Effects of actinomycin D on the lens regenerating system

by TUNEO YAMADA and MARION E. ROESEL

From the Biology Division, Oak Ridge National Laboratory

WITH THREE PLATES

INTRODUCTION

A U T O R A D I O G R A P H I C studies of transformation of iris into lens during Wolffian lens regeneration of adult Triturus viridescens indicate an enhancement of RNA synthesis in the nucleus of the iris cells in the early phase of regeneration (Yamada & Karasaki, 1963; unpublished). Activation of protein synthesis and production of lens antigens follow the enhancement of RNA synthesis (Yamada & Takata, 1963; Takata et al., 1964). This sequence of events suggests the possibility that lens removal elicits synthesis of specific RNA's which in their turn induce synthesis of proteins essential for transformation of the tissue. If this is the case, it should be possible to suppress lens regeneration by subjecting the system to an inhibitor of RNA synthesis. In the present study the possibility was tested, using actinomycin D as the inhibitor. This antibiotic has been shown to be a specific inhibitor of RNA synthesis dependent upon DNA as the primer (Goldberg et al., 1962; Hurwitz et al., 1962; Reich et al., 1962), and to interfere with morphogenesis of various developmental systems (Brachet & Denis, 1963; Flickinger, 1963; Gross & Cousineau, 1963; Lallier, 1963; Markman & Runnström, 1963; Pierro, 1962; Wallace & Elsdale, 1963).

In our experiments actinomycin was injected into the body cavity of adult T. viridescens, in which the tissue transformation had been initiated by lens removal. The effects of such treatment on the tissue transformation were studied in serial sections of the regenerates. By adjusting experimental conditions, it was possible to suppress lens formation or to demonstrate differential susceptibility in various cell types which are undergoing differentiation.

1Authors' address: Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A.
Materials and Methods

Material

Adults of both sexes of *T. viridescens* adapted to laboratory conditions were used for this study. The left lens was surgically removed through a cut in the cornea of an animal anesthetized with MS 222. The animals were kept at 21–22°C and fed with the white worm, *Enchytraeus albidus*.

Actinomycin treatment

Actinomycin D was furnished by Merck Sharp and Dohme Research Laboratory. According to Waksman et al. (1958), actinomycin D contains actinomycin D₁V (= actinomycin C₁) as the main component with trace amounts of actinomycin D₁, D₁I, D₁III, and D₁V. The antibiotic in aqueous solution was injected into the body cavity at doses of 1 µg./g. body weight. Injection was repeated every day or every other day for a period which is specified for each series. Animals were kept for different time intervals after the end of treatment under the conditions described above.

Method of observation

The effect of treatment was judged by microscopic observation of the operated eye of the treated and non-treated animals in histological sections. These sections were made by fixing the isolated eyeball in Bouin and sectioning it serially at 10 µ. Staining was done in Mayer’s hemalum and nigrosin-picric acid.

Controls

In the main control series, animals were also unilaterally lentectomized and reared under similar conditions without injection. At different time intervals after lens removal, the animals were killed and their left eyes were sectioned for study. A special series of controls was made in which distilled water was injected in the same quantity used for the experimental series.

In some cases the right non-operated eye of actinomycin-treated animals was sectioned and the histological condition of non-regenerating iris and lens was investigated.

Staging of lens regeneration

The morphogenesis occurring in the regenerating system was staged according to Sato (1940). His staging system originally proposed for lens regeneration in *Triturus taeniacus* and *pyrhogaster* was later adopted by Stone & Steinitz (1953) and Reyer (1954) for that of adult and larval *T. viridescens*, respectively. Some representative stages used frequently in the present work are illustrated in Plate 1.
PLATE I

FIGS. A–C. Normal regenerates of the control series in the middle phase of lens regeneration. The anterior optic chamber above, and the posterior optic chamber below in the picture. Left, the dorsal margin of the pigmented iris. × 280.

FIG. A, Stage IV. The depigmented cells are arranged into the external (upper) and internal (lower) layers. A few free pigmented cells surrounding the regenerate are the phagocytic amoeboid cells described by Eguchi (1963).

FIG. B, Stage VI. The internal wall of the lens vesicle is thickened due to growth of the cytoplasm of individual cells (primary lens fibers). Mitotic figures in the external wall.

FIG. C, Stage VII. Further growth and differentiation of the primary fiber cells, which now indicate cytoplasmic stainability with picric acid. The external wall (lens epithelium) shows mitotic figures and is still pseudostratified.

FIGS. D–E. Normal regenerates of the control series in the later phase of lens regeneration. × 280.

FIG. D, Stage X. The lens epithelium is now single-layered. In the center of the lens fiber area are the primary lens fibers, in which some nuclei are becoming less stainable. At the equator, transformation of the lens epithelium cells into the fiber cells is occurring (the intermediate zone).

FIG. E, Stage XI. In the fiber area, fragmentation of nuclei and increase in the cytoplasmic mass are evident. Note further production of fiber cells (secondary lens fibers) in the intermediate zone.

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Estimation of the size of the lens fiber area

Under the microscope two axes of the largest section of the lens fiber area of a regenerate were measured: the dorso-ventral axis and the longest axis perpendicular to it. The area of the ellipse having these two axes \((a, b)\) was then calculated according to the formula: \(\pi ab/4\). This provided an estimate of the size of the lens fiber area.

RESULTS

Progress of lens regeneration in the non-treated animals

To establish the inhibitory effect of actinomycin D on the progress of lens regeneration, information concerning the normal course of lens regeneration is indispensable. Two hundred and twenty-four unilaterally lentectomized animals were kept under the conditions specified above and killed at intervals between 12 to 51 days after lens removal. The operated eyes were studied in serial sections and the Sato stage of the regenerate was determined. The data summarized in Text-fig. 1 show that lens regeneration was in progress in all operated eyes during the period studied. However, a considerable variation in the stage was evidenced within each time interval group. The results were in reasonable agreement with those obtained earlier by Stone & Steinitz (1953) in the adults of \(T.\ viridescens\).
Injection of distilled water for 11 days did not cause appreciable effects on the progress of lens regeneration in fourteen animals.

Long-term treatment

Nine to eleven daily injections of standard doses of actinomycin were given during the early and middle phases of lens regeneration. The results are summarized in Table 1. The data of series I and II indicate that the daily injection for 10 to 11 days beginning from 1 day after lens removal suppresses the progress of regeneration beyond the stage of depigmentation. Depigmentation occurs in control regenerates at 7 to 10 days after lens removal. No further progress of morphogenesis was noticed when the animals were left alive for 2 to 5 days after the end of treatment. A clear suppression was also obtained in the series of nine daily injections (series III) beginning in the depigmentation phase. No development beyond the stage of the depigmented dorsal iris was evidenced.

The long-term treatment frequently caused death of the animals during the experimental period. The incidence of death per day tended to increase with repeated daily injections. For example, the incidence was 1/34 after seven daily injections and 4/16 after 11 daily injections. An increase of the mortality during the post-treatment period was also indicated. High mortality interfered with analysis of the data and forced the abandonment of further experiments using this dosage range.

Short-term treatment

Injections of the standard doses of actinomycin D were repeated daily for 4 days at different phases of lens regeneration. Description of the data will be grouped according to these phases.
Figs. A–B. Suppression of the progress of regeneration after a treatment with actinomycin during 8 to 11 days. Fixed at 17 days. The anterior optic chamber on the left and the posterior optic chamber on the right side of the pictures. ×280.

Fig. A, Stage III. Several amoeboid cells with pigment granules.

Fig. B, Stage IV.

Figs. C–D. Treatment during 15 to 18 days and fixation at 23 days. Disintegrating lens epithelium and well-differentiated lens fibers.

Fig. C. Oriented as Figs. A and B. ×280.

Fig. D. The lens epithelium on the right and lens fibers on the left side of the figure. ×700.

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PLATE 3

FIGS. A–D. Abnormal lenses formed after a treatment with actinomycin during 13–16 days. Fixed at 26 days. The anterior optic chamber on the left and the posterior optic chamber on the right side of pictures. Many amoeboid cells with pigment granules are surrounding the regenerate. ×280.

FIG. A. A group of lens fiber cells indicating an advanced stage of fiber differentiation.
FIG. B. Another section through the same regenerate showing the degenerating lens epithelium.
FIG. C. A group of lens fiber cells with a large amount of acidophilic cytoplasm.
FIG. D. Another section of the same regenerate. The fiber aggregate is accompanied by a small number of lens epithelium cells (right below).

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Treatment at the earlier phase of regeneration

Four series of experiments were conducted in which animals were injected during 3 to 6 days, 8 to 11 days, 10 to 13 days or 11 to 14 days after lentectomy. The animals were killed 1 to 12 days after the end of injection. Text-fig. 2 summarizes the data on the progress of regeneration in the treated animals. When the experimental regenerates were compared with the control regenerates of the same regeneration day, two types of differences were observed (Table 2). One is the simple delay of morphogenesis which was also observed in the long-term treatment (Plate 2, Fig. A, B). The other type is morphological abnormality of the regenerates. The lens epithelium cells formed an aggregate which lacked epithelial characteristics and is attached to an aggregate of lens fiber cells. These fiber cells show a stage of differentiation relatively far advanced compared to the size of the regenerate. In Text-fig 2, such abnormal regenerates were classified according to the stage of their lens fiber cells. From the diagram thus prepared, it is clear that the delay of progress of regeneration occurs in all series of this group, whether the course of regeneration is normal or abnormal.
Table 2
Effects of short-term treatment with actinomycin on various stages of lens regeneration*

<table>
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<tr>
<th>Period of treatment†</th>
<th>Total</th>
<th>Simple delay</th>
<th>Abnormal regenerates</th>
<th>No effect</th>
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<td>3-6</td>
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<td>17</td>
<td>0</td>
<td>0</td>
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<td>39</td>
<td>18</td>
<td>15</td>
<td>6</td>
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<tr>
<td>11-14</td>
<td>15</td>
<td>10</td>
<td>4</td>
<td>1</td>
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<tr>
<td>Intermediate phase</td>
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<tr>
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<td>9</td>
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<tr>
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<td>16-19</td>
<td>11</td>
<td>2</td>
<td>8</td>
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<td>Total</td>
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<td>63</td>
<td>93</td>
<td>14</td>
</tr>
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</table>

* Experiments with post-treatment period of 6-12 days alone are included.
† Figures indicate the day after lens removal.

Abnormal regenerates were restricted to the series in which treatments were started later than 10 days after lentectomy.

Treatment at the intermediate phase of regeneration.

Four series of experiments were conducted in which the treatment was performed from 12 to 15 days, 13 to 16 days, 15 to 18 days or 16 to 19 days after lentectomy. When the animals were killed 5 to 11 days after the end of treatment, regenerates indicated abnormalities of morphogenesis in sixty-one out of sixty-eight cases. Simple delay was very infrequent. The most conspicuous abnormality was a degenerative suppression of the lens epithelium. This was characterized by disaggregation of individual cells, pycnosis of nuclei, and cytoplasmic vacuolization (Plate 2, Figs. C, D; Plate 3, Fig. B). In extreme cases, the lens epithelium was totally lacking. These effects on the lens epithelium were often accompanied by size reduction of the lens fiber area (Text-fig. 3). Whether this reduction occurred or not, the lens fiber area showed a well-advanced state of cellular differentiation, as indicated by morphology and stainability of nuclei and cytoplasm (Plate 2, Figs. C, D; Plate 3, Figs. A, C).

In Text-fig. 4 the abnormal regenerates are staged according to the degree of fiber differentiation, disregarding the size and morphology of the lens epithelium. The diagram thus prepared indicates that the treatment did not significantly impair the progress of differentiation of the lens fiber cells. Except in five cases, the lens fiber area of the abnormal regenerates did not show degenerative
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Changes which characterized the lens epithelium. In contrast to the fiber area, the intermediate zone (cf. legends for Plate 1) often showed pycnotic nuclei.

In ten additional cases, the animals were killed 1 day after the end of the actinomycin treatment. Some delay in the progress of regeneration was observed (Text-fig. 2, column 15 days). In nine out of ten regenerates, pycnosis was observed in the lens epithelium. However, in none of them was disintegration of the lens epithelium evident.

Text-fig. 3. Diagram to illustrate the size of the lens fibers area in the control and some of experimental series. Signs are common to Text-figs. 2 and 4.

Treatment at the later phase of regeneration

Two series of treatments were carried out during 34 to 37 days and 42 to 45 days after lens removal, respectively. At the time of treatment, the regenerates had already attained an advanced degree of differentiation, close to that of the normal lens. Studied at 5 days after the treatment, the lens epithelium of the treated regenerates was either absent or degenerative (Table 2). The intermediate zone
also showed strong degenerative signs. By contrast, the condition of the lens fiber area was quite normal, except for infrequent cytoplasmic vacuoles in the cells lying close to the degenerating lens epithelium.

Throughout the reported series of short-term treatments the frequency of death was relatively low. During the injection period of 4 days, no death in 219 treated animals occurred. The death incidence per day increased slowly during the post-treatment period: 1/175 (2nd day); 1/156 (6th day); 2/59 (9th day); 3/28 (11th day).

TEXT-FIG. 4. Diagram to illustrate the effect of short-term treatment in the later phase of regeneration.

- ●: control regenerates; ▽: regenerates treated from 12 to 15 days after lens removal; ▽: regenerates treated from 13 to 16 days after lens removal; ●: regenerates treated from 15 to 18 days after lens removal; ▽: regenerates treated from 16 to 19 days after lens removal.

Effects of treatment on the non-regenerating lens and iris

In eight cases, the normal right lens of the animals which received nine or ten daily injections was studied in sections 1 to 3 days after the end of injection. No abnormality was observed in the morphology of these lenses. In ten additional cases, the normal right lens was studied after four daily injections and an 8-day post-treatment period. Again the lens showed a normal histological condition.

A microscopic study of the normal iris tissue of the right eye of animals treated as above did not reveal signs of tissue damage. The same results were obtained with the non-regenerating part of the iris of the left eye of the animals injected with antinomycin.

Effects of 2-day interval injection

In twenty-one cases actinomycin was injected at a dose of 1 μg./g. body weight every other day instead of each day for a period of 5 to 9 days during the inter-
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mediate phase of lens regeneration. In eight cases abnormal regenerates of the type described earlier and in four cases simple delay were obtained, while in the remaining cases no effects were observed.

DISCUSSION

The data presented show that injection of actinomycin can inhibit regenerative transformation of iris into lens without affecting the tissue integrity of normal iris and lens. If we assume that in our experiment actinomycin is primarily inhibiting the synthesis of RNA, which is dependent upon DNA, as demonstrated in other systems (Goldberg et al., 1962; Hurwitz et al., 1962; Reich et al., 1962), then the data indicate that a change in pattern of transcription of genetic information is involved in the mechanism of regenerative tissue transformation. This is in harmony with the idea that enhancement of RNA synthesis observed in the iris nucleus after lens removal is one of the essential steps of the tissue transformation (Yamada & Karasaki, 1963). However, the possibility cannot be excluded that the actinomycin inhibition of regeneration is caused indirectly through suppression of synthetic activity of a tissue other than the regenerate.

Comparison of the different series of short-term treatments indicates that different phases of regeneration have characteristic susceptibilities to the antibiotic. In the phases of depigmentation (Stages I–II) and lens vesicle formation (Stages III–IV), the actinomycin treatment causes a delay in the progress of regeneration without causing abnormalities in the morphology of the regenerates. When applied during the initial phase of lens fiber formation (Stages V–IX) the antibiotic causes inhibition of lens epithelium development. Cellular differentiation of the lens fiber cells, on the other hand, is not significantly affected, but the size of the lens fiber area is often reduced. This reduction can be interpreted by assuming an inhibitory effect of the antibiotic on the transformation of the lens epithelium cells into the lens fiber, which is occurring during the treated phase. The cytotoxic effect of the antibiotic on the lens epithelium is also evident in the later stage of regeneration (Stage XII), in which the histology of the regenerate closely approaches that of the normal lens. Again differentiation of the lens fiber cells was not affected.

The phase, Stages IV–VI, during which a change in susceptibility toward actinomycin occurs, is a critical time with respect to a number of subcellular events essential for cellular differentiation. Autoradiographic and electron microscopic data suggest production of new ribosomes in the cytoplasm of prospective lens fiber cells during this phase. Simultaneously, ultrastructural changes occur in the nucleoli indicating a rise in their synthetic activities (Karasaki, in press). This phase also coincides with the time at which the lens antigens first become detectable with immunofluorescence (Takata et al., 1964) in the prospective primary lens fibers which now cease to synthesize DNA (Sara Eisenberg, unpublished). The temporal coincidence of the appearance of lens antigens in the cytoplasm of lens fiber cells and the acquisition of resistance...
toward actinomycin by those cells is impressive and may suggest that messenger RNA's of fiber cells exert a prolonged control over cellular differentiation, including synthesis of lens antigens; and that once they are produced in a sufficient amount, the cell can proceed with differentiation even in the presence of the antibiotic. However, it should be pointed out that our experiments demonstrate only a relative resistance of lens fiber differentiation. This is in accordance with our unpublished autoradiographic studies which show some RNA synthesis occurring in actinomycin-treated lens regenerates. Therefore our data do not necessarily suggest long-lived messenger RNA's controlling lens fiber differentiation. Nor can we entirely exclude the possibility that the observed resistance of lens fiber cells is due to their impermeability toward the antibiotic. However, the latter possibility is rather unlikely in view of the recent data of P. J. Kohonen (unpublished): A lens abnormality similar to the one observed here can be produced by X-irradiation during the intermediate phase of lens regeneration. This finding complements our present results in strongly suggesting a capacity of differentiation of the cytoplasm of lens fibers, independent of nuclear activities.

If we assume that under certain conditions actinomycin preferentially suppresses synthesis of ribosomal RNA, which occurs in the nucleolus (Perry, 1963), the present data should imply that actinomycin-inhibition of lens regeneration is mainly caused by suppression of ribosome production and not by suppression of messenger RNA synthesis. This interpretation does not contradict the idea that messenger RNA's which are synthesized in regenerating cells are carrying the specificity of lens tissue and are involved in synthesis of lens antigens. It is probable that the original population of ribosomes present in the iris tissue is insufficient for active synthesis of proteins needed for the progress of regeneration, so that production of a new ribosomal population is the prerequisite for new messenger RNA's to function. Whether the latter is short- or long-lived does not play a decisive role in this connexion.

The temporal and spatial distribution of actinomycin susceptibility in the present system coincides not only with that of active RNA synthesis (Yamada & Karasaki, 1963; Yamada, unpublished) but also with that of DNA synthesis and cell replication (Sara Eisenberg, unpublished). Hence the possibility is open that some of the actinomycin effects observed in the system are caused by the inhibition of DNA polymerase, which was demonstrated in the actinomycin-treated regenerating mouse liver (Guidice & Novelli, 1963). This inhibition may cause suppression of cell replication and indirectly lead to developmental disturbances.

Treatment of the same lens-regenerating system with puromycin, an inhibitor of protein synthesis, does not cause the localized disintegration of lens epithelium (Yamada, unpublished). This provides circumstantial evidence for the idea that differential susceptibility of the lens epithelium toward actinomycin is due to its high level of RNA synthesis.
The experiments of Wessell (1964) suggest that zymogen synthesis in the cultured pancreatic rudiment becomes resistant to a certain level of actinomycin when DNA synthesis ceases. Thus the correlation between appearance of resistance toward actinomycin, production of tissue-specific substances, and cessation of DNA synthesis may be common to different tissues.

SUMMARY

1. Effects of actinomycin D on transformation of iris into lens in Wolffian lens regeneration of adult *T. viridescens* were studied by injection into the body cavity of lentectomized animals. One microgram of the antibiotic per gram body weight was injected every day or every other day for a 3- to 12-day period at various phases of lens regeneration.

2. Administration of actinomycin during the early phase of lens regeneration for a period longer than 4 days suppressed the progress of regeneration. Suppression was complete or partial depending upon the experimental conditions.

3. Four daily injections of the above indicated amount were given at various phases of lens regeneration. When the treatment began after appearance of primary lens fiber cells, differentiation of those cells proceeded normally, while developmental arrest and disintegration were observed in the lens epithelium. The actinomycin-treatment given after the regenerate has formed a well-differentiated lens caused a degenerative inhibition of the lens epithelium, but no appreciable change in the morphology of lens fibers. The non-regenerating iris and lens did not show any morphological sign of abnormalities after the same treatment.

4. Data are discussed in connection with information on syntheses of RNA, proteins, and DNA occurring in the lens-regenerating system. A change in the pattern of transcription of genetic information is suggested as a part of the mechanism of tissue transformation.

RÉSUMÉ

Les effets de l'actinomycine D sur le cristallin en régénération

1. Les effets de l'actinomycine D sur la transformation de l'iris en cristallin dans la régénération du cristallin Wolffian de *T. viridescens* adulte, ont été étudiés par injection dans la cavité péritonéale d'animaux dont le cristallin a été préalablement enlevé. Un microgramme de l'antibiotique par gramme poids du corps a été injecté chaque jour, ou chaque deuxième jour, pendant une période de 3 à 12 jours, à des stades différents de la régénération du cristallin.

2. L'administration de l'actinomycine pendant la phase précoce de la régénération du cristallin, pendant plus de 4 jours, supprime le progrès de la régénération. Cette suppression est complète ou partielle selon les conditions expérimentales.

3. Quatre injections journalières de la dose indiquée ci-dessus, ont été faites...
à des phases différentes de la régénération du cristallin. Lorsque le traitement commence après l'apparition des cellules fibreuses primaires du cristallin, la différenciation de ces cellules continue normalement, tandis que dans l'épithélium du cristallin on observe l'arrêt du développement, et la désintégration. Si le traitement à l'actinomycine commence après la formation d'un cristallin bien différencié, il y a une inhibition dégénérative de l'épithélium du cristallin, sans changement appréciable dans la morphologie des fibres du cristallin. Après le même traitement de l'iris et du cristallin qui ne sont pas en régénération, on n'a pu démontrer aucun indice morphologique anormal.


ACKNOWLEDGEMENTS

We would like to express our gratitude to Mrs Lola M. Kyte for technical assistance. This research was supported by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

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(Manuscript received 1st June 1964)