Trout Leukocytes: Growth in Oxygenated Cultures

Abstract. *A reproducible method of culturing leukocytes from rainbow trout* (Salmo gairdneri) *has been found. Using an atmosphere of 100 percent oxygen, we stimulated peripheral blood leukocytes to divide at 19°C. Cultured cells were used for a karyological study; they had a modal chromosome number of 60 and a constant arm number of 104, a value previously reported for other tissues in these fish.*

Advances in the field of animal cytogenetics have been rapid since Nowell's (*1*) discovery that mitosis of leukocytes could be stimulated and arrested at metaphase, thus producing a methodology for a nondestructive means of karyotyping. Nowell's basic technique was successful with a wide range of vertebrates with one notable exception; despite numerous attempts, efforts to cultivate fish leukocytes were unsuccessful (*2*).

Recently, however, Heckman and Brubaker (*3*) and Ojima *et al.* (*4*) have successfully cultivated leukocytes of goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*). Apparently, the choice of species had much to do with this success because our use of the procedures used by these investigators was unsuccessful in mitotic stimulation of Salmonidae leukocytes.

Rainbow trout (*Salmo gairdneri*), which inhabit cold water, require greater environmental oxygen levels than do either goldfish or carp, which usually inhabit warmer, less-oxygenated waters. The possibility that cells also might require threshold oxygen tensions was explored. Cultures were set up with oxygen tested as an important factor.

Anticoagulant (0.5 ml, Difco blood separation vial) was drawn into a sterile 10-ml syringe. Blood (4 to 8 ml) was then withdrawn by cardiac puncture from rainbow trout located at the Pennsylvania Fish Commission's Benner Spring Fish Research Station, Bellefonte, Pennsylvania. The samples were refrigerated for transport to our laboratory, and then centrifuged (100*g* at 9°C) to sediment the erythrocytes. Samples of plasma rich in leukocytes were collected at 5- to 10-minute intervals. Culture medium consisted of a mixture of 100 ml of Difco TC minimal medium Eagle spinner modified, 10 ml of fetal calf serum [Grand Island Biological Company (GIBCO)], 2 ml of antibiotic-antimycotic mixture (100 times the concentration, GIBCO), and 0.2 ml of L-glutamine (200 mmole/liter, GIBCO). Four milliliters of this mixture was placed in culture bottles (2 cm in diameter, 7 cm deep); in cultures to be treated with oxygen the medium was saturated with pure oxygen. At this time 0.4 ml of the collected plasma and 0.3 ml of Difco phytohemagglutinin M (PHA) were added to each bottle. The atmosphere in the bottles which had been previously saturated with oxygen was also replaced with pure oxygen and sealed.

The maximum mitotic activity occurred after 120 hours of incubation at 19°C. Alkalinity of the cultures increased from an initial *p*H of 7.0 to an average *p*H of 7.4 after 5 days of incubation. Four hours before harvesting of the cultures, colchicine mixed in Hanks balanced salt solution was added at a final concentration of 0.005 mg/ml. Cells were harvested by the method of Heckman and Brubaker (*3*) except that the hypotonic solution used was either double distilled water or 0.067 g of $CaCl_2$ and 0.2 g of sodium citrate in 100 ml of water. The slides were stained with a mixture of one part of saturated alcoholic crystal violet and four parts of 1 percent ammonium oxalate.

Growth in the cultures that had been saturated with oxygen was consistently good compared with that of cells in unoxygenated cultures. This relationship held true when other factors such as the initial *p*H, the amounts of plasma and leukocytes, or the concentration of PHA were varied to obtain maximum growth. The effect of increasing the oxygen tension in the cultures is easily observed; 10 to 1000 mitotic spreads were obtained per single slide from those cultures to which oxygen was added. Only in rare instances could up to ten mitotic figures be obtained from unoxygenated cultures.

In the culture of leukocytes of most vertebrate species low oxygen tensions favor transformation (*5*). In addition, CO_2 is essential for growth of mammalian cells (*6*). However, Fryer *et al.* (*7*) showed that small amounts of CO_2 which did not affect the *p*H had an inhibitory effect on the growth of coho salmon embryonic cells. These cultures had an initial increase in *p*H from 7.3 to 8.0 followed by a very slow decline, but never a *p*H below 7.0. In contrast, mammalian cells produce toxic acidic *p*H levels in culture. In goldfish leukocytes cultured by the technique of Heckman and Brubaker (*3*), which does not require addition of oxygen, the medium was quite acidic at the time of harvest. Differences in the CO_2 requirements of salmonid cells and mammalian cells have also been reported by Kleeman *et al.* (*8*). Our findings, together with those of the above authors, indicate important metabolic differences between salmonid and mammalian cells.

Numerous counts of well-spread metaphase figures showed a modal diploid number of 60 in the cultured leukocytes of rainbow trout. The chromosome number in these figures varied between 58 and 62. In all instances arm numbers totaled 104, distributed as 44 metacentrics and 16 acrocentrics in those cells with 60 chromosomes. These results are consistent with those of Bungenberg de Jong and Wright (*9*), who reported 60 chromosomes in squash preparations of embryo disks, and with those of Ohno *et al.* (*10*), who reported diploid numbers ranging from 58 to 65 in cells from numbers of different tissues. Thus somatic segregation which Ohno *et al.* proposed to account for these varying chromosome numbers is extant in cultured leukocytes of rainbow trout.

The discovery that oxygen is an important factor in culturing leukocytes from certain fish should stimulate research in the field of fish cytogenetics. Such research should aid in clarifying the mechanisms, particularly of Robertsonian translocations and polyploidy (*11*), considered to have played roles in fish evolution. With leukocyte cultures use of karyotypic differences as racial markers or the differentiation of pathological from normal karyotypes (*2*) should be easier. In addition, as pointed out by Wolf and Quimby (*2*), continued work in this area should clarify some of the uncertainty about the development, phylogeny, and nomenclature of fish leukocytes as well as aid studies of physiology and virology in fishes.

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Nuclear Localization of Histamine in Neonatal Rat Brain

Abstract. *The concentration of histamine in the brains of neonatal rats is considerably higher than that in adults. Subcellular fractionation studies revealed that about 90 percent of the histamine content of neonatal rat brain is confined to the crude nuclear fraction obtained by differential fractionation. Purified nuclei prepared from these fractions retained 90 percent of their histamine content. The nuclear localization of histamine in the brains of neonatal rats suggests a function for histamine in modulating the growth processes of the neonatal brain.*

Histamine appears to have various functions in different parts of the body. In many peripheral tissues most of the histamine is localized in mast cells where it presumably is involved in allergic and inflammatory processes (*1*). In the stomach of several species histamine is localized in unique chromaffin-like cells where it may function in the secretion of gastric acid (*2*). In the adult mammalian brain subcellular fractionation indicates that histamine is localized in particulate fractions enriched in pinched-off nerve endings ("synaptosomes") and may therefore have a synaptic function (*3*).

Histamine synthesis is greatly enhanced in some rapidly growing tissues, especially fetal rat liver, and it has been suggested that histamine may have an important function in certain cases of rapid tissue growth (*4*). However, in some rapidly growing tissues, such as regenerating rat liver, there does not appear to be an enhancement of the activity of histidine decarboxylase, the histamine synthesizing enzyme, although polyamine synthesis is enhanced (*5*).

In the neonatal rat brain amounts of histamine are more than five times higher than they are in the adult, and are correlated well with periods of neuronal growth (*6*). Knowledge of the intracellular localization of a chemical often helps explicate its function. Accordingly, we have examined the subcellular localization of histamine in the neonatal rat brain. We report here that in the neonatal rat brain histamine is almost wholly localized within the nuclear fraction.

Sprague-Dawley rats (*7*) were obtained at 15 days' gestation and kept in individual cages. After birth neonatal rats were maintained with their mothers until they were killed by decapitation. Brains were rapidly removed and homogenized, and crude nuclear, mitochondrial, microsomal, and supernatant fractions were obtained by differential centrifugation. In some experiments purified nuclei were prepared by sucrose gradient sedimentation according to the method of Blobel and Potter (*8*) modified so that the most dense layer contained 1.8*M* sucrose. Histamine was measured by a modification of the enzymatic-isotopic procedure of Snyder *et al.* (*9*) in which the sensitivity of the procedure is enhanced so that as little as 0.2 ng of tissue histamine can be reliably estimated (*10*).

In initial experiments histamine was measured in the telencephalon, diencephalon, and rhombencephalon of rats at six ages from 1 hour to 17 days after birth. In the telencephalon and diencephalon, the time course of changes in histamine content was closely similar to that reported by others (*6*, *11*). Histamine concentrations, expressed per unit of wet brain weight, were about 200 ng/g at birth, with a maximum of about 250 to 300 ng/g at 5 to 10 days; the amount then gradually declined by 17 days to about 50 ng/g, the same as that amount found in adult rat brain. In the rhombencephalon fluctuations in histamine concentrations were not as marked. From birth to 10 days, the concentration in the rhombencephalon was about 100 ng/g, which gradually declined by 17 days to adult levels of about 50 ng/g. These findings may relate to well-known patterns of growth in different parts of the brain. Brain maturation proceeds in a caudocephalic direction so that the rhombencephalon may have already undergone in fetal life rapid growth processes which in the telencephalon and diencephalon take place during the neonatal period.

In subcellular fractionation studies in which differential centrifugation is used, we found in the diencephalon

Table 1. Change in the subcellular localization of histamine during development of the rat diencephalon. Rats were decapitated at various ages, and brains were dissected into telencephalon, diencephalon, and rhombencephalon. The diencephalon regions from rats at various ages were homogenized with a Teflon pestle in 15 volumes of ice-cold 0.32*M* sucrose. Differential centrifugation was employed according to the following scheme. The P_1 pellet was obtained by centrifuging the homogenate for 10 minutes at 1000*g*. The resulting supernatant fluid was centrifuged for 35 minutes at 18,000*g* to obtain the P_2 pellet. The supernatant fluid from the P_2 fraction was centrifuged for 1 hour at 100,000*g* to obtain the P_3 pellet and a soluble supernatant fraction (S). Recovery values obtained when exogenous histamine was added to subcellular fractions ranged from 90 to 100 percent. The sum of the histamine content of the four subcellular fractions was 90 to 99 percent of the total histamine content of the appropriate homogenate. Data presented are the mean values obtained from four different experiments whose results varied less than 20 percent. The reaction product of the histamine assay in P_1 pellets of 3-, 7-, and 21-day-old rats had the same R_F value as 1,4-methylhistamine in three paper chromatographic systems.

Age (days)	Histamine (ng/g of tissue)					Total histamine content of tissue (%)			
	P_1	P_2	P_3	S	Total	P_1	P_2	P_3	S
3	129	12	0	0	141	91	9	0	0
5	188	7	2	3	200	94	4	1	1
7	210	23	2	2	237	88	10	1	1
13	139	16	0	7	162	87	10	0	3
21	14	18	2	26	60	23	30	3	44