The Significance of Cell Death

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Why is there cell death? Why, especially during development, is there such a luxuriant waste of cells? Why doesn't the embryo "do things right in the first place?" The very fact of cell death raises fundamental questions about the way in which developmental organization is created and maintained. Does development, in fact, proceed in a fashion that we would consider to be logical and efficient, or might the generation of the many different types of cells that appear early in life be a fundamentally chaotic process, which requires cell death to create order?

Till has pointed out that there are two types of cellular events which occur during development, to which he has given the names "Lamarckian" and "Darwinian" (Till 1981). The term Lamarckian is used to describe those occasions in which cells respond to signals in their environments and modify the expression of their genomes, whereas Darwinian describes those processes in which cells, having already become committed to particular patterns of gene expression, are selected to live or die. Both Darwinian and Lamarckian processes can generate the sort of developmental order that is seen to arise during embryonic life. The research that my colleagues and I have carried out in the last few years has led us to the belief that Darwinian processes provide the dominant organizing force in development (Michaelson 1987, 1989, and in prep.). As such, we have come to the view that cell death is more a cause, than a result, of developmental organization.

In our laboratory, we have been interested in the synthesis of the proteins of the blood, the so-called plasma proteins. These are produced either in the lymphoid organs of the immune system, which produce the immunoglobulins, or in the

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parenchyma of the liver, which makes most of the rest. The synthesis of the immunoglobulins has a number of features, which, as I describe below, are used not only by the immune system, but may also be at work in regulating the expression of the plasma proteins produced by the liver. Two features of antibody production are central to the control of the immune response. *First*, antibody synthesis is the prototype of a Darwinian process, that is, a developmental process organized by cellular selection. The presence of a foreign substance (an "antigen") in the body causes the division of those few lymphocytes, which happen, by chance, to express a complementary immunoglobulin. Antibody-producing cells are selected by the immune system, and this process is known to immunologists as clonal selection (Jerne 1955; Burnet 1959; Lederberg 1959). *Second*, immunoglobulin gene expression is a stochastic, or random, process. Whether an individual lymphocyte expresses this or that V-region gene is essentially a matter of chance.

The random or stochastic property of immunoglobulin gene expression is now an accepted feature of the immune response. Historically, this has been detected by a number of methods, but one of the first indications of this randomness was the identification of the phenomenon of allelic exclusion (Pernis et al. 1965; Cebra et al. 1966). Being diploid organisms, vertebrate animals contain two homologous copies of each autosomal gene. However, for immunoglobulin genes, the two homologous copies are never expressed simultaneously in the same cell. Technically, allelic exclusion is detected with antisera that identify genetic variants, or allotypes, of immunoglobulin genes. Allelic exclusion can be seen in animals heterozygous for an immunoglobulin allotypic polymorphism. By using antiallotypic sera, individual lymphocytes in these animals are found to express one or the other allotype, but never both. Allelic exclusion follows from the stochastic feature of immunoglobulin gene expression, by the following reasoning: If the expression of each immunoglobulin gene is random, there is no reason to expect the two homologous alleles of a gene to be activated coordinately. If we hold two dice, one in each hand, and throw them both, there is no reason to expect both to come up the same.

The stochastic property of immunoglobulin genes may seem

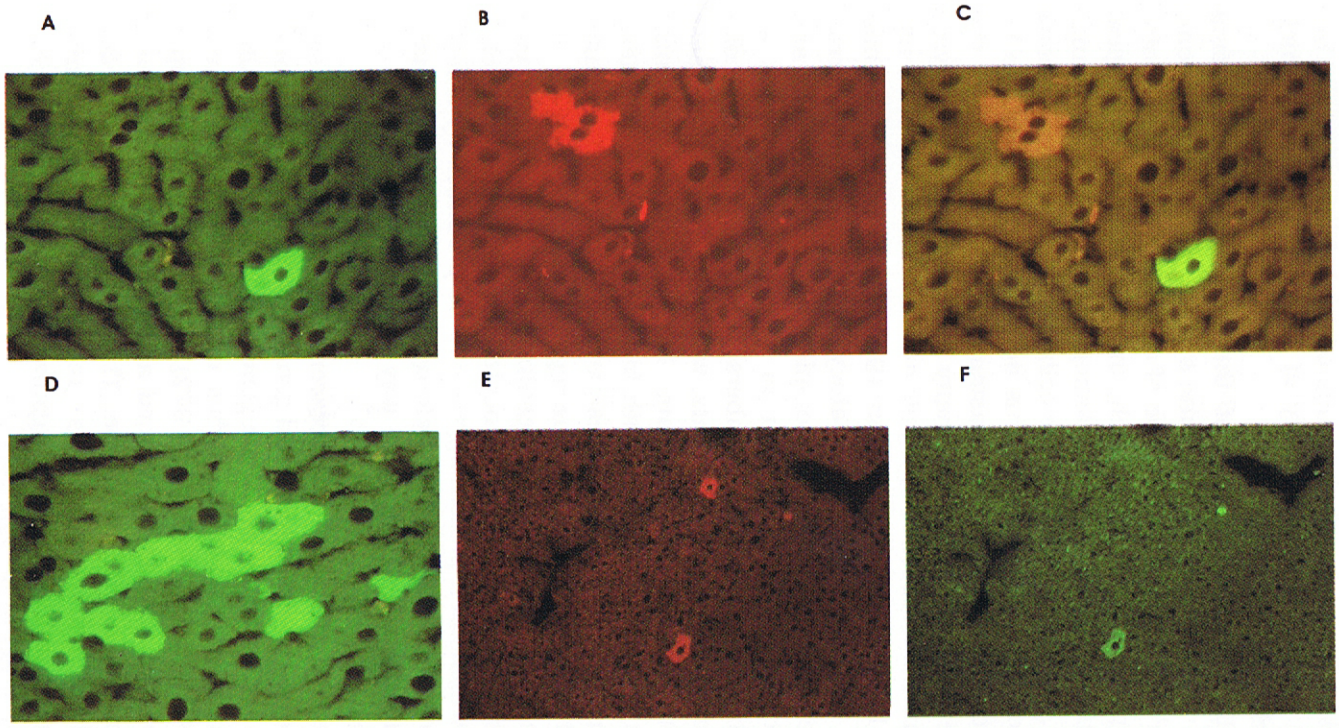

FIGURE 1 Immunofluorescence of mouse liver. (*A*) Albumin-containing cell (green) identified by immunofluorescence with rabbit anti-mouse albumin. (*B*) C3-containing cell in same field (red) identified by goat anti-mouse C3. (*C*) Double exposure of same fields as *A* and *B*, showing simultaneously albumin (green) and C3 (red) containing cells. A large series of similar experiments established the separate identity of cells containing albumin, transferrin, C3, fibrinogen, and α_2-macroglobulin. (*D*) Cluster of albumin-containing cells. (*E,F*) Immunofluorescence of liver from an Alb^a/Alb^c heterozygous mouse. The reactivity of rabbit anti-mouse albumin is revealed in red; the reactivity of mouse anti-Alb^c is seen in green.

When we examined the livers of Alb^a/Alb^c heterozygous mice, we found a very remarkable thing: The albumin locus appears to display allelic exclusion, exactly like that known for immunoglobulin genes. This could be seen in the observation that only about half of the albumin-containing cells (identified by rabbit anti-mouse albumin antiserum) express the Alb^c allele (identifiable by anti-Alb^c) in the livers of Alb^a/Alb^c mice (Fig. 1). The most straightforward, but perhaps most surprising, conclusion from this observation is that the expression of the albumin gene is a stochastic process.

As in the case of immunoglobulin genes, the stochastic feature of albumin gene expression strikes us as wasteful and illogical. However, as in the case of immunoglobulin gene expression, the usefulness of this random property can be seen in its capacity to generate the heterogeneity of hepatocytes present in the liver.

As mentioned previously, plasma protein-containing cells are present in the liver both as individual cells and in groups of adjacent cells, which we suspected might be clonal. The analysis of these groups of albumin-containing cells in Alb^a/Alb^c heterozygous mice substantiated the idea that these clusters are clones. We reached this conclusion from the observation that although some clusters in these mice are Alb^{c+} and some are Alb^{c-}, *within* each cluster there is no variation. We examined over 80 clusters containing approximately 400 cells from four separate heterozygous mice and did not find even one cluster that contained both Alb^{c+} and Alb^{c-} cells. Presumably, each cluster is a clonal group, derived from a single cell committed either to express the Alb^c gene or not, and this property is inherited by the progeny of that cell.

To summarize, these observations suggest two conclusions. *First*, the activation of the albumin gene appears to be a stochastic process. *Second*, the state of gene expression appears to be cell-heritable; once a hepatocyte has become committed to express an albumin gene, this property is passed along to the clonal progeny of that cell. The cell-heritable feature of gene expression is, of course, not unique to the albumin gene but is a widespread observation in biology, and there have been a number of suggestions as to the mechanism of this process. Among the hypotheses that have been put for-

ward to explain cell-heritable changes in gene expression are methylation, supercoiling, protein-DNA interactions that are perpetuated through replication, and self-propagating hypersensitivity sites (Riggs 1989). As to the role of the stochastic activation step in the final determination of phenotype, I suspect that the stochastic step is only one of several components of gene expression. It seems likely to me that after gene activation occurs, there are additional levels of control, mediated by regulatory proteins interacting with *cis*-acting sequences, as have been suggested by many studies in the last few years.

The apparent stochastic feature of albumin gene activation, surprising as it may be, has precedents in other systems. Allelic exclusion has been observed, not only for immunoglobulin genes, but also for genes coding for phenotypes as diverse as skin coat color and erythrocyte hemoglobin. For coat color genes, this allelic exclusion is seen as a patchy or variegated appearance of the pelt of heterozygous animals. Although in several of these cases the coat color genes are on the X chromosome and the variegation can be ascribed to random X-chromosome inactivation, most examples of coat color variegation are autosomal. This variegation has been observed for coat color genes on half a dozen autosomes in the mouse, as well as for a variety of autosomal genes in guinea pigs, cattle, rabbits, dogs, and pigs (for references, see Michaelson 1987). For globin genes, allelic exclusion has been observed in erythroid precursors from children heterozygous for one of the fetal globin genes. Kidoguchi and his colleagues made this observation when they found that each of the two allelic forms of a fetal globin gene are not expressed coordinately in the progeny of single cells (Kidoguchi et al. 1980).

Stochastic gene expression has also been observed in a number of cases where the process of gene activation has been followed in single cells. This has been observed for the generation of hematopoietic stem cells by Till and McCollough (1980), for melanogenesis by Bennett (1983), for globin gene expression by Housman, Levinson, Gusella, Orkin, and Leder (Orkin et al. 1975; Gusella et al. 1976; Levinson and Housman 1981), for myogenesis by Nadal-Ginard (1978), and for terminal differentiation by Smith and Whitney (1980).

I turn now from the apparent functional heterogeneity of

hepatocytes, and the cause of this heterogeneity, to the manner in which this heterogeneity appears to be utilized by the liver to control the overall output of plasma proteins. We have collected a great deal of data on the quantity of cells of each

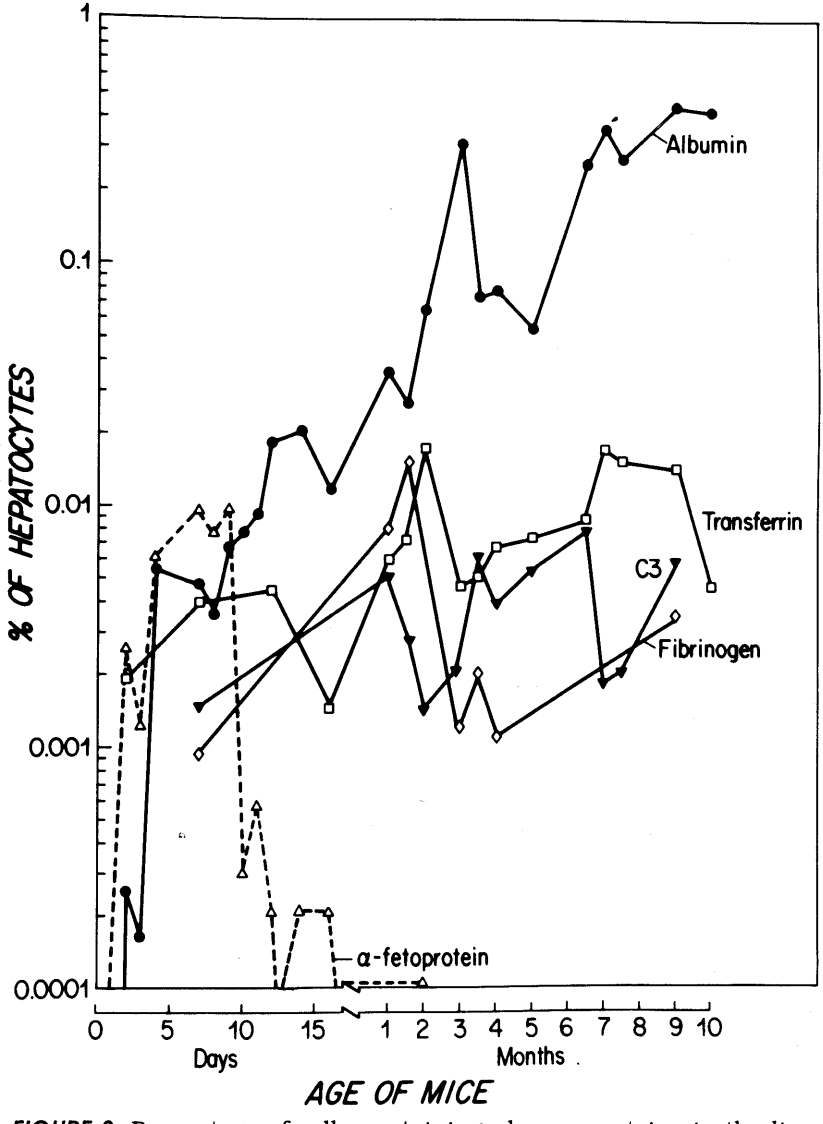

FIGURE 2 Percentage of cells containing plasma proteins in the livers of mice of various ages. (Data from J. Michaelson, in prep.).

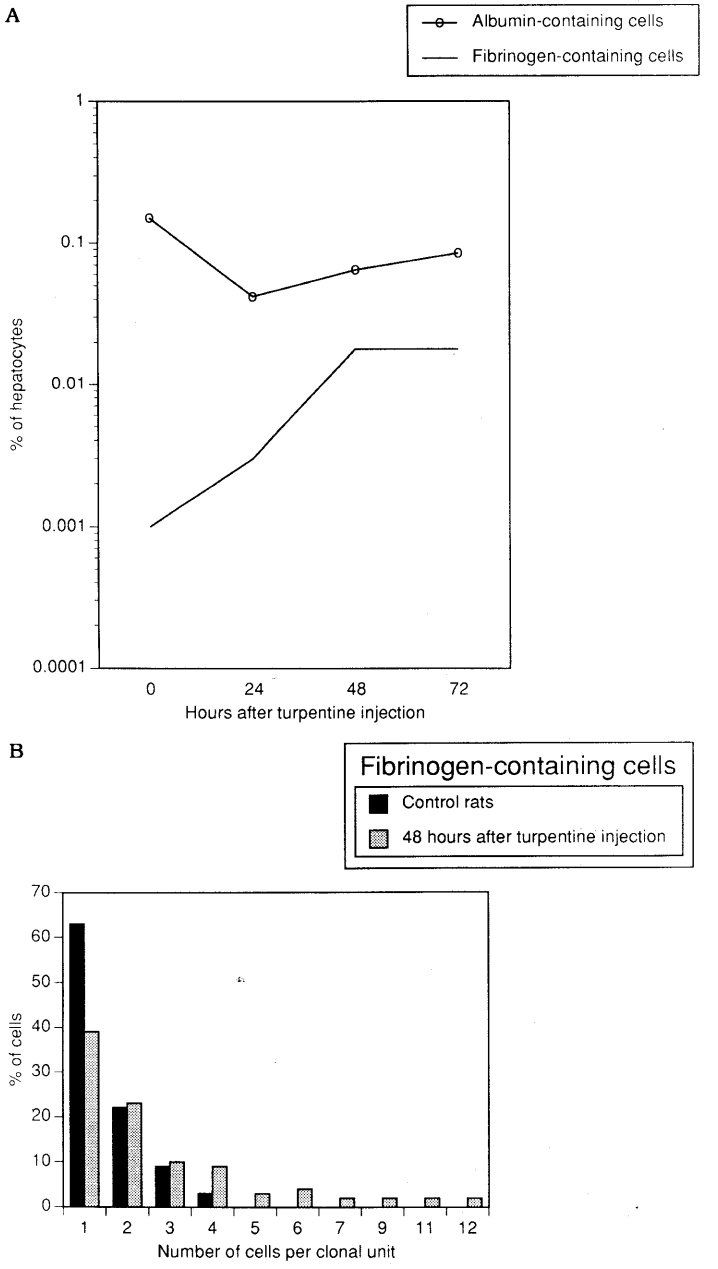

FIGURE 3 *(See facing page for legend.)*

type (Figs. 2 and 3) (Michaelson 1989 and in prep.). These data suggest that the functional status of the liver is determined by the cellular mix among the specialized types of hepatocytes and that changes in function may be mediated by changes in this mix by selective cell growth and death. For example, we have found that in the adult mouse liver, albumin-containing cells constitute somewhat less than 1% of hepatocytes. About one-tenth of this number of cells produce transferrin, and one-thirtieth of this number produce C3 (Fig. 2). These values roughly reflect the relative rates of synthesis of these three proteins.

The liver appears to be able to change its plasma protein output by changing the mix of specialized cells by cell growth and death. For example, early in life, α-fetoprotein is the principal plasma protein, but soon after birth, there is a gradual switch from α-fetoprotein synthesis to albumin synthesis (Abelev 1971; Nahon 1987). We have found that there is an equivalent change in the number of cells containing each protein (Fig. 2). There are considerable numbers of α-fetoprotein-containing cells in the livers of neonatal rats and mice; the number of these cells declines as the animals mature, while the number of albumin-containing cells rises from being very rare to being the most prevalent plasma protein-containing cell in the liver. This is apparently due to the fact that α-fetoprotein-containing cells divide most rapidly in the livers of newborn animals, as could be seen by the average number of cells contained in clonal clusters of α-fetoprotein-containing cells. Apparently, these cells divide more slowly as mice mature, as shown by a smaller number of cells in those clonal groups. The converse is true for albumin-containing cells. Thus, the switchover from a predominance of α-fetoprotein-containing cells to a predominance of albumin-containing cells appears to be due to differential cell growth.

FIGURE 3 (*A*) Change in the number of albumin- and fibrinogen-containing cells after an acute-phase response induced in rats by an injection of turpentine. (*B*) Distribution of fibrinogen-containing hepatocytes into single cells and clusters of various sizes in control rats and in rats 48 hr after induction of the acute-phase response. These data illustrate the increase in the average cluster size of fibrinogen-containing cells, apparently by cell division.

During adult life, there are also changes in plasma protein synthesis, particularly during inflammation. For example, Schreiber has shown that the inflammation induced in rats by an injection of turpentine causes, over a 2-day period, a slight decrease in albumin synthesis and a five- to tenfold increase in fibrinogen synthesis (Schreiber et al. 1989). We have found an equivalent slight reduction in the number of albumin-containing cells and a sixfold increase in the number of fibrinogen-containing cells (Fig. 3A). Furthermore, it appears that the sixfold increase in the number of fibrinogen-containing cells is due to cell division, as shown by the increase in the average size of the clusters of fibrinogen-containing cells (Fig. 3B). We have recently found direct evidence that the acute-phase response is accompanied by considerable cell growth in the liver, by visualizing dividing cells that take up the DNA precursor bromodeoxyuridine.

What can we conclude from the data collected so far? Our picture is far from complete, but we can make some generalizations, if only to frame testable hypotheses. The hepatocytes that comprise the liver appear to be heterogeneous in that each plasma protein is seen in a separate set of hepatocytes. This heterogeneity appears to be generated by stochastic gene activation. Change in the overall synthesis of each plasma protein may be mediated by selective growth and death among these specialized cells in the liver. In short, plasma protein synthesis appears to fit our description of a Darwinian process; that is, a process organized by cellular selection among a diverse collection of specialized cells.

What are the general lessons to be drawn from these observations? Does the process of plasma protein synthesis exemplify a general phenomenon in biology? The signs of cell growth and death are seen everywhere in embryology (Kerr et al. 1972, 1987; Beaulaton and Lockshin 1982). Glucksmann enumerated 74 separate examples of embryonic cell death alone (Glucksmann 1950). The cell death that accompanies development is not incidental but represents a highly refined cellular mechanism (Kerr et al. 1980; Tomei et al. 1988). Might the differential cell growth and death seen in development not just be the result of morphogenesis, but the *cause* of morphogenesis? Selective growth and death of cells is such a com-

mon feature of embryogenesis that it is easy to overlook the capacity of such processes to mold the outcome of development itself. For example, the liver starts out as just a few cells in the hepatic diverticulum of a 7-day-old mouse embryo; these cells grow much more rapidly than the rest of the embryo, generating, by this selective cell growth, an organ which by day 10 is recognizable as the liver by size and shape (Theiler 1989). In short, the liver comes into being by the selective growth of its precursors. Such events occur repeatedly in development (Michaelson 1987).

Within organs themselves, the process of the generation of form, or morphogenesis, can often be ascribed to cell selection. One of the best illustrations of such a process is the development of one system, the vertebrate limb (Fig. 4) (Michaelson 1987). Limb development begins in the embryo as a small outgrowth called the limb bud, composed of a boundary of ectoderm enclosing mesodermal cells (Hinchliffe and Johnson 1980). After a period of growth, condensations of bone-forming and muscle-forming cells appear roughly in the general pattern of bones and muscles that will constitute the mature limb. It appears unlikely that limb morphogenesis occurs by a Lamarckian process, in which the undifferentiated mesoder-

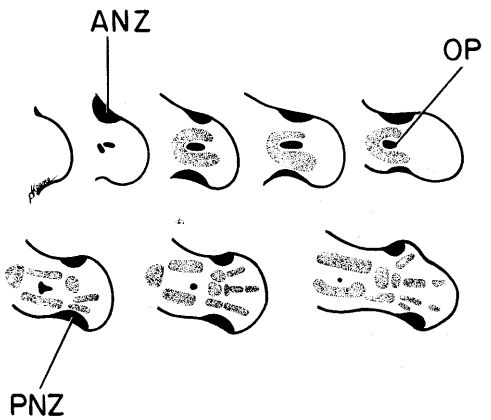

FIGURE 4 Development of the chick wing, showing areas of highest cell death (black) and areas of chondrogenic condensation (gray). (ANZ) Anterior necrotic zone; (PNZ) posterior necrotic zone; (OP) opaque patch. (Reprinted, with permission, from Michaelson 1987).

mal cells are instructed to become muscle or bone (by diffusible "morphogens" or other signals), for the simple reason that undifferentiated mesenchymal cells with potential to become either myogenic or chondrogenic do not exist in the limb. In fact, the two cell types appear to be derived from entirely separate, noninterconvertible cell lineages that migrate into the limb from different regions of the embryo (Abbott et al. 1974; Christ et al. 1977; for additional references, see Michaelson 1987). It seems that, like the parenchyma of the liver, the mesoderm of the limb bud is composed of a salt-and-pepper mixture of cells with separate potentialities.

What then, accounts for the appearance of limb structure? As in the case of the liver, we can account for limb morphogenesis by a Darwinian process of cellular selection. We see the signs of this selective process in the massive amounts of cell death throughout the developing limb. Large areas of this cell death are seen at the front and back of the limb bud (the anterior and posterior necrotic zones) and in the central area (the opaque patch) (Fig. 4) (Hinchliffe and Johnson 1980). We suspect that this cell death is the selective elimination of chondrogenic cells, since bone will later appear only in those areas where cell death is not seen. It might be said, then, that cell death does not arise as a result of limb development; limb development arises as a result of cell death (Michaelson 1987).

The immune system illustrates how a biochemical response can be finely tuned by a selective process, but is such a process capable of shaping development in three dimensions, as is seen in the limb? Fortunately, there have been several theoretical studies that suggest that such an outcome is, in fact, very much a possibility. In one of the nicest of such studies, Swindale asked whether a striped pattern, such as seen in a zebra's skin or in the visual cortex, can be generated by cellular selection alone (Fig. 5). He developed a computer-simulated model of cellular competition and, starting from a random mixture of cells interacting through diffusible substances, found that a highly patterned developmental outcome may result (Swindale 1980).

The features of morphogenesis driven by cell selection, and particularly cell death, are not unique to the limb, the liver (Bursch et al. 1985; Columbano et al. 1985), or the immune

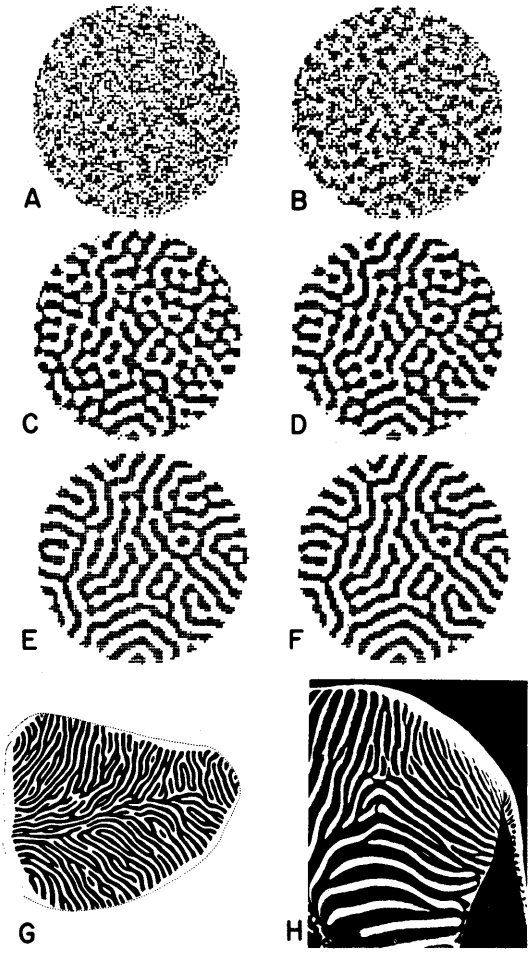

FIGURE 5 Computer simulation of the spontaneous emergence of striped form in a field of cells or synapses arising through a process of selection (*A–F*). Comparable examples from the natural world: (*G*) ocular dominance stripes in the visual cortex; (*H*) stripes on the rear flank of a zebra. (Redrawn from Swindale 1980.)

system (Duke 1989; Trauth et al. 1989), but appear to be a widespread feature in development. Common examples of this include regression of the Wolfian or Müllerian ducts, destruction of larval structures during tissue reformation in insect and amphibian metamorphosis (Wadewitz and Lockshin 1988), closure of the palate, invagination of the optic cup,

shaping of the nose, creation of the digestive tract and heart, formation of the nervous system (Oppenheim and Nunez 1982; Ashwell and Watson 1983; Johnson et al. 1989), and remodeling of the uterine epithelium (Rotello et al. 1989) and prostate (Buttyan et al. 1989).

I began this chapter by posing the question, "Why is there cell death?" Perhaps we can begin to formulate an answer to this question. We can look to the role that cell selection can play in the generation of developmental order. The process of selective growth and death has great simplicity, but it is also capable of great versatility and precision. If, as we suspect, development is a Darwinian process of cellular selection, then the meaning of cell death is clear: Cell death may act to create order out of the diversity of cell types, which emerge during embryonic life.

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