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The Leech as a Laboratory Animal for the biomedical Research

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We have collected a great quantity of leeches, comprising five species, from six different ponds and rice paddies in the south-western part of Korea. The leeches were cultured in our laboratory with special interests on the blood-suckers (*Hirudo nipponia*) in fresh water supplemented with essential ions. A number of experiments was carried out with biomedical aspects. We have observed traits which can be used as a laboratory animal. The paper reports general features observed from leeches with preliminary study results carried in culture systems. The paper also describes study aspects of leeches in biochemistry, neurology and developmental biology.

KEY WORDS: leech, laboratory animal, culture system

Four species of fresh water leeches have reported from southwestern region of Korea (Yoon *et al.* 1989). The phylogenetic feature of leeches reflect a monophyletic link between clitellate and uniramia with internal fertilization, egg protected by cocoon, development without larval stage, formation of fate areas in early embryo (Sawyer, 1986). Leeches are carnivorous clitellate with a characteristic sucker mode of locomotion. There are approximately 300 species which inhabit fresh water or humid environments and carnivorous or parasitic on other organisms, and occur virtually all over the world. The species we have found from ponds, rice paddies and rivers of Taejon area belong to two genera (*Hirudo* and *Whitmania*). The length of mature leeches in our laboratory varies from species but in general attained 3-5 cm in the contracted state.

There are a number of well-known laboratory organisms for research investigation; *E. coli*, fruit flies, guinea pigs, rabbits and monkeys. Very few species from invertebrate animals, particularly from fresh water, however, have been known for use of research experiments. We have discovered a number of desirable attributes from leeches that have been raised in laboratory setting for a period time.

The paper is to present the interesting aspects of these leeches as laboratory animals for study model of biomedical research investigations with some preliminary experiments.

Materials and Methods

Leeches were collected from 6 different ponds located within 100 km from Taejon. The collection was started from early spring with once or twice in a month until the organism can be caught. The leech was transferred into distilled water tanks that were constructed with glass in our laboratory (Fig. 1). The tanks were mimicked natural habitat by the supplement of grasses, sands and mud. The leech was fed with live amphibians for the young and blood in mammalian bladder for adults (Fig. 2). The leeches are very adaptive animals and can survive for months or even over a year without the food. We have found at least 5 species in our culture tanks and four of which have previously been described in the systematic classification (Yoon *et al.* 1989), but one or more have not been reported in Korea.

Observation

Segmented worms annelid which include leeches, are of importance to man. Large earthworms (*L. terrestris*) are cultivated and sold as fish baits. The sludge worms and marine worms

Fig. 1. Leech culture tanks. Fresh water is circulated with hydraulic pump. Most of leeches are attached to the bottom of the tanks.

(polychaetes) are as water pollution indicators and in turning over sediment on ocean bottom respectively.

The medical use of leeches, which dates from antiquity, reached its peak in the past century. European species *Hirudo medicinalis*, which is quite similar to our native *H. nipponia* (regardless whether this is an adequate name or not) was exported throughout the world. Hirudin, an extract from leeches is used as a blood anticoagulant (pharmaceutical aspects will be described later).

All leeches are hermaphroditic and reproduction is always sexual. The leeches examined in our laboratory have several pair of testes arranged by segments beginning with segment 12. The female reproductive system consists of a pair of ovisacs containing ovaries which located in front of the testes. Gnathobdellae families to which our four species belong, transfer sperms into the vagina of another by the penis. The eggs, numbering from 30-50 are deposited in oval cocoon and are generally attached to rocks or in mud. Development of leeches takes place within the cocoon. The cocoons contain some amount of albumin, which nourishes the developing embryo.

The first four cells (blastomeres) give rise to micromeres at one end of the egg and

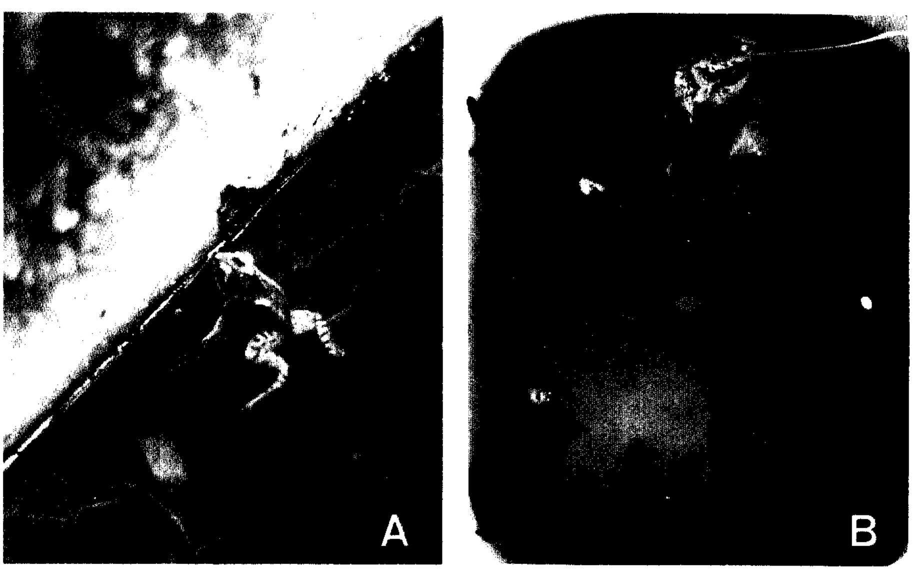

Fig. 2. Feeding leech at different life stage. A. Living amphibian is an important source for the first feeding stage in that juvenile leech teeth are underdeveloped. B. Mature leeches were fed with mammalian blood in porcine bladder.

macromeres at other end. The first somatoblast-a cell that gives rise to differentiated tissue, from a division of the macromeres at the 16 cell stage, give rise to components including the nervous system, known as ectoderm. The second somatoblast forms at the 64 cell stage and give rise to future mesoderm, (such as muscle) and endoderm (digestive tract).

The elongated gastular has a ventral mouth at the front end and a posterior anus. The mesodermal hands hollow out to form the body cavity or coelom, as the number of mesenchymal cell increases, and young leeches hatch from cocoons feeding upon albumin. Very little is reported about the life-span of leeches inhabit in the country. The span of a laboratory setting is under observation in our laboratory (Fig. 3). The leeches are one of the most highly organized animals and lack power of complete regeneration.

Locomotion in the leech can be compared to that of the inchworm; the anterior and posterior suckers serve as points of contact. When the posterior sucker attaches to a surface, the leech elongates and the anterior sucker fastens to the surface, and the posterior sucker is released, a wave of contraction move in a forward. During swimming, undulations of body up and down are produced by wave of contraction and push forward (Gray, 1938; Magni and Pellegrino, 1978).

Leeches of Hirudo family feed primarily on mammals but also suck blood from snakes, frogs and other pond animals. A leech (Hirudo) has 34 segments, may increase in length as a result of subdivision and elongation of the annuli (ring). The typical number of annuli in a segment of well developed is four or five. The anteriorly located eyes vary in number from species. The clitellum that present during reproduction, extends from segment 10 through 24.

Internal features are clearly distinguishable on the dissective microscopes (Fig. 4). The coelom is filled with connective tissue. The mouth surrounded by the anterior sucker opens into the gut; the crop and intestine follow then the rectum. The reproductive organs and salivary glands are prominent as in most worms for survival of species. The nervous and circulatory systems are well developed and good for the understanding of general mechanisms of a higher animal.

Digestion, respiration, excretion are interesting properties associated with the mode of life of leeches which are adapted to environments. Development of brain, ganglion, nerves, muscles and other organs are readily distinguishable and intricately coordinate the body. Every organs can be investigated in a manner similar to that of other animals.

On-going studies in our laboratory and suggested studies

Studies of Genetics and developmental biology: as described earlier, reproduction is sexual and mating is observed in culture and can be isolated as a pair. Inheritance of mutation and various traits are observable from the hatching. All leeches begin as a fertilized egg. But there is a question as to why a particular egg develops into *Haementeria ghilianii* instead of Hirudo (Sawyer, 1986). We have observed a few *glossiphoniid*-like animals in our laboratory (Fig. 5). Because native species, however, laid eggs in cocoon under the mud, it is sometimes difficult to trace all processes of development of eggs. Leeches have 8 to 16 pairs of chromosomes. The species we have observed has 8 pairs. Cytological study will be very helpful for clarification of species.

Biochemistry and pharmaceutical studies: Since nineteenth century, antibiotic properties of leech has been studied. For example, anthrax, relapsing fever (Weiler, 1949), tetanus, meningitis, streptococcal infections (Weiler, 1949; Hirst, 1941) have been studied. The antibiotic in *Hirudo medicinalis* is produced by *Aeromonas hydrophila* (*/hirudinis*) which lives endosymbiotically in its gut. This bacterium cultured in vitro kills microbes of tuberculosis, dysentery, diphtheria, *Staphylococcus* aureus and other diseases. Salivary secretions from blood-sucking leeches are primary concerned aspects of biochemistry and physiology. The bite of all blood sucking leeches is painless and to prolong bleeding. These are due to an anaesthetic effect as well as anticoagulants secreted by the leech. The proteolytic inhibitors were isolated and purified: hirudin, bdellin and eglin. These inhibitors are

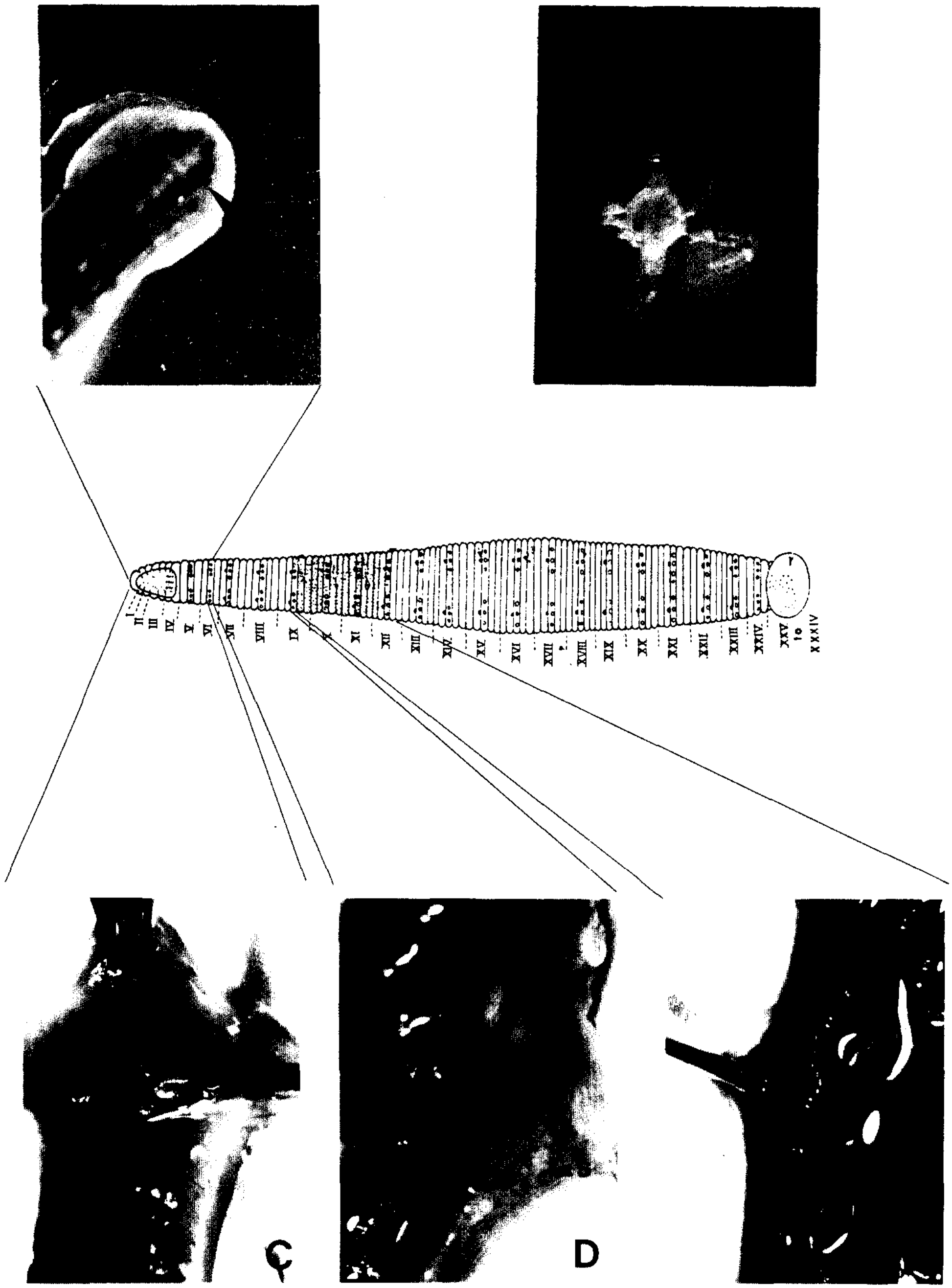

Fig. 3. The internal and external feature of leeches grown in the laboratory. A. Arrow indicates the eye of leech. The leech has 5 pairs of eye at 2, 3, 4, 6, 9th anulus. B. The ventral side of a stripped segmental ganglion of leech. The 21 ganglia are joined in chain fashion to each other. Each ganglion (B) containing neurons and glial cells is anchored independently to the wall of lacuna by paired peripheral nerve roots (C). Scale = 500 μm. C. Blood-sucking leeches have well developed three jaws. Each jaw has a large number of projected teeth like the saw. D. Internal structure of the leech. Segmental ganglion (g) and salivary glands (s) are shown. E. Reproductive organs. Prostate (p), epididymis (e), ovisac (o), vagina (v) and testis (t) are located in segment XI-XVI.

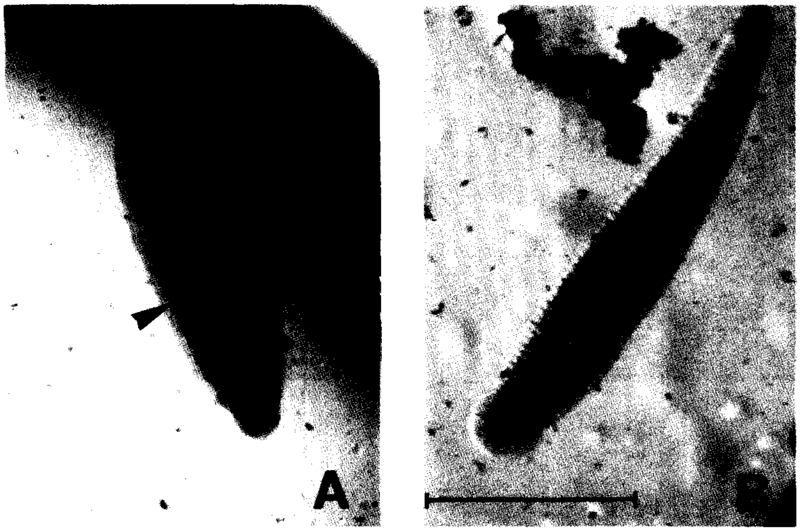

Fig. 4A. Glossiphoniid found in our laboratory. The arrow indicates eggs attached directly to ventral surface of parent (characteristic of Haementeriinae). Length of the leech is 6 mm. **B.** The newly-hatched young leech from glossiphoniid. Scale = 1 μm.

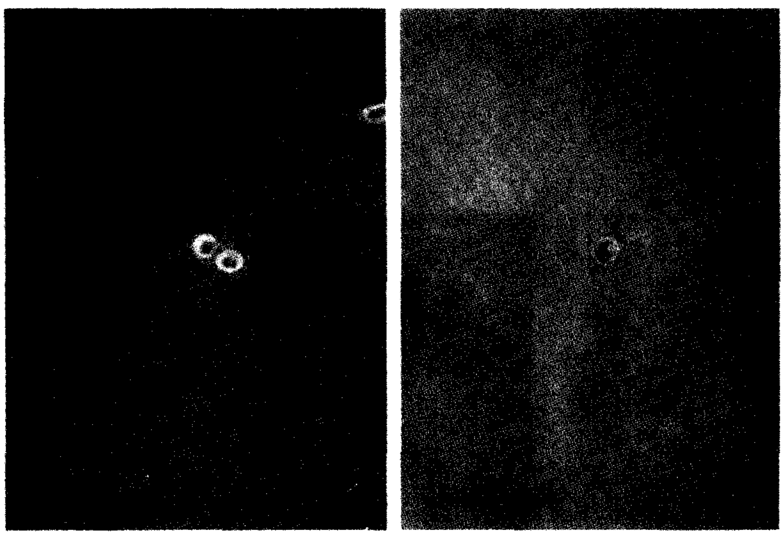

Fig. 5. Outgrowth of isolated leech neurons on plant lectin Concanavalin A substrates. Neurons were cultured for 2 days (A) and 3 days (B) in L-15 media with 2 % fetal bovine serum in our laboratory. The arrow shows extended neurites. Scale= 100 μm.

immunologically distinguishable and considerable medical potential. Hirudin, for example is a thrombin specific inhibitor. Intense studies have devoted to the purification and structure of it (Markwardt, 1956, 1970; Triebel and Walsmann, 1966; Magnusson *et al.*, 1975; Bagdy *et al.*, 1973, 1976). Recently, numerous works on interaction with thrombin and three dimensional structure of hirudin-thrombin complex (Stone and Hofsteenge, 1986; Grutter *et al.*, 1990; Rydel *et* *al.*, 1990; Jackman *et al.*, 1992) have been performed. For mass production of hirudin, recombination and expression of hirudin in *E.coli* and in yeast has been studied (Harvey *et al.*, 1986; Loison *et al.*, 1988; de Tazis du Poet *et al.*, 1991).

Bdellin with a molecular weight of about 5000 is the smallest known naturally occurring inhibitor of trypsin, plasmin and acrosin and may be useful in medical science where plasmin inhibition is indicated.

The low molecular weight eglin is a potent inhibitor of elastase, cathepsin G, chymotrypsin and subtilisin. This polypeptide is of medical interest for several reasons. Eglins effectively block the inflammatory response induced after localized trauma or surgery (Dergane and Zdravic, 1960) and eglins are effective against some kinds of collagenolytic, ulcerous conditions of the gut endothelium (Crohn's disease), in addition, against non-specific proteolysis of clotting factors associated with septicaemia. Recently marketing, spreading factor, Orgelase hyaluronidase is mucolytic and specific for hyaluronic acid (Meyer *et al.* 1940; Hirst, 1941; Damas, 1974). Leech hyaluronidase has powerful antibiotic properties as well as good effect in the treatment of glaucoma.

The chemicals secreted in leech saliva, important to the surgical procedures, are also being explored as therapeutic agents against several diseases, including atherosclerosis, thrombosis and cancer.

Neurological study: The central nervous system (CNS) of leeches is paid great attention for neurological study recently. Instead of having one brain as in most animals, leeches have 34 segmental brains called ganglia, all of which are more or less alike. Each ganglion has fewer than 200 pairs of neurons each of which is unique and subserves a particular function. Many of these neurons have already been identified according to size, position, and electrical properties (Nicholls and Baylor, 1968). The relatively small number and large size of the neurons has made leeches, along with sea hares and crayfish, favorite invertebrate subjects of neuroanatomists and neurophysiologists. In fact, a great body of information has accumulated about leech neurons to the point that as much is known about the overall workings of the leech nervous system as that of any other animal, including humans. Furthermore, owing to its simplicity and experimental accessibility the leech nervous system is proving to be exceptionally useful for the ultimate understanding of the neuronal basis of behaviour. In conjunction with the remarkably advanced behaviour displayed by these animals in nature, leeches promise to become a standard animal for the still incipient field of neuroethology.

Current understandings of physical and chemical aspects of the leech nervous system were reviewed (Muller *et al.* 1981). The rapid sprouting and outgrowth of cultured leech neurons has been studied to develop a system suitable for investigating how single identified leech neuron recognizes specific substrates such as the plant lectin Con A (Chiquet and Acklin, 1986). Thrombin (serine protease) modulates and reverses neuroblastoma neurite outgrowth (Gurwitz and Cunningham, 1988). It might be useful to observe from cultured neurons by the treatment of thrombin, because these neurons are a large size.

In our laboratory, the rapid sprouting experiments were carried out with leech neurons to investigate how a single neuron behaves differently to various substrates. A preliminary result shows components extracted from leech extracellular matrix with reported substrates produce neurites in 2 days (Fig. 6).

In fields of neurocell biology containing signal transduction and neurotoxicology (for example Botulinum toxin and Tetanus toxin), the simplicity and experimentally accessibility of cultured leech neuron described above would stimulate many researchers having dealt an arduous task with any other neuron cell.

Discussion

The leeches are brought in the laboratory, because of desire to understand the broad outline of cell biology at beginning. We have constructed culture tanks that were mimicked for natural environments of leeches in the pond and able to observe their habitats for a period time. We have found that there were voluminous literatures on leeches with special fascination for systematics, anatomy and behavior. In the meantime, a number of preliminary experiments were conducted from them on observations of development and morphological variations, feedings and biochemistry of protease inhibitors. We have also carried out neurological experiments with ganglia and neuron in culture systems. We have observed more species than previously described in Korea. Data accumulated at present are too small for a separated publications each. We have however, experienced enough for use of the leech as laboratory system.

As R.T. Sawyer stated, the leech is living pharmacopoeia which is the mirror image of human physiology and there is a legitimate biological rationale behind the use of leeches in medicine. Recently Biopharm (U.K.) Ltd has marketed live leeches of several species to aid in microsurgery and to stimulate further research into exciting new biomedical field.

We have attempted to provide readily accessible animals in laboratory with inexpensive manner and to understand own natural resources.

Appendix

Systematics

Hirudo nipponia

1. Three well developed jaws, but entirely lacking salivary papillae
2. A large number (approximately 60-70) of tooth projections like saw
3. No median longitudinal furrow on ventral surface of upper lip
4. There are 22 annuli between 5th eye and male genitalia
5. Distinctive reproductive organs with the anatomical examination
6. Five to seven centimeters long in mature size
7. 34 segments with more than 100 annuli
8. 5 pairs of eyes within 20th anterior annuli

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생명과학 연구를 위한 실험동물로서의 한국산 거머리에 관한 제고

홍석진 · 김동령 · 정효일 · 이상기* · 조철오 · 강계원(한국과학기술원 생물공학과,
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우리나라에 분포하고 있는 거머리를 서남쪽 지방에 위치한 6곳의 연못과 논에서 주기적으로 채집, 사육하였다. 채집한 5종 이상의 거머리 중 흡혈 거머리인 *Hirudo nipponia* 에 관심을 갖고 이온이 첨가된 배양용액에서 대량으로 배양하면서 여러 생물의학적인 실험을 수행하였으며 이로부터 실험동물로 사용될 수 있는 우리나라 거머리의 여러 특징을 관찰하였다. 지금까지 수행한 예비 실험의 결과와 여러 자료를 토대로 생물학 특히 생물의학, 신경학, 발생학 분야에 거머리가 좋은 재료로 이용될 수 있다는 점을 논하였다.