mL of EDTA (concentration from the standard reagents list) to the flask. This chelates calcium and magnesium ions which would interfere with the action of trypsin, and also rinses away residual FBS. Check the cells under phase contrast to ensure that cells are not lost with this step. Swirl flask gently, leave 1 minute, and remove EDTA by aspiration.

6. Add 1.5 mL of trypsin solution to the flask, and observe under the phase contrast microscope. The cells will begin to detach from the culture surface. The speed of detachment will depend on the particular cell type, and also on the age of the culture. Ideally, the cells will detach individually, and form a single cell suspension.

7. The cells should not be left in trypsin too long. After 10 minutes, if the cells have not detached, use a sterile cell scraper to scrape them from the culture surface.

8. Alternatively, steps 6 and 7 can be avoided completely, and the cell scraper alone can be used to detach the cells. This will avoid the selective loss of cells which are sensitive to trypsin. It may be advantageous to perform both methods of detaching the cells on duplicate cultures, as each method may favour the survival of different cell types. Other enzymes such as collagenase or dispase may also be used to detach cells.

9. If trypsin was used, add 8.5 mL of serum containing medium to the flask, as a source of trypsin inhibitors. Pipet the medium up and down, directing a stream of medium towards the bottom of the flask, to make sure that all cells are dislodged and resuspended in the medium.

10. Transfer the cell suspension to a sterile 15 mL centrifuge tube, and centrifuge in the table-top centrifuge at 1,000 rpm for 5 minutes.

11. Aspirate the supernatant from the centrifuge tube, being careful not to aspirate the cell pellet. Leave a small amount of supernatent , about 0.25 mL, over the cell pellet.

12. Flick the centrifuge tube with your finger to break up the cell pellet and resuspend the cells in the small volume of medium. Add 10 mLs of fresh medium to the centrifuge tube, and transfer 5 mL to each of two culture flasks (the old one and a fresh one). Add 5 mL of medium to each. This results in a 1:2 passage of the original culture, which appears optimal for fish cell lines.

PREPARATION OF RAINBOW TROUT SERUM OR PLASMA

Materials and equipment

- ☐ large rainbow trout - in excess of 200 g
- ☐ MS222
- ☐ Vacutainer needle holder (Becton Dickinson)
- ☐ blood sample needles (Becton Dickinson)
- ☐ Vacutainer venous blood collection tubes, no additive (red cap) for serum, or with EDTA (lavender cap) for plasma (Becton Dickinson)

1. Anaesthetize fish with MS-222, as described previously, or stun fish by a blow to the head. Blood must be drawn quickly, as it rapidly coagulates inside of the fish.

2. Insert needle into the caudal vein. This is achieved by inserting the needle just behind the anal fin until it hits the spinal cord, then retracting a bit. When droplets of blood are visible, attach vacuum tube to needle.

3. Collect 4-5 mLs of blood per tube. Gently agitate tube, place on ice, and replace with another tube if blood continues to flow, without moving the Vacutainer needle holder.

4. To prepare plasma, place the tubes on ice until centrifugation. Process as quickly as possible.

5. Centrifuge for 10 minutes at high speed in the table top centrifuge, to pellet the blood cells.

6. Carefully remove the supernatant, which is the plasma. Plasma should be sterile if handled carefully, but can be filter sterilized.

7. Freeze plasma at -20°C if not used immediately.

8. To prepare serum, collect the blood into a tube without anticoagulant. Allow the tubes to sit for 30 or more minutes at room temperature before centrifuging.

9. Loosen the clot from the test tube wall.

10. Centrifuge for 10 minutes at high speed in the table top centrifuge.

11. The supernatant in this case is serum. Store under the same conditions as plasma.

DISCUSSION

Collagenase dissociation and maintenance of the resulting primary cultures in high FBS concentration consistently leads to cell lines from rainbow trout. With this procedure, cell lines have been generated from brain, pituitary, Corpuscles of Stannius, gill, spleen, gonads, and liver. The techniques appear to work for Coho and pink salmon and lake trout, as well as rainbow trout. Cultures have also been successfully initiated from other species such as the smallmouth bass and lake sturgeon. Therefore, this method might be generally applicable to at least the salmonidae and perhaps fish in general.

GENERAL CHARACTERIZATION

The paucity of cell specific markers for fish cells means that cell lines can be characterized in only general ways. Morphology, as judged by phase contrast microscopy, is commonly used. The rainbow trout cell lines that have arisen by the method described here have polygonal shapes or are epithelial-like. Initially, the primary culture contains cells with a variety of shapes. As the cultures are repeatedly passaged, they will become more uniform (figure 2).

The FBS concentration influences the cellular shapes that predominate. The high FBS concentration used (30%) appears to inhibit fibroblast proliferation and maintain the epithelial character of the cultures. For some cell lines, the cultures take on a more fibroblast-like appearance when the FBS concentration is lowered to 10 %.

The general characterization of cell lines should include screening for microbial contamination (Module 7A), determining karyotype (Module 9A:1) and cryopreserving cells (Module 4C) by the methods that have been described for mammalian cells. Cell lines are also often screened for their ability to support the growth of a variety of fish viruses.

Maintaining quality control of fish cell lines has been very well described in a recent review (Lannan, 1994).

CRITICAL PARAMETERS

For successful generation of continuous cell lines, some steps appear to be more important than others. The dissociation step is critical. Primary cultures develop better from a cell suspension prepared by collagenase A digestion than with collagenase H or type I. A single cell suspension is not as likely to result in a cell line as are cell clumps and tissue fragments, like those shown in figure 2 (a) (Ganassin & Bols 1996).

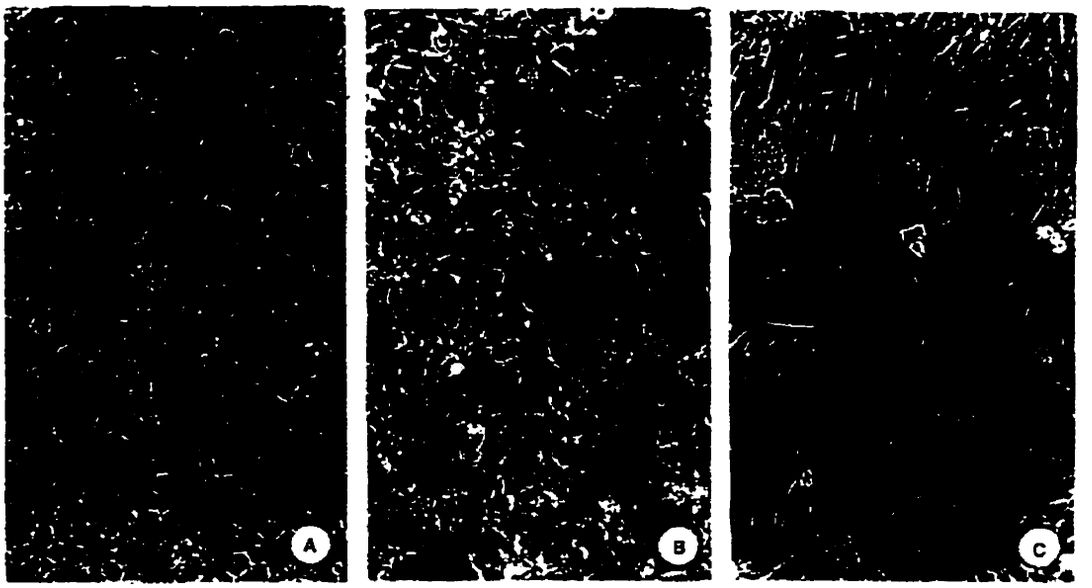

Figure A1: Cell lines are most successfully initiated from tissue which is dissociated into single cells and small clumps of tissue with collagenase (a). As these cells and fragments settle and attach to the bottom of the flask, a variety of cell types are evident (b). With repeated passage, one cell type becomes dominant (c). The cultures shown are from rainbow trout spleen. The oval shaped nucleated cells in (a) are red blood cells, which disappear after a week or two of culture. Phase contrast, Mag. 200x

The first subcultivation should be done on confluent, or even superconfluent, primary cultures. Maintaining the primary culture at confluence for several weeks to many months prior to subcultivation appears to make subsequent subcultivations more successful. Confluent primary cultures can be maintained by changing the medium infrequently (~ every four weeks). However, once the culture has been subcultivated, more frequent medium changes are required. Passaging cultures too early or subcultivating at more than a 1:2 ratio can lead to cultures that grow too slowly to be of use.

The high concentration of FBS appears to be important. Although success can be achieved with 10 % FBS, cell lines are more consistently obtained with a supplement of 30 % FBS. The relatively high FBS concentration seems to inhibit the growth of fibroblastic cells which, when they appear, tend to crowd out all other cell types present. However, if the concentration of FBS is too high (50 %), proliferation in general appears inhibited. Other sera, such as horse or calf, do not appear to support cell growth in primary cultures.

The basal medium does not appear to be a critical factor. Although most of our experience has been with Leibovitz's L-15, success has also been achieved with CO_2-Independent Medium (Gibco).

TIME CONSIDERATIONS

The time from the initial preparation of the primary cultures to the development of sufficient flasks to cryopreserve cells is variable. It would appear to depend on the tissue and on the FBS concentration. In the case of the gill and 10 % FBS, this was approximately 2 years (Bols *et al.* 1994). With the use of 30 % FBS, this process is shortened considerably and can be reduced to the order of 5-6 months.

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