INFLUENCE OF CELL LOSS DURING *IN VITRO* CULTURE OF *DROSOPHILA* WING DISCS

by

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INTRODUCTION

Tissue culture is an effective method for study of the developmental capacity of embryonic tissue. The imaginal discs of Drosophila larvae are determined as to the structure in the adult to which they will give rise, but these discs remain undifferentiated throughout larval life. Hadorn (1966) found that by interrupting the development of one of these discs between its determination and differentiation, he could modify the amount of determination and the ability of the disc to differentiate at any given time. His experiments were carried out in vivo; that is, a determined blastema was taken from a larva and injected into the abdomen of an adult where it was allowed to proliferate. After any length of time, the tissue could be recovered and transplanted into a prepupal larva. After emergence of the adult the amount of differentiation could be determined by direct observation. Hadorn reported the occurrence of transdetermination, a phenomenon in which a determined imaginal disc differentiates into an unrelated structure (Hadorn, 1966). Futhermore, he and his students concluded that the amount of transdetermination is related to proliferation; that is, the longer the disc is allowed to grow in the adult, the more transdetermination will take place (Tobler, 1966; Garica-Bellido, 1966).

In contrast to in vivo culture, in vitro technique involves the culturing of tissue in defined media. This method has developed only recently for insect tissue (see Schneider, 1967 for review). In effect, in vitro culturing of an imaginal disc extends the period of time between determination and differentiation without allowing tissue growth (Schneider, 1967). Using whole and half wing discs McCrady (1968) found that if material cultured in this manner was transplanted into a larva, the amount of subsequent differentiation depended in part on the length of culture time. Whole discs transplanted without in vitro culture yielded poor wing development and good thorax development, while split discs transplanted

without *in vitro* culture yielded better wing development and similar thorax development. Three days *in vitro* culture before transplantation produced intermediate development; the wing developed more, the thorax less. After seven days of culture of the whole disc, the wing differentiated even more, but not as much as the wing half of the disc cultured alone. On the other hand, the thorax of the whole disc developed very poorly and the thorax half of the disc alone even more poorly.

It was noticed that a large number of cells were lost from the thorax half during *in vitro* culturing. On the other hand, very few were ever seen to migrate from the whole disc. These observations led to two alternative explanations of the decrease in the ability of the thorax half to differentiate. Either loss of cells or loss of the wing influence could be responsible. This study has correlated cell loss and subsequent development of the disc part, in order to determine which of these alternatives is correct.

MATERIALS AND METHODS

The larvae of D. virilis were used as hosts and as the source of wing disc tissue. All stages were raised on sterile David's medium (1962) at a constant temperature of $25^{0} \pm .25$ centigrade.

Food trays inserted into half pint milk bottles containing thirty or more flies served as the means for egg collections. The flies were allowed to deposit eggs for approximately four hours before removal of the tray. The eggs were transferred to a solution of 2% sodium hyphochlorite and subsequently dechorionated by emersion for ten minutes (Poulson and Waterhouse, 1960). Dechorionation serves to rid the eggs of bacteria. After transferral by a sterile Pasteur pipette to food-containing petri dishes, the eggs were permitted to reach late third instar.

Wing discs were taken from donor larvae in sterile Schneider's (1964) medium, then rinsed in a second container of sterile Schneider's.

Half discs were suspended in a .05 ml. drop of medium on the underside of a sterile coverslip. In order to check spreading of the drop, coverslips were treated with a silicone solution (Beckman Desicote). The coverslip with the drop was placed on a depression slide and sealed with vasoline.

Counts of cell migration from the thorax and wing halves were taken daily beginning twenty-four hours after the discs were removed from the donor and ending twenty-four hours before transplantation. Counts were made under bright field illumination using the 10X or 25X objective.

Seven days after in vitro culturing was begun, the discs were transplanted into larvae which were within six hours of pupation. Using the transplantation techniques of Bodenstein (1950) the discs were injected into the posterior ventral abdominal areas of ether anesthetized larvae. For the injection, a fine-tipped glass needle, fitted to a syringe, was used (see also Ursprung, 1968).

The larvae were allowed to undergo metamorphosis. After the adult flies emerged, they were etherized and submerged in Waddington's insect saline. The ventral abdominal area was opened and inspected for a transplant. If present, transplants were removed, fixed and mounted.

The dissection of disc tissue, the in vitro culture and the trans-

plantations were performed by Dr. McCrady.

The anterior part of the wing disc gives rise to the thorax area on which normally develop eleven large bristles called macrochaetae and one hundred or more smaller bristles, or microchaetae. The criterion for development of the thorax half was the number of macrochaetae and microchaetae.

The posterior half of the wing disc develops into a wing on which are located numerous small bristles called span bristles. The length of the span bristles was the criterion used for measuring the amount of wing development. A standard ocular micrometer was used to measure bristle length.

The t test was employed to determine the statistical significance of all data.

RESULTS

The following table presents the number of cultures set up and the number of transplants performed. The discrepancy between number of discs in culture and number of transplants is due to disc loss during the culture period.

TABLE I

Disc	Number Discs in culture	Number Transplants	Number Hosts Survived	Number Transplants recovered
Thorax	123	99	65	37
Wing	121	86	62	40

DESCRIPTION OF EMIGRANT CELLS

The most consistent observation concerning the type of cells lost by the two halves was that the cells lost by the thorax were much smaller than those lost by the wing. Those leaving the thorax measured 3.9μ to 4.9μ compared to 12μ to 17μ for those leaving the wing. However, there were a few exceptions. Twenty-four of the thorax halves lost large cells along with small cells while thirty wing halves lost small cells, and eleven of these lost no large cells. Often the large cells would assume a spindle shape; these spindle shaped cells averaged 34μ in length. It was also obvious that the number of cells that migrated from the thorax half was greater than the number from the wing half. Figures 1 and 2 are graphs of daily cell migration from thorax and wing halves.

CORRELATION OF AMOUNT OF CELL LOSS AND SUBSEQUENT DEVELOPMENT

Figure 1 indicates that the standard deviation from the mean is quite large in the thorax half on each day. On the final day of counting some thorax halves had lost no cells, while a few lost as many as 500. The amount of cell loss was divided into three categories: high, medium, and low. The middle category was designated as the mean ± one-half standard deviation (163.6 ± 67.9). Any loss by the final day above this range was considered

FIGURE 1

Number of cells seen outside thorax area of wing disc during *in vitro* culture period.

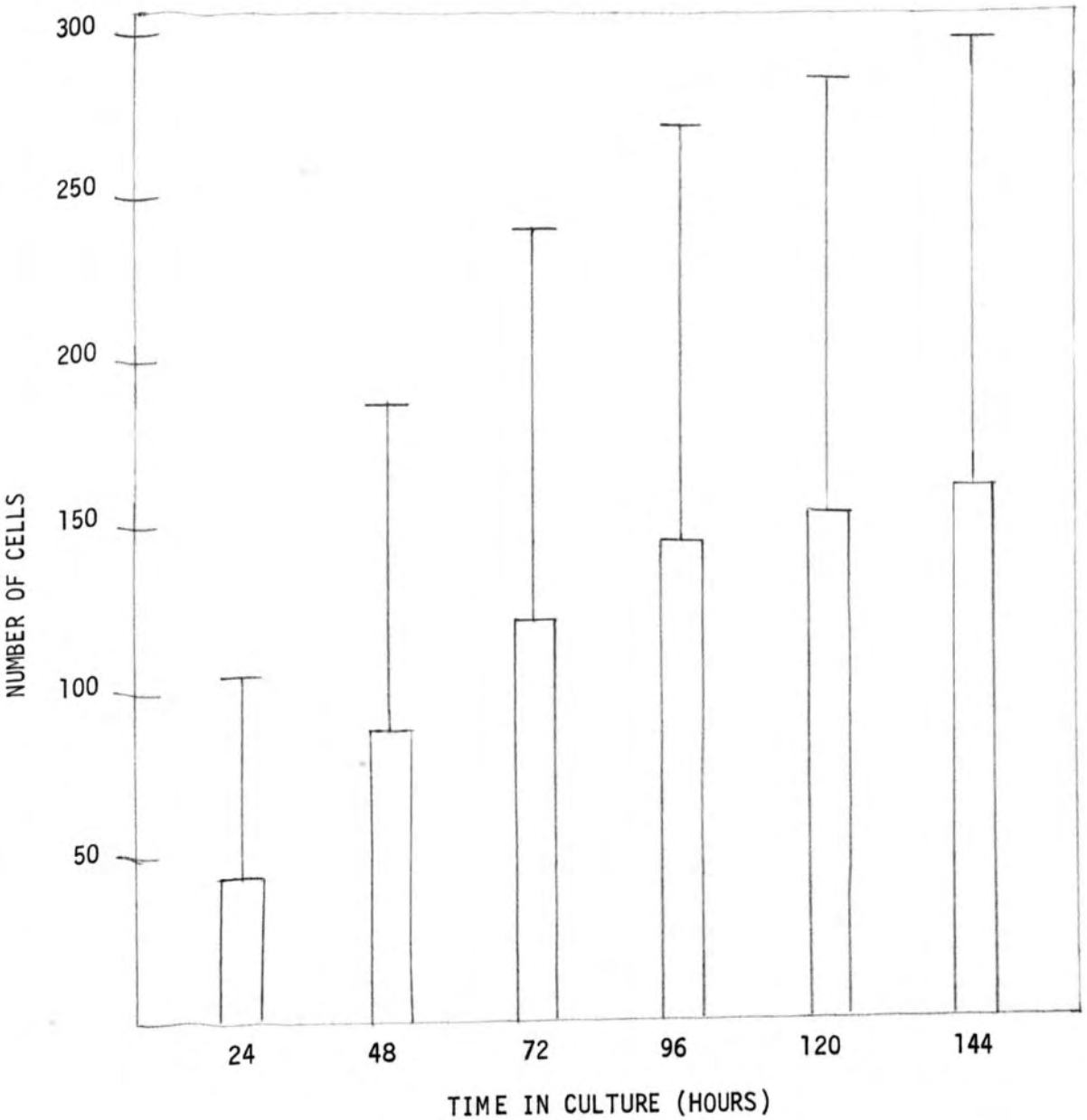
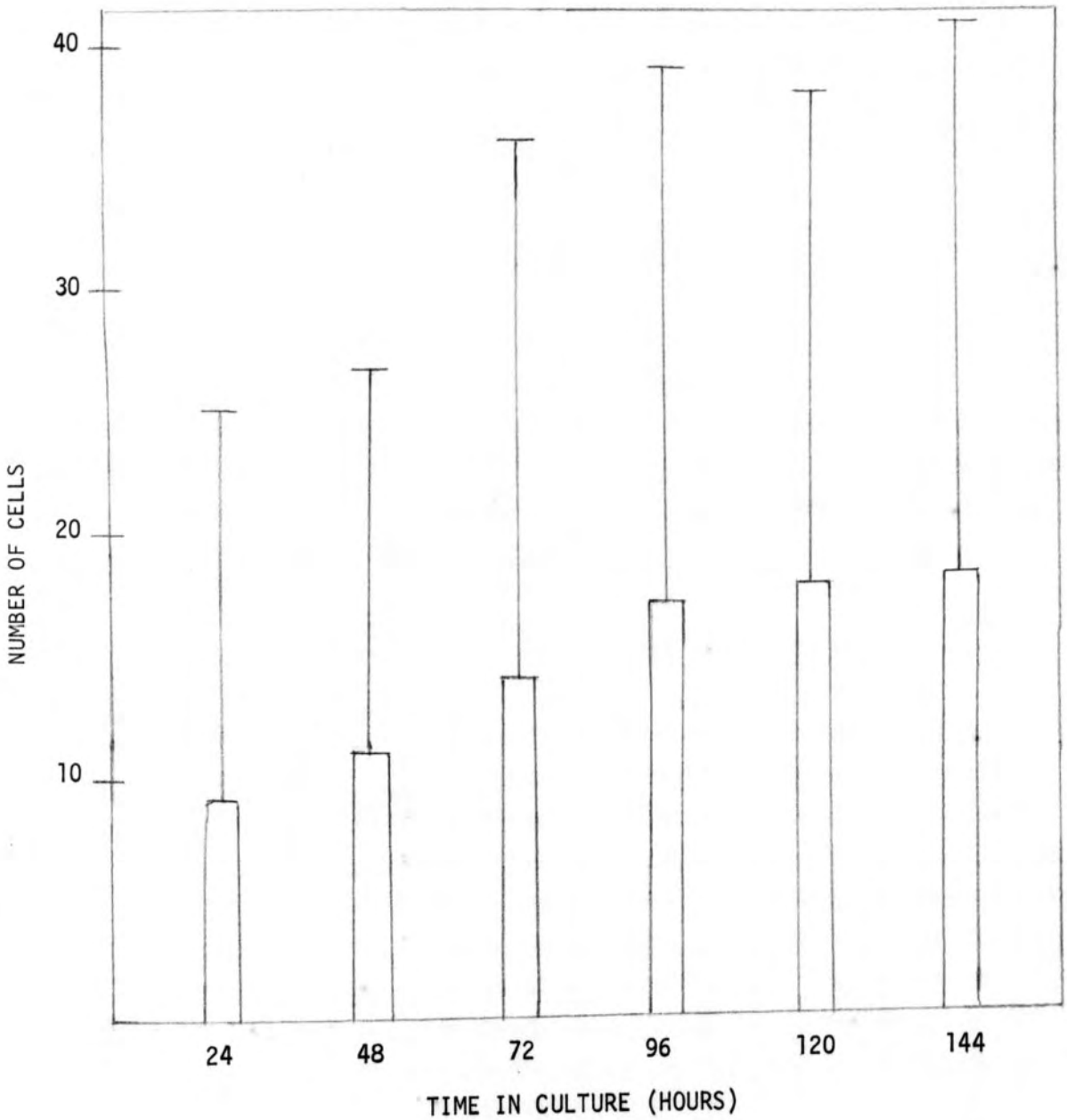

300
250
200
150
100
50
NUMBER OF CELLS
24
48
72
96
120
144
TIME IN CULTURE (HOURS)

FIGURE 2

Number of cells seen outside wing area of wing disc during *in vitro* culture period.


40
30
20
10
NUMBER OF CELLS
24
48
72
96
120
144
TIME IN CULTURE (HOURS)

high (> 231.5) while loss below this range was considered low (< 95.7). Development of thorax halves with cell loss in the low category was compared to development with cell loss in the high category. In these cases, number of macrochaetae and microchaetae was the criterion for gauging the amount of development. There were ten cases in the low range; the average number of macrochaetae was 2.0 ± 2.4, of microchaetae, 17.2 ± 16.9. For the twelve cases in the high range, the average number of macrochaetae was 1.9 ± 2.3 while the average number of microchaetae was 14.0 ± 10.4. The t value for the difference between the two means for macrochaetae is .08, with $p = 0.9$. The t value for microchaetae is .49; $p > 0.5$.

At the end of the six day period, the average number of cells lost by the wing half was 18.3 ± 11.3. The amount of cell migration was classified into high, medium or low categories in the same manner as the thorax halves (18.3 ± 11.3 constituted the medium loss group). The length of the span bristles for wing areas with low cell loss was $22.0\mu \pm 3.7\mu$. For wing areas with high cell loss the span bristles measured $22.4\mu \pm 4.3\mu$.

CORRELATION OF TIME OF CELL LOSS AND SUBSEQUENT DEVELOPMENT

A comparison was made between the number of bristles found in thorax halves that lost cells at a more or less constant rate over the six day period and the number of bristles found in thorax halves that lost their total amount of cells within the first three days. In sixteen cases, there was a loss of cells after the third day. For these cases, the average number of macrochaetae was 1.8 ± 2.5 and the average for microchaetae was 16.3 ± 14.7. Fifteen discs lost no cells after the third day. These averaged 3.5 ± 3.0 for macrochaetae number and 20.5 ± 16.6 for microchaetae number. For macrochaetae, t value for the difference between the two means was 1.63, with $p > 0.1$. Corresponding values for microchaetae are $t = .73$, $p > 0.3$.

In the wing halves early cell loss, that is, before the end of the third day, included sixteen cases with an average bristle length of $21.2 \pm 3.8\mu$. The ten cases in which cell migration took place after the third day had an average bristle length of $23.4 \pm 2.5\mu$. The difference between the means are $t = 1.7$, $p > 0.1$.

DISCUSSION

McCrady's study (1968) indicated that thorax areas of wing discs cultured *in vitro* for seven days developed less well than the thorax areas of whole discs cultured *in vitro* for the same length of time. In general, the longer the thorax halves were cultured, the less they developed. In contrast the wing halves developed well either alone or when cultured as whole discs, and there was more development than in the thorax, at least in so far as wing development can be measured by bristle length. The fact that the thorax exhibited more development when cultured as part of a whole disc rather than a half disc could be an indication that the wing half has some sort of influence on the thorax half that contributes to its development. The wing could exert its influence by preventing cell loss or by some other means. The large number of cells observed leaving the thorax halves during *in vitro* culturing as opposed to the low number leaving the wing half implied that the decrease in the ability of the thorax halves to differentiate could be due to this excessive loss of cells and that the wing half did indeed contribute to thorax development by preventing this occurrence. Consequently, in this investigation emphasis was placed on the effect of cell migration on differentiation.

It was noted in the results that, in contrast to the general rule, some thorax halves lost large cells and some wing halves lost small cells. This discrepancy may be the result of a difference in the level of cut. When the whole discs were cut in half, some cuts were higher than others. If the large cells are normally located in the wing area and small cells in the thorax area, a cut higher than usual would result in loss of small cells from the wing while a cut lower than usual would result in migration of large cells from the thorax half of the disc.

Shatoury and Waddington (1957) reported that at the end of the second and third instars, the lymph glands of larvae became very active and released free cells into the body cavity. They divided these cells into three main categories: spheroids, platelets and hexagons. Immediately after hypertrophy of the lymph glands, they found cells, similar in structure to the lymph cells, clustered around certain imaginal discs.

They concluded that these cells were lymph cells and that they acted as stimulants for further development of these discs. They based their conclusions on the fact that after these cells, which they called "oikocytes," appeared, the imaginal rudiments were brought into a state of readiness for metamorphosis. After this differentiation occurred, the "oikocytes" degenerated. It should be noted that these studies were carried out on sectioned tissue, which might alter the structural appearance.

In this investigation the cells that were lost by the thorax and wing halves fit into the three categories discribed by Shatoury and Waddington (1957). This study concentrated on the time of cell loss and the number of cells lost. In the thorax half there was no consistent pattern of cell loss. As a rule, the majority of thorax halves lost cells within the first twenty-four hour period. Although the average cell loss in this period was 43.7, it was not uncommon to find a thorax that lost from 150-200 cells. In one case a thorax half lost 400 cells during the first day. After the first day, there was no definite pattern. Some thorax halves lost all their cells within a three day period. These halves were placed in the group considered early in cell loss. Other halves lost a moderate number of cells every day; others had no loss in the beginning days and a heavy loss on the fourth or fifth day. Generally, cell loss increased very little near the end of the counting period. An attempt was made to correlate the number of bristles developed with early or late loss of cells by the thorax halves. As the results indicate, the difference was not significant.

The large variations in the number of cells lost by individual thorax halves led us to believe that this might influence development, but again the statistical difference was not significant.

In the wing halves, the pattern of cell loss was no more consistent than in the thorax halves. The average number of cells lost was much lower, but once more some halves lost their total amount in the first three days and others lost a few day by day. In some cases, there was no loss until the fourth day. However, a comparison of span bristle length of those wings exhibiting late cell loss to those showing early loss indicated

no significant variation. Wing halves losing a large (> 30) number of cells were not found to differ significantly in bristle length from those losing less than 10.

In short, when each half of the disc was considered, no correlation was found between time of cell departure and development or between number of cells lost and subsequent development. Therefore, if these cells are Shatoury's "oikocytes", their influence has already been exerted. This is a possibility since the discs are not taken from the larvae until the latest period of third instar and Shatoury observed "oikocytes" at the end of second and third instar.

Since there were no correlations found, it must be concluded that the wing half exerts its influence by means other than preventing cell loss. The lack of development could be due to lack of minimal tissue. Many previous studies have established the principle that there must be a definite minimum amount of tissue for an in vitro culture to survive. In the case of in vitro culture of wing discs, the whole disc may be the minimum amount of tissue necessary for normal development. Another possibility is that the wing acts as a sort of structural base on which the thorax may begin development. Lack of differentiation could be due to the absence of this assistance. The need for such a structural base must be acquired by the thorax region of the disc at some time in the latter half of the in vitro period, since thorax half transplants without in vitro culture develop quite well.

SUMMARY

1. Preliminary studies indicated that failure of the thorax half of wing disc to differentiate normally when cultured *in vitro* separate from wing half was due to some influence exerted by the wing on the thorax that contributed to its development. The large number of cells lost by the halves suggested the influence of the wing half was by prevention of cell loss.

2. Wing and thorax halves were cultured separately *in vitro* for a seven day period and daily counts of cell loss were recorded.

3. The majority of thorax halves lost numerous small cells while most wing halves lost a smaller number of large cells.

4. The time of loss and amount of loss were compared to amount of subsequent development in the wing and thorax halves after transplantation into prepupal larvae. Number and length of bristles were used as criteria for development.

5. No correlation was found between time and amount of loss and development.

6. The results indicate that the wing exerts its effect by some means other than prevention of cell loss.

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