against 0.05 M acetate buffer, pH 5.5 with 0.2 M NaCl and was then chromatographed on a Sephadex G-75 column equilibrated with the same buffer. The active fractions were pooled and run on a column of organomercurial-Sepharose under conditions employed by Barrett[8], then the cathepsin B1 preparation was rechromatographed on Sephadex G-75 column.

Results and discussion. As can be seen in the table the activity of homogenates from early placentas was higher than the activities of term and post-term homogenates, respectively. Samples of purified enzyme (about 0.03 units) were run in isoelectric focusing gels. The distribution of activity was demonstrated and the pI determined. Present data (table) indicate the presence of 3 forms of cathepsin B1 in the developing or mature placenta and 4 in the involuting one. The forms differ in isoelectric points, the pI-values ranging from 5.1 to 5.7. Cathepsin B1 isolated by Evans and Etherington[2] from human placenta had a pI of 5.4. The authors have not detected isozymes on isoelectric focusing of the crude samples of the placental enzyme.

Previously we demonstrated 3 isozymes of cathepsin B1 in human fetal placenta membranes and chorion, and only 1 isozyme in amnion[3]. The nature of the microheterogeneity of cathepsin B1 is not clear at this time. Takahashi et al.[9] suggested that the charge difference between isozymes may be the result of deamidation and the forms of cathepsin B may represent a part of the activation and degradation of the enzyme. On the other hand, Tawatari et al.[10] working with the crystalline cathepsin B1 from rat liver ascribed this microheterogeneity to the changes in the carbohydrate content during the solubilization step. Changes in the cathepsin B1 activity as well as the variety in isozyme pattern may reflect the alteration in the metabolism of the developing placenta. Early in gestation the placenta carries on many metabolic functions that are later taken up by the fetal organs. The endopeptidase activity of cathepsin B1 is well established[11], and peptidylodipeptidase activity against glucagon[12] and fructose-1,6-bisphosphate aldolase[13] has recently been observed.

1 A.A. Swanson, B.J. Martin and S.S. Spicer, Biochem. J. *137*, 223 (1974).
2 P. Evans and D.J. Etherington, Eur. J. Biochem. *83*, 87 (1978).
3 M. Warwas and W. Dobryszycka, Biochim. biophys. Acta *429*, 573 (1976).
4 M. Warwas, A. Lisowski, J. Malinowski and J. Sward, Diagn. Lab. *16*, 267 (1980); Polish text.
5 A.J. Barrett, Analyt. Biochem. *47*, 280 (1972).
6 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
7 I.W. Drysdale, P. Righetti and H.F. Bunn, Biochim. biophys. Acta *229*, 42 (1971).
8 A.J. Barrett, Biochem. J. *131*, 809 (1973).
9 K. Takahashi, M. Isemura and T. Ikenaka, J. Biochem. *85*, 1053 (1979).
10 T. Tawatari, Y. Kawabata and N. Katunuma, Eur. J. Biochem. *102*, 279 (1979).
11 A.J. Barrett, in: Proteinases in Mammalian Cells and Tissues, p. 81. Ed. A.J. Barrett. North-Holland, Amsterdam 1977.
12 N.N. Aronson and A.J. Barrett, Biochem. J. *171*, 759 (1978).
13 J.S. Bond and A.J. Barrett, Biochem. J. *189*, 17 (1980).

Microdensitometric analysis of denervation effects on newt limb blastema cells

R.M. Loyd and T.G. Connelly

Department of Anatomy and Center for Human Growth and Development, The University of Michigan, Ann Arbor (Michigan 48109, USA), 27 November 1980

Summary. This report examines the fate of cycling cells in normal and denervated blastemas of adult newts. Cells are found to accumulate in G_1 in blastemas which are nerve independent. No stage specific accumulation different from controls is found in limbs with nerve-dependent blastemas.

Regeneration of an amphibian limb is dependent upon nerves for its completion[1]. Early phases of dedifferentiation and initiation of DNA synthesis occur in the absence of nerves, but normal mitotic activity, blastema formation, and growth fail to occur in denervated limbs[2,3]. Limbs are considered to be nerve-dependent until 15 days after amputation (8 days for larvae)[4,5], yet are generally capable of producing morphologically normal but smaller regenerates following denervation at 17 days post-amputation; i.e., become nerve-independent. Therefore, the period from 15-17 days has been termed the transition period[4] when the limb switches from an absolute to a conditional dependence on innervation. The fate of cells in nerve-dependent blastemas after denervation is unclear. Tassava and Mescher[6] proposed a model suggesting that the nerve facilitates passage of dedifferentiated cells through the G_2 phase of the cell cycle and allows them to divide in M. This hypothesis suggests that following denervation cells might accumulate in G_2. Mescher and Tassava[2] were unable to demonstrate the presence of such cells in sections of blastemas using microspectrophotometric techniques. Recently, Maden[7] has reported that cells in denervated axolotl limbs may block in G_1. These alternative results are clouded by differences in experimental methodology which seriously affect their interpretation. In order to reconcile the differences between these authors, we undertook a detailed search, using microdensitometry, for discrete populations of cells in amputated, denervated limbs of adult newts which might differ from those found in amputated, normally regenerating limbs.

One of the differences in previous experimental methodologies is that Maden measured whole nuclei in dispersed preparations while Mescher and Tassava measured nuclei in sectioned material. A more important factor is that Maden denervated well developed blastemas which may have been nerve-independent. Mescher and Tassava, on the other hand, did denervations at the time of amputation, before blastema formation, when the limbs were clearly nerve-dependent.

Right and left limbs of adult newts were amputated midway between the wrist and the elbow. In 1 series of experiments left limbs were denervated at 15 days post-amputation, when blastemas had reached the early bud stage. In a 2nd, left limbs were denervated at 19-21 days post-amputation, when regenerates had reached the late bud stage[1] (comparable to the cone stage referred to in the

experiments of Maden). Denervations were performed by severing the 3rd, 4th, and 5th, spinal nerves at the shoulder. Several animals in each group were not further disturbed but were observed to see if their blastemas were capable of producing regenerates following denervation. At least 3 control and 3 denervated blastemas of each series were fixed daily on days 0-6 following denervation. The limbs were processed in toto for the Feulgen reaction and cells of the blastema were placed on a slide. Microdensitometric measurements of the dispersed nuclei were made with a Quantimet 720 image analyser. A minimum of 100 cells were measured in each of the 3 limbs for a total of 300 cells at each time point. Results were analyzed statistically using the Kolmogorov-Smirnov and Smirnov tests, and the Mann-Whitney U-test and were performed using the MIDAS statistical package of the University of Michigan Statistical Research Lab. The combined use of the non-parametric tests allows one to tell not only if there are differences between groups, but also in which direction the differences lie.

Of the blastemas denervated at 15 days and observed for an additional 21 days, 90% showed inhibition of regeneration (nerve dependence). On the other hand, 80% of the blastemas denervated at the cone stage (19-21 days) continued to regenerate into morphologically normal but smaller limbs. Thus 19-21-day regenerates were generally nerve-independent at the time of denervation. Figure 1 shows that most of the cells in such a denervated blastema appear to possess the G_1 complement of DNA by day 5 following denervation. There is no significant shift toward density classes which could be associated with cells possessing the G_2 complement of DNA. Figure 2 shows that in limbs denervated on day 15 following amputation, there is no discernible shift toward either a G_1or a G_2 population. Since G_1 and G_2 take up approximately the same percentage of the total cell cycle[8], we feel we should be able to detect cells having a G_2 complement of DNA if they accumulate since we do see a shift to G_0-G_1 in the nerve-independent limbs. Thus, there is no significant difference in density classes between the denervated left and innervated right limbs when limbs are denervated at nerve-dependent stages. No significant changes were found in the cell population of either group earlier than day 5 post denervation.

The results shown in figure 1 agree well with those of Maden[7]. However, the fact that control limbs in this group continued to regenerate (i.e., were nerve-independent) and the results shown in figure 2 (measurements from clearly nerve-dependent limbs) suggest to us different interpretations from his. Since denervation of nerve-independent limbs causes precocious redifferentiation of cycling blaste-

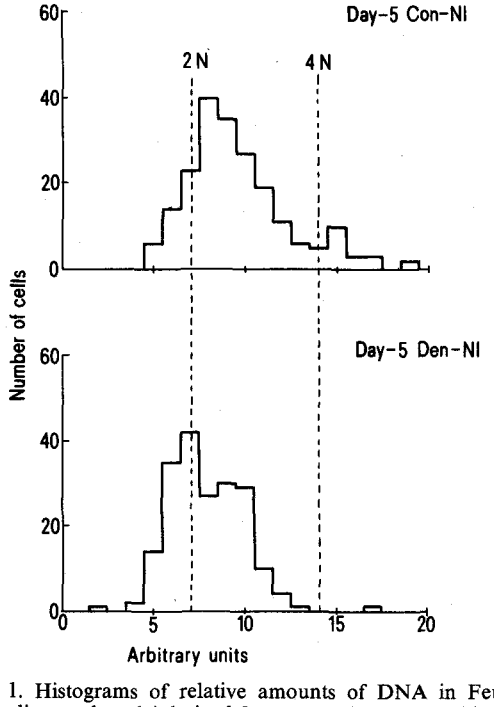

Figure 1. Histograms of relative amounts of DNA in Feulgen-stained, dispersed nuclei derived from normal cone stage blastemas (Con) or from those denervated (Den) 5 days previously (19-21 days post-amputation). Total time since amputation is 24-26 days. Since 80% of limbs denervated at identical times and stages as these, were able to produce small but morphologically normal regenerates, we feel that these regenerates were nerve-independent (NI) at the time of denervation. Measurements of integrated optical density of the nuclei were made using a Quantimet 720 Image Analyzer. A minimum of 100 cells from each of 3 control (Con) and 3 denervated (Den) limbs were counted. The arbitrary density units reflect relative amounts of DNA within a group of stained nuclei. All nuclei were stained and measured the same day. Statistical tests show that the histogram for denervated limbs is shifted to the left of that of control limbs (toward the G_0-G_1 complement of DNA).

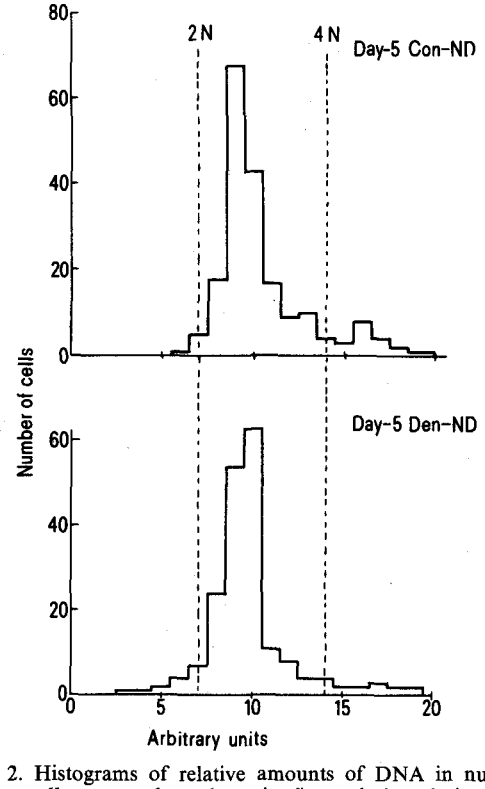

Figure 2. Histograms of relative amounts of DNA in nuclei of blastema cells prepared as those in figure 1, but derived from normal 20-day blastemas or those denervated 5 days previously (day 15). No statistically significant difference is detectable between these 2 histograms. One should not attempt to compare these histograms with those of figure 1 since the blastemas for the 2 experiments were of markedly different chronological and developmental age at the time of fixation. Since all the blastemas on such limbs which we observed for 21 days after denervation regressed or ceased regeneration we feel that these limbs were clearly nerve-dependent (ND). The peak on the graph represents the phase of the cell cycle (S) which occupies the greatest period of time in an actively dividing cell population and therefore contains the greatest percentage of cells.

ma cells, one would expect an increase in the number of cells with the G_0-G_1 complement of DNA. Although Maden does not report controls for nerve dependence similar to those in our experiment, we suspect that the cone stage blastemas in his experiment were nerve-independent. As figure 2 shows there is no discernible change from the innervated limbs in the overall pattern of the microdensitometric curve following denervation of 15-day regenerates. These results are consistent with those of Mescher and Tassava who have failed to find any abnormal accumulation of cells in denervated limbs. An important point to be made is that the controls continue to proliferate and exhibit their characteristic curve for DNA amounts. The experimental blastemas of day 15, even though denervated, exhibit a curve essentially identical to the controls. In these limbs, mitosis decreases or stops altogether and the blastema regresses to the level of amputation. The fate of blastema cells during regression remains elusive and there is continuing controversy in the literature over where in the cell cycle the nerve has its influence. Carlone and Foret[9] have provided in vitro evidence to support the idea of Tassava and Mescher that the cells of a denervated limb block in G_2. When they added newt brain homogenate to blastemas cultured without nerves, they found a burst of mitotic activity after only 8 h. Since the G_2 phase of the blastemal cell cycle lasts from 4 to 8 h[8,10] this suggests that the cells may have been blocked in G_2. On the other hand, Globus[11] and Olsen and Tassava[12] have suggested that the neurotrophic effect may be a more general one, facilitating the movement of cells from one phase to another until they complete the total cell cycle. In the absence of nerves, cells would stop at all stages rather than one discrete phase. In addition, as has been previously postulated[2], the cells may then breakdown and/or be phagocytized, or otherwise leave the limb field since the blastemas of denervated, nerve-dependent limbs regress. In these cases, detection of separate populations of cells in denervated limbs would be impossible by the techniques presently employed.

1 M. Singer, Q. Rev. Biol. *27*, 169 (1952).
2 A.L. Mescher and R.A. Tassava, Devl Biol. *44*, 187 (1975).
3 R.A. Tassava, L.L. Bennett and G.D. Zitnik, J. exp. Zool. *190*, 111 (1974).
4 M. Singer and L.J. Craven, J. exp. Zool. *108*, 279 (1948).
5 O.E. Schotte and E.G. Butler, J. exp. Zool. *87*, 279 (1941).
6 R.A. Tassava and A.L. Mescher, Differentiation *4*, 23 (1968).
7 M. Maden, J. Embryol. exp. Morph. *50*, 169 (1979).
8 R.S. Grillo, Oncology *25*, 347 (1971).
9 R.L. Carlone and J.E. Foret, J. exp. Zool. *210*, 245 (1979).
10 H. Wallace and M. Maden, J. Cell Sci. *20*, 539 (1976).
11 M. Globus, Am. Zool. *18*, 855 (1978).
12 C.L. Olsen and R.A. Tassava, Midwest Reg. devl Biol. Conf. 1980, abstracts.

Visualization of DNA in various phages (T4, χ, T7, ϕ 29) by ethidium bromide epi-fluorescent microscopy[1]

T. Kuroiwa, S. Nishibayashi, S. Kawano and T. Suzuki

Department of Cell Biology, National Institute for Basic Biology, Okazaki 444 (Japan), 13 January 1981

Summary. DNA-containing areas in various phages (T4, χ, T7 and ϕ29) could be observed at the light microscopic level using ethidium bromide epi-fluorescent microscopy. The flourescent intensity per phage was in linear proportion to the DNA content in each phage.

Williamson and Fennel[2] and James and Jope[3] have succeeded in staining T4 phages with a DNA-specific fluorochrome 4'6-diamidino-2-phenylindole (DAPI). However, the DNA-DAPI complex must be excited by only UV-light[2]. On the other hand, DNA-ethidium bromide (EB) complex in cells could be excited by both UV and green light[4]. Green light had a lower fading effect on fluorescence (see results) and a lower lethal effect on organisms than UV[5].

We succeeded in visualizing ϕ29 phages containing very small amounts of DNA (11.5×10^6 daltons)[6] by use of EB, a new staining solution reducing the fading of fluorescence and epi-fluorescent microscopes equipped with green excitation light.

Materials and methods. Stocks of T4, χ and T7 phages and *Escherichia coli* K-12 were kindly given by Dr Komeda at the National Institute of Genetics in Mishima, and the ϕ29 phages by Dr Hirokawa at the Sophia University in Tokyo.

A drop of suspension containing bacteria or phages at a concentration of 10^6-10^8 per ml was put on a glass slide, then a drop of the buffer-NS (20 mM Tris-HCl at pH 7.6, 0.25 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 0.1 mM $ZnSO_4$, 0.1 mM $CaCl_2$, 0.8 mM phenylmethylsulfonylfluoride, 0.05% β-mercaptoethanol) or distilled water containing EB at the concentration of 50 µg per ml was added, and they were throughly mixed. A cover slip was then placed on the mixture. The materials were stained for 30 min.

In order to study fluorescence decay, under a continuous excitation with UV or green light, the fluorescent intensity emitted from the EB stained *E. coli* was examined at 15-sec intervals for 120 sec by use of a Zeiss-MPM 03-Fluorometer equipped with a high-pressure mercury lamp XB0-100 W, filter combination No. 48 77 11 or No. 48 77 15 and a Neofluor 100/1.30 oil objective. The areas of the individual bacterial nuclei were optically isolated by the 1-µm MPM diaphragm. DNase and RNase treatments were done on the EB stained *E. coli* cells on a glass slide according to the procedures described previously[7].

The photographs were taken at a magnification of $\times$625 on 35-mm Fuji 400 film with a 50-sec exposure time using an Olympus BH epi-fluorescent microscope, which was equipped with a high-pressure mercury vapor lamp HBO 100 W, 545-nm excitation filter (IF 545 + BG 36), a 580-nm dichroic mirror (DM 580 + O 590), a 610-nm barrier filter (R 610) and an UVFL 100/1.30 objective. The exposed films were developed at 20 °C for 7 min in Fuji Pandol developer. Negatives of phages were scanned at 620 nm with a Joyce-Loebl MK3C microdensitometer. The light spot, which sited the negative of single phage, projected through an objective of magnification 10 onto a slit of