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昆虫细胞系的发展：历史的回顾

The Development of Insect Cell Lines: A Historical Perspective

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I wish to express my gratitude to the Society for Invertebrate Pathology for inviting me to deliver this year's Founder's Lecture, honoring Dr. Thomas D.C. Grace. It is not only a great honor and a distinct pleasure to deliver this lecture. For several decades I had hoped to get a chance to come to Australia and now finally this dream could be fulfilled.

When I thought about a proper title for my lecture, at first I considered the following: "Chain reaction effect of collaboration and human contacts in the development of invertebrate cell culture". As you will see, this title does not fit the contents of my presentation, because the work of Dr. Grace started in Australia independently of contact with tissue culture workers elsewhere. I shall try to present the historical background and some of the art of scientific investigation that has been responsible for the past and current development of invertebrate cell culture and focus on the impact of Tom Grace's work on the whole field. It will be a biased and very personal account as well.

In 1915 Richard Goldschmidt came to the Osborn Laboratories at Yale from the Kaiser Wilhelm Institute in Berlin. He met Harrison and became familiar with vertebrate tissue culture. This gave him the idea to try to maintain explants of the *Cecropia* moth and silkworn spermatozoa in vitro (1915, Proc. Natl. Acad. Sci. U.S.A., 1, 220—222). He succeeded and thus became the first to culture insect tissues in vitro—next door to Harrison's lab and certainly influenced by Harrison's cultivation of vertebrate cells. However, no adequate media were available and insect cells were maintained for but limited periods

in saline solutions with sugar as energy source and with insect or vertebrate tissue extracts. Efforts to develop insect tissue culture were continued by Glaser in 1917(Psyche,24,1—7). In 1922 Glaser became head of parasitology at the Rockefeller Institute's animal pathology labs in Princeton, New Jersey, where he influenced several coworkers to develop axenic cultures. In 1934 he invited William Trager to his laboratory and Trager began to work with silkworm tissue culture and grasserie virus (1935, J.Expt., Med., 61,501—514). This was followed by mosquito tissue culture in which the multiplication of equine encephalitis virus was galantly demonstrated (1938, Am.J.Trop. Med.Hyg., 18, 378—393). Trager made a systematic attempt to develop media based on the chemistry of insect hemolymph. Although cells survived only a few days in Trager's early media, he was able to grow insect and mammalian viruses in insect cells in vitro.

Trager was the first to study carefully the growth conditions of insect cells in culture, to demonstrate the ability of individual cells to survive for several days in vitro, and to use insect tissue culture for the study of insect and mammalian viruses. Among the formidable difficulties encountered by him, was the lack of antibiotics which made it almost impossible to avoid eventual contamination of the majority of cultures.

In 1956 Silver Wyatt, Gerard R. Wyatt's wife,greatly improved the culture medium used for silkworm pupae and she succeeded in maintaining ovarian tissues for 14 days (J.Gen.Physiol.,39, 841—852). Her medium contained 21 amino acids,5 salts, 3 organic acids,the sugars fructose,trehalose and glucose, in concentrations corresponding to those of insect hemolymph. The pH and osmotic pressure were also adjusted correspondingly. The antibiotics, penicillin and streptomycin were used to prevent bacterial contamination.

I had the good fortune to meet Harrison in 1947 when I was studying for my Ph. D. degree at Columbia University and attended the annual meeting of the Society for Growth and Development at Storrs, Connecticut, A year later, after the next meeting of the same society, in Burlington, Vermont I happened to return with Harrison by car from Burlington to New Haven, and during the long drive he told me about his early tissue culture work. Also, in 1948 I met William Trager, who was still working in Princeton but was about to move to the Rockefeller Laboratories in New York. We became close friends when I joined the Plant Pathology lab at Rockefeller Institute in 1949 and a few years later we became neighbors in Scarsdale,a suburb of New York City. For 12 years, while I worked at Rockefeller Institute-Rockefeller University, I spent many hours with Trager and in 1955 he and his colleague Maria Rud-

zinska helped me in my own attempts to maintain leafhopper tissues in vitro for limited periods (1956, Virology, 2, 369—376).

It so happened that I also met personally Goldschmidt in 1951 at a Cold Spring Harbor symposium but at that time I only knew of him as a famous geneticist and I did not know of his early work with insect tissue culture.

Completely independently from the work in the United States and Canada, three years before the publication of Wyatt's paper, a young Australian entomologist, Thomas D.C. Grace at the C.S.I.R.O. in Canberra became interested in invertebrate cell culture. In 1954 he published a short paper in Nature in which he described his efforts to grow insect cells in vitro (Nature, London, 4421, 187—188). He also stressed the difficulties encountered and the possible ways to overcome these difficulties. When Wyatt's paper appeared in 1956, Tom further improved his medium and soon was able to grow cells from ovaries of 4th instar silkworm larvae in hanging-drop cultures. These cells underwent frequent mitosis for 19 days and survived for 29 days. The medium consisted of Wyatt's solution with the addition of cholesterol, extracts of endocrine, and ovarian tissues and ten components of the Vitamin B Complex (1958, J. Gen. Physiol., 41, 1027—1034).

At that time, Grace's boss, Max F. Day, became Australia's scientific attachee in Washington, D.C. . He kept in close contact with Tom and received frequent reports about the progress of the work in Canberra. Day visited the Rockefeller Institute and when we met, he told me that Tom could get a leave of absence for two years to come to New York to continue his attempts to improve insect tissue culture. My N.I.H. grant provided the necessary funding and at Dr. Day's suggestion I invited Tom to come to my laboratory. From February 1957 till February 1959 Dr. Grace worked at the Rockefeller University. His work progressed quite well and he succeeded in improving insect culture media and maintaining certain insect organs alive for many months in vitro (1959, Trans., N.Y.Acad.Sci. Ser. II, 21, 237—241). Frequent consultations with Willlam Trager at Rockefeller were stimulating and we traveled together to NIH in Bethesda, where we visited Dr. Harry Eagle who was heating the Laboratory of Cell Biology at the National Institute of Allergy and Infectious Diseases at that time.

Grace succeeded in maintaining in vitro the ovarian tissue of *Callosamia promethea* for 350 days (1958, J.Gen.Physiol., 41, 1027—1034). Diapausing pupae and a modified Wyatt's medium were used for these cultures. Active growth was obtained during the first 165 days, which represented a great advance toward the achievement of continuous growth of insect tissues. Subse-

quently Tom was able to subculture insect cells. Explants from promethea moth ovaries were set up in hanging-drop cultures. After 48 hours cells moved out from these tissues and either floated freely in the liquid medium or became attached to cover glass. A few days later, after numerous mitotic divisions had been seen, the cells and pieces of tissues plus fresh explant from ovaries were shaken in an Erlenmeyer flask at 80 revolutions per minute for 4 days. Then the cultures were placed in a roller tube and rotated at 1 revolution per 5 minutes. Cells migrated from explants for 5 days. On the 6th day the first subculture was made, on the 16 th day the second subculture, and four further subcultures were completed during 6 months. Cells continued to migrate and increased in number by division. These experiments clearly demonstrated that long-term culture of insect tissues was finally achieved by Grace. Yet it was not yet possible to grow dispersed cells continuously.

While Tom's scientific work at Rockefeller progressed well, not everything in his personal life was rosy. Difficulties started when part of his belongings, shipped by sea, could not be found for several months, because of a spelling difficulty of the name GRACE ("G.R.I.C.E." I, like in IBC!). There were serious medical problems in his closest family, but Tom took the adverse events in stride and never missed a day of work. Upsetting was sometimes the menu at lunch, when such dishes as sweet potato which Tom abhored were being served. The second summer Tom won a Lalor Foundation Award that enabled him and his family to spend a couple of months at the Cold Spring Harbor Laboratories on Long Island and avoid the very hot and humid summer in New York City.

Grace's goal was to grow a monolayer of insent cells and when he returned to Australia he continued his efforts, starting this time with 15000 pupae of *Antherea pernyi* (1962, Nature, London, 195 : 788—789). The thrilling news of his success came in 1962, when Max Day came to the First Int. Conference on Invertebrate Tissue Culture in Montpellier and presented Tom's breakthrough experiments and a movie of the first 4 insect cell lines from *Antherea pernyi*. This was the indisputable highlight at the conference, and it changed insect tissue culture forever. At the International Congress of Entomology in Kyoto, Japan in 1980, Grace described how he succeeded in obtaining continuous growth of insect cell lines (Grace, 1982, in Invertebrate Cell Culture Applications, K. Maramorosch and J. Mitsuhashi, eds.,Academic Press, 1—8). At first he changed his medium approximately every 7 days but noticed that a couple of days after the medium was changed, cell multiplication slowed. Therefore he decided to change only half the medium and did so

at 10—14 day intervals. This method resulted in better growth but after 2 months cell growth and migration dropped to very low levels. Tom decided to maintain the cultures, instead of discarding them, and he called his procedure "organized neglect". He examined the cultures about every 2 days and when muscle contractions were regular and some cells appeared healthy, he changed half of the medium. This "organizd neglect" was continued for over a year, when, to Tom's surprise and joy he noticed a sudden, rapid increase in the cell number and divisions. At that time he started to subculture the cells every 6 days and the first 4 insect cell lines became a reality.

The successful cultivation after a year of "organized neglect" was perhaps due to polyploidy of the cells, an adaptation to the medium, or some unknown factors. In 1964 Grace established the first mosquito cell lines. His work was followed by others and soon Drosophila cell lines were obtained by Echalier and Ohanessian (1970 In Vitro, 6, 162—172), leafhopper cell lines by Chiu and Black (1967 Nature, London, 215, 1076—1078); tick cell lines by Rehacek (1971, Acta Virologica, 55, 32—41) to name just a few who contributed to the fast expanding field. Invertebrate cell culture conferences have been organized every four years, following the first one in France, and culture media have been vastly improved.

The importance of Grace's achievement can hardly be overestimated. A few years earlier, in 1955, Dr. Paul Weiss of Rockefeller and the U. S. National Academy of Sciences organized a conference at Macdonald College of McGill University in Canada, to discuss the feasibility of invertebrate tissue culture. I received a copy of the mimeographed proceedings from Max Day, who was among the invited participants. Gerry Wyatt was also one of the participants. Paul Weiss wrote the concluding remarks, stating that contrary to mammalian cells, insect cells will most likely never be grown in continuous culture because in adults the number of insect cells is determined and no new cells are produced once this number is attained. Other participants thought that succes would perhaps be achieved as soon as the chemistry of insect hemolymph became known and media with a composition similar to insect hemolymph became available. The Wyatts found that trehalose is among the essential components of insect hemolymph and insect cell culture media and during the following years analysis of hemolymph and modifications of culture media to match the hemolymph of respective invertebrates in composition, pH, and osmotic pressure became a trend. Only later, in 1971, was it found that the composition of a culture medium does not have to match the composition of the insect's hemolymph (Grace, 1971, in Invertebrate Tissue Culture, C. Vago, ed.,

Academic Press, Vol. 1, 171—209). Hemolymph was found inadequate as the sole source of tissue culture nutrient and it could be substituted conveniently with animal sera.

Grace's breakthrough was greeted with understandable enthusiasm because it proved the feasibility of growing invertebrate cells in continuous culture.

The influence and suggestions of Glaser at Rockefeller Institute in Princeton were responsible for Trager's successful work with silkworm and mosquito tissue culture. Glaser, who died in 1947, as well as Trager, who,at 80 is still very active at Rockefeller, also influenced another scientist, who died only a few months ago but should be mentioned prominently today. In the 1930s in the laboratory of Wendell M. Stanley at Rockefeller Institute in Princeton worked as a postdoctoral fellow a Yale Ph.D. graduate, Dr. Zan-Yin Gaw. Inspired by Harrison, Glaser and Trager, and trained as a virologist, Dr. Gaw decided to try the cultivation of the grasserie virus in *Bombyx mori* cells *in vitro* after he returned to his native China. At Wuhan, Gaw became the chairman of the microbiology department. In 1959, three years before Grace's successful cultivation of *Antherea* cells, Gaw published his results in English, (1959, Acta Virologica, Vol.3., 55—60). Gaw(also spelled Kao), was indisputably the first to grow insect cells continuously. At the time of his 1959 report, he had carried his cell line of *Bombyx mori* epithelial gonad cells through 22 passages and used cell monolayers for his virus studies. Although his report appeared in English, China's isolation and the subsequent cultural revolution prevented the wide dissemination of his work. Gaw's very important achievement did not influence invertebrate tissue culturists in the way Tom Grace's success did. Thus Gaw's work in no way detracts from the achievement and influence exerted by Grace on the whole field of invertebrate cell culture over the past 30 years. During a search of the literature in 1959, I came across Gaw's report and at the International Congress of Entomology I mentioned his results. Although my paper was published by the Congress, (1962, Proc.XI Int.Congress Entomol. Vienna, 2, 801—807) Gaw's breakthrough continued to be overlooked by invertebrate cell culture workers outside China. In 1982 prof. Gaw invited me to visit him at Wuhan, where I met him and his coworkers, who are continuing in the direction started by their great mentor.

Several important papers were published by Grace,following the thorough review of insect tissue culture prepared jointly with Max Day for the Annual Reviews of Entomology in 1959. Grace's papers that appeared in 1958, 1959, 1962, 1963 (1963, Ann. Epiphyties, 14, 27—28; 1968, Expm. Cell Res., 52,

451—458)1968 and 1971 made his name a houshold word of invertebrate tissue culturists. Currently, in 1991, his medium is still prominently listed by commercial tissue culture media manufacturers.

More than a quarter century has elapsed since Grace established the first continuous cell lines from *Antherea pernyi* in Australia. Today more than 200 cell lines of invertebrates exist. Invertebrate cell lines are being used for various purposes and attempts are being continued to develop cell lines of invertebrates other than insects. Media are being improved and great progress has been achieved in the development of serum-free invertebrate culture media. Often unexpected results are being obtained, as was the case with the M&M medium, developed by Mitsuhashi and Maramorosch (1964, Contr. Boyce Thompson Inst., 22, 435—460), initially for leafhopper tissue culture, but found best suitable for mosquito cell cultivation and for studies of arboviruses in mosquito cells.

During the past decade, from 1980 till 1990, invertebrate cell culture became widely used in biotechnology and in basic research in genetics, molecular biology, endocrinology, biochemistry, physiology and virology. Use of invertebrate cells is rapidly gaining popularity for the production of recombinant proteins, viral insecticides, and the production of vaccines. In vitro techniques are indispensable for studies of insect virus expression vectors. What will the following decade bring?

We can only speculate about future applications of invertebrate cell culture. possible applications and developments include efficient and inexpensive mass cultivation procedures. Further improvements will most likely be made in the development of genetically engineered baculoviruses for nonchemical pest control. Vaccines will be produced as well as drugs, using baculoviruses and invertebrate cells. Genes from other organisms will be inserted into cultured invertebrate cells for a variety of purposes, including, perhaps, improved oysters that produce cultured pearls.

We could speculate that invertebrate cells of certain species will provide new sources of food, for fish, domestic animals and even humans.

Application of invertebrate cell culture and molecular biology will lead to significant progress in the understanding of phsiological and developmental mechanisms and of cellular and molecular interactions between invertebrate cells and fungi, microsporidia, bacteria and viruses. Numerous physico-chemical and developmental-genetic principles remain to be worked out in endocrinology, gene expression, specificity, resistance to infection, the mechanism of resistance to drugs and variety of control agents.

Too bad that we do not have multiple lifetimes to enjoy and observe what the next 100 years of research will bring to the field to which Tom Grace and Zan-Yin Gaw provided a solid, everlasting foundation.