The Ability to Differentiate, and the Size of Regenerated Cells, after repeated Regeneration in *Spongilla lacustris*

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Summary

The findings presented here have a bearing on regeneration in general. They show that even in simply organized animals, the number of totipotent cells which are able to differentiate decreases as the individual ages. In older specimens it takes longer for the totipotent cells to differentiate than in younger ones; at the same time the nuclear and cytoplasmic volume of these cells is reduced.

The expanded basal epithelium of sponges germinating from gemmules is an organ necessary to establish the tension in the body which is indispensable for the functioning of the sponge. Sponges that have germinated from gemmules can be forced to regenerate a basal epithelium. The materials for this regeneration is furnished by the archaeocytes, which are embryonic, totipotent amoebocytes. The number of archaeocytes that are able to perform this regeneration decreases with time, i.e. as the differentiation of the entire body proceeds.

On the other hand, the ability of the sponge to expand repeatedly on its own basal epithelium after being pushed away from it is limited only by the onset of cytolysis.

The ability of the archaeocytes to regenerate new *typical* basal epithelial cells is reduced after repeated regeneration.

The size of both nucleus and cytoplasm is reduced more and more during repeated regeneration. The nucleo-cytoplasmic ratio is thus kept fairly constant.

Introduction

AN organism having the ability to regenerate lost parts must draw upon a stock of undifferentiated cells to restore its integrity when injured. This stock is a limiting factor in the organism's ability to regenerate. It is still an unsettled question whether such undifferentiated cells have always been embryological in character or whether some of them, owing to forces released by mutilation, attain this primitive state after dedifferentiation from more specialized conditions. Evidence is, however, more and more in favour of the first alternative. In lower organisms, such as sponges, the regenerative power seems to be almost unlimited; it has been assumed that the archaeocytes (amoebocytes wandering in the exoplasm of the sponge (Brøndsted, 1936)) are able to transform themselves almost unrestrictedly into any kind of sponge-cell. In the earlier paper (1936) it was shown (and this was independently confirmed by Brien (1937)) that after dissociation of the sponge body by pressing it through boulting cloth, the four main cell-types again participate in building up small functioning sponges, taking up their proper positions in relation to one another. At that time I pointed out that we cannot expect the

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necessary proportion between the four cell-categories to be established in the small reunited bodies that have come into being by random assembly of wandering cells; it must therefore be assumed that the apparently least-differentiated category of cells, the archaeocytes, are able to differentiate into missing cells of other categories.

If this viewpoint is correct, the question arises: Is the presumed potency of the archaeocytes unlimited? The present paper is an attempt to answer this question.

In the autumn the freshwater sponges, *Spongilla*, *Ephydatia*, &c., break up into gemmules, 0·3–0·8 mm. in diameter; these consist only of yolk-laden archaeocytes surrounded by a horny capsule. In the spring the archaeocytes creep out through a preformed micropyle in the capsule, and slide out on to the skeleton, which consists of siliceous spicules cemented together with a horny substance, the spongin. The archaeocytes differentiate in a few days. The process has been described by several authors, especially by Brien (1932), who gives full evidence that the archaeocytes differentiate into other cell-types. I have shown (Brøndsted, 1943) that a certain tension in the little organism is necessary to ensure organization of a functional water-canal system. This tension is set up by the basal epithelium, which spreads out on the substratum with exceedingly flattened cells that glue themselves on to it. By means of the electron microscope, Brøndsted and Carlsen (1951) have shown that this very strong adhesive power of the cells of the basal epithelium is due to a submicroscopical cortical feltwork of fibrils (presumably of spongin), built by the cells that cover the substrate.

It is possible to push away a little sponge from its substratum in such a manner that the basal epithelium remains upon it (Brøndsted, 1943); the sponge will round up in a few minutes and spread out again in the course of 1 or 2 days when placed on another slide. If, however, the rounded-up sponge is replaced on its own basal epithelium it will again spread out upon it, and with a speed many times greater than that of a sponge placed on a clean slide (Brøndsted, 1943).

We see here the counterpart of the well-known fact that the epithelial cells bordering a wound rapidly spread out and try to cover the wound. In our experiments, the remaining basal epithelium represents the wound and the body-cells have to spread out to cover it, and this they do with great speed. When the wound is closed, the integrity and tension of the sponge is restored, and very soon the proper organization for functional activity is attained.

The situation is different when the sponge, after being pushed away from its basal epithelium and having rounded itself up, is placed on the clean surface of a slide. Here the situation resembles that of the germinating gemmule, for a new basal epithelium has to be made.

The question arises: Are archaeocytes able to differentiate into basal epithelial cells indefinitely (or as long as the cells are able to move about)?

Experiments

Preliminary investigations showed that 4 expanded individuals were able to make new basal epithelia 5 times each when pushed away soon after expansion terminated, whereas only 4 individuals out of 12 were able to make new basal epithelia 3 times when they had been expanded for some days and had begun their natural functions (the oscula and water-canal system had developed). The other 8 individuals, however, were still very much alive; they were in the state of globular cell-aggregates, wandering round on the slides without ability to expand.

A more extensive experiment was planned to study the now obvious question whether a time factor governs the ability of the sponge to regenerate the kind of differentiated cells which make up the basal epithelium.

Fifteen gemmules were brought to germinate on 3 slides, 5 on each. The first 5, group A, were pushed away from their basal epithelium and placed on new slides as soon as they had expanded fully. The next group, B, was allowed to differentiate for 2 days after expanding before they were pushed away. The third group, C, was pushed away after differentiating for 4 days; the sponges had at that time differentiated fully and were functioning. Four slides with several gemmules on each served as controls. The sponges were pushed away on the fourth day after expanding, when they were functioning fully; they were placed back on their own basal epithelia. When a sponge had expanded after being pushed away, it was again pushed away to test its expanding force. Table 1 gives a survey of the results.

The experiments show that the ability to make new basal epithelia diminishes rapidly when the sponges have had time to differentiate before they were

Table 1*a*

Survey of the ability of gemmules to make new basal epithelia at different times after the first expansion (hence at progressive stages of differentiation). After the sixteenth day no more expansions occurred

	Days from beginning of the experiment																			
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*19*	*18*	*19*	*20*
A1	exp. o	+	+	+		+	+		+						+						
A2	exp. o	+		+			+				+						+				
A3	exp. o	+		+	+				+				+								
A4	exp. o	+						+	+			+									
A5	exp. o	+	+	+	+				+		+		+								
B1	exp.		o		+					+											
B2	exp.		o					+			+										
B3	exp.		o		+				+	+			+								
B4	exp.		o		+			+	+				+		+						
B5	exp.		o														+				
C1	exp.				o																
C2	exp.				o																
C3	exp.				o		+							+							
C4	exp.				o																
C5	exp.				o																

exp. = first expansion. o = first removal from basal epithelium. + = expansion after every removal; the new removal took place when expansion was completed.

TABLE 1*b*

Controls to 1*a. Expansion after removal and replacement on basal epithelia. The individual gemmules have not been followed up. The figures are numbers of expanded specimens after every new removal. After the sixteenth day no more expansions occurred*

Control slides with	*Days from beginning of the experiment*																			
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
10 spec.	all exp.				0	9	8	8	7	7	7	6	5	5		4	3				
9 spec.	all exp.				0	9	9	9	8	9	9	9	9	9		9	8				
14 spec.	all exp.				0	14	14	14	14	14	13	14	13	12		7	7				
10 spec.	all exp.				0	10	10	10	10	10	10	10	9	10		8	6				

pushed away from their basal epithelium; as Brien (1932) shows, the various differentiated cell-types (choanocytes, canal cells, spicule-forming cells) are derived from the archaeocytes in the gemmule, and therefore in the functioning sponge the original stock of archaeocytes has been drawn upon heavily. The 5 specimens of group A were able to rebuild a basal epithelium 28 times, which gives an average of nearly 6 per individual. The specimens of group B show an average of about 3 per individual, and those of group C only an average of less than 0·5. The controls were able to expand on an average 7 times per individual; but this figure is in reality too low because several of them were in a state of dissolution. The experiments indicate that the ability to produce a certain category of differentiated cells diminishes with the age and state of differentiation of the sponge in regeneration.

The question presents itself whether the ability to regenerate is correlated with the size of gemmules, i.e. the number of cells in the organism.

Fifteen specimens of various sizes were studied as to their ability to regenerate new basal epithelia. Fifteen gemmules regenerating on their own basal epithelia served as controls. All individuals were pushed away from their basal epithelia after they had differentiated (4 days).

TABLE 2

The ability of gemmules of various sizes to make new basal epithelia 4 days after the first expansion. The size of the gemmules is indicative of the number of cells constituting the gemmule. The experiment shows that the number of cells is not correlated with ability to differentiate new basal epithelia

Size of gemmules in μ.	300	380	400	450	500	500	550	600	600	600	600	600	650	650	700
Number of expansions.	4	0	0	0	3	0	1	4	5	0	0	6	0	5	1

Controls. Here the gemmules had also expanded 4 days before they were removed, but after removal they were placed on their own basal epithelium; therefore they did not need to differentiate new epithelia. Size of gemmule (and therefore the number of cells) is not correlated with ability to expand

Size of gemmules in μ.	350	350	400	400	450	450	450	500	500	550	550	600	600	650	700
Number of expansions.	10	11	11	11	10	10	11	9	11	10	11	9	9	10	10

It appears from this experiment that the size of the individual, and therefore the number of cells, is not correlated with the ability to differentiate basal epithelial cells. Furthermore, most controls that are made to expand on their own basal epithelium can do this up to 11 times each, and perhaps even more; if they are not cytolysing they can do this regardless of their size.

The typical basal epithelial cell has the appearance shown in fig. 1. Apart from the extremely flattened shape and the vacuolization in the cytoplasm

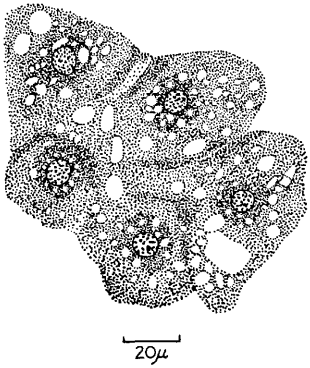

FIG. 1. Five cells of the normal basal epithelium of *Spongilla lacustris*.

around the nucleus, the basal epithelial cell can be recognized by its lack of nucleolus.

The basal epithelia of the sponges in the above-mentioned experiment were fixed in Zenker's fluid and stained with haemalum. A count of the better part of the first basal epithelium of sponge no. 13 in table 2 showed 14 cells provided with nucleoli out of 309 cells, i.e. 4·5 per cent. In the sixth and the last basal epithelium from the same sponge, 73 cells out of 186 cells, i.e. 39 per cent., had a nucleolus. Fig. 2 shows basal epithelial cells after five regenerations in the same individual as fig. 1.

In another individual, no. 15, from the same experiment, the first basal epithelium had 7 cells each with a nucleolus out of 557 cells, i.e. 1·3 per cent., whereas the third basal epithelium had 45 cells with nucleolus out of 137 cells, i.e. 33·5 per cent. In still another experiment, one individual in the first basal epithelium had 4·8 per cent. cells with nucleolus, but 13·2 per cent. in its third epithelium; and in a fourth individual the corresponding figures were 3·3 per cent. and 10·3 per cent.; in a fifth the figures were 1·2 per cent. and 10 per cent.

A question of considerable interest is whether or not the size of the regenerated cells diminishes after repeated regeneration. The sponge considered in this paper lends itself to the solution of this problem.

An extensive account of the size of the nucleus in the basal epithelium in the living state and after fixation by some 30 different methods will be given elsewhere (Brøndsted, A., and Brøndsted, H. V., in press). It will be shown that the measurement of 100 nuclei is quite sufficient to give a reliable average of their size; the influence of the fixatives on nuclear volume is also discussed. In specimen no. 15 the nuclei in the first epithelium have a diameter averaging 8·78μ, in the third 8·41μ, and in the sixth 7·91μ; i.e. during six regenerative

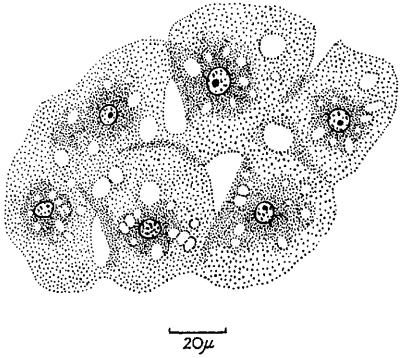

FIG. 2. Six cells of the basal epithelium of *Spongilla lacustris* after five regenerations. The specimen was fixed and stained in the same way and for the same times as the specimen of fig. 1. Note the lighter appearance.

processes, the diameter of the nuclei had diminished by about 10 per cent. and their volumes by about 30 per cent. In another specimen, no. 13, there is a decrease of nearly 50 per cent. in volume through six passages.

It is not only the nuclei that diminish in the course of repeated regeneration. The areas of the expanded basal epithelial cells were measured by counting the number of cells covering a certain area. In the first basal epithelium 939 nuclei were found; the area of each basal cell here averages 1959μ^2; whereas in the third, in which 1,174 nuclei were counted, each cell has an area of 1469μ^2, so that the cells have diminished their volumes by at least 25 per cent. during the whole regeneration period.

DISCUSSION

Several points in these investigations have a bearing on important problems of regeneration.

Even such simply organized animals as the sponges have rather a limited power of regeneration; the ability to regenerate differentiated cell-types is inversely proportional to the state of differentiation of the entire animal. That means that the organism attains its functional organization at the cost of embryonic developmental possibilities. This agrees well with the diminishing

regenerative power of (for example) amphibian embryonic cells, which shift to new differentiation patterns; it is a well-known fact that the regenerative power diminishes from the early gastrula stage onwards: 'the doors are being closed', to use an expression of J. Needham.

The archaeocytes represent the stock of embryonic undifferentiated cells; as Brien has shown, these cells differentiate to the various categories of cells necessary to build a functioning sponge; a certain equilibrium between the number of cells of the various types is attained in normal development, but a certain stock of archaeocytes is available for new differentiation in spite of the fact that in sponges that are no longer able to expand, several archaeocytes are still found. There exists clearly a minimum amount of archaeocytes below which renewed differentiation to other cell types is not possible. It remains obscure what are the forces governing this equilibrium.

In our experiments the expansion of the sponge on its own basal epithelium resembles the phenomenon of the closing of a rather big wound; this process requires energy; this is apparent from the following fact. When rounded-up sponges at last fail to expand after they have been placed back on their own epithelium, they die, showing cytolysis; whereas sponges, which at last fail to expand on new slides when pushed away from their basal epithelium, are still alive for some days and able to move about; therefore they do not require as much energy as for expanding.

The ability of the archaeocytes to differentiate to true basal epithelium cells decreases with time. The first time basal epithelium is normally made, the archaeocytes very soon transform themselves, and in doing so lose their nucleoli; in repeated regeneration the transformation is harder to accomplish; this is seen from the fact that more and more cells still retaining their nucleoli are found when the basal epithelium is regenerated on later occasions. It is obvious that our material offers an opportunity of studying the question of cytochemical processes involved in, at least, one course of cell-differentiation. Investigations are proceeding in our laboratory.

The question whether by repeated regeneration the cells retain or diminish their size is, of course, of interest. The answer is important in connexion with the question whether the organism strives to maintain the vigour of some working cells at the expense of other cells or if all cells suffer alike under the strain. In the experiments with *Spongilla* it has been shown that all cells involved in regeneration diminish their size; i.e. all cells share the common burden.

The reduction in the size of the regenerating cells affects the nucleus as well as the cytoplasm. The measurements do not give precise information about the nucleo-cytoplasmic ratio, but they suggest that it remains constant. It is assumed that cyto-chemical investigations, together with those mentioned above, may prove fruitful for the understanding of some of the physiological interrelations between nucleus and cytoplasm. In this connexion reference should be made to the very interesting paper of Schrader and Leuchtenberger (1950).

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