Effects of ultraviolet light on the activity of an avian limb positional signalling region

By Lawrence S. Honig

From the Department of Biology as Applied to Medicine, Middlesex Hospital Medical School, and Laboratory for Developmental Biology, University of Southern California

SUMMARY

Cells from a region on the posterior margin of the developing avian limb bud (the polarizing region) can signal positional information to responding anterior cells. Polarizing activity may be assayed by disaggregation of the tissue into single cells, followed by reaggregation and grafting of cell pellet. This method enables treatments of the cells while in single-cell suspension. The effects of ultraviolet radiation (254 nm) were studied to determine the role of nucleic acids in polarizing activity. Ultraviolet radiation eliminated quail polarizing activity over the same range of doses as it reduced cell spreading (20–60 J/m²). In contrast to the published effects of γ-radiation in which polarizing activity is affected only at doses much higher than those that are cell lethal, ultraviolet apparently abolishes cell survival and polarizing activity at comparable doses. Compared to other biological systems, polarizing activity is quite sensitive to ultraviolet light: the D₃₇ is 18 J/m²; the extrapolation number, n, is 3.6. From comparison of the effects of ultraviolet radiation to those published for ionizing X- or γ-radiation, one may conclude that ultraviolet and γ-radiation abolish limb polarizing activity at equivalent nucleic acid dosages, not at equivalent cell lethal doses.

INTRODUCTION

During chick limb embryonic development there is a region on the posterior margin of the limb bud which, when transplanted to an anterior site on a host limb, causes mirror-image duplication in the definitive limb pattern. This zone of polarizing activity was originally identified by Saunders & Gasseling (1968) and A. B. MacCabe, Gasseling & Saunders (1973). Evidence suggests that the polarizing region is responsible for signalling positional information along the limb anteroposterior axis (Tickle, Summerbell & Wolpert, 1975). It is active in normal development (Summerbell, 1979), signalling over distances of tens of cell diameters (Honig, 1981a). The signal seems universal in the sense that homologous regions from other amniote embryos including reptiles and mammals also induce chick limb reduplications when grafted into chick limb.

Author's present address: University of Southern California, Laboratory for Developmental Biology, GER 323, University Park, Los Angeles, California 90089–0191, U.S.A.
buds (Fallon & Crosby, 1977; J. A. MacCabe & Parker, 1976; Tickle, Shells- well, Crawley & Wolpert, 1976). In a study of the nature of polarizing activity, Smith, Tickle & Wolpert (1978) examined reduplications following grafts of polarizing regions subjected to various doses of $^{60}$Co $\gamma$-radiation. Very large amounts of radiation, $\sim 960$ Gy ($1$Gy = 100 rad) were required to abolish positional signalling by the polarizing region, doses much larger than these were sufficient to abolish cell multiplication (Smith, 1982) or normal DNA synthesis (Honig, Smith, Hornbruch & Wolpert, 1981). These experiments showed that polarizing activity does not require cellular contribution by the graft; similar signalling behaviour has been shown during regeneration in *Drosophila* imaginal discs (Adler & Bryant, 1977), and the amphibian limb (Holder, Bryant & Tank, 1979; Maden, 1979). However, since $\gamma$-radiation adversely affects a multitude of cell functions (e.g. Honig *et al.* 1981), the cellular nature of the polarizing region signal could not be determined. Interest in assessment of the role of the cell nucleus in positional signalling instigated use of ultraviolet irradiation which would have at least 10-fold greater selectivity for nucleic acids than other cell components. The penetrating power of actinic u.v. radiation (~ 254 nm) is quite limited: the half-attenuation thickness for tissue is $\sim 10$ $\mu$m. Therefore, irradiation of whole eggs, embryos, or even isolated polarizing regions ($\sim 200$ $\mu$m in dimension) is not an effective procedure. To determine the u.v. sensitivity of polarizing region cells, a different assay had to be used. This assay, recently discussed by Tickle (1981) and Honig & Hornbruch (1982) utilizes the ability of polarizing region cells to undergo complete disaggregation into single cell suspension, followed by centrifugal reaggregation yielding a cell pellet which then shows polarizing activity (Saunders, 1972). Here I show the results of treatment of polarizing region cells with u.v. light using this assay. Quail polarizing regions are used to facilitate comparison of these results with those of Smith *et al.* (1978) and Smith (1982) who examined the effects of $\gamma$-radiation on quail tissue. Quail tissue is also advantageous since the cells form more cohesive pellets following the disaggregation–centrifugation procedure (Honig & Hornbruch, 1982).

**MATERIALS AND METHODS**

Fertile chick eggs were obtained from a local flock (Needle Farm, Enfield, U.K.) and fertile eggs of Japanese quail (*Coturnix coturnix japonica*) were obtained from a colony kept at the Middlesex Hospital Medical School.

To assay polarizing region activity, about thirty quail embryos at stages 21–23 (Hamburger & Hamilton, 1951) were placed in cold calcium- and magnesium-free Hank's Balanced Salts Solution (HBSScf, Gibco). The wing and leg buds were excised, rinsed, and incubated in 2% trypsin (Gibco, 1:250) in HBSScf at $0^\circ$ for 2 h. After four rinses with cold cmf Dulbecco's phosphate-buffered saline (PBScf; Oxoid) the limb mesenchymes were freed of their ectodermal jackets by dissection at 5–10 $^\circ$C. Polarizing regions were cut out
Table 1. Effects of u.v. irradiation on polarizing activity

<table>
<thead>
<tr>
<th>Dose (J/m²)</th>
<th>0</th>
<th>4</th>
<th>14</th>
<th>28</th>
<th>35</th>
<th>59</th>
<th>71</th>
<th>130</th>
<th>230</th>
<th>2700</th>
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<tr>
<td>No. operations</td>
<td>32</td>
<td>7</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>13</td>
<td>13</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>4-reduplication</td>
<td>24</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3-reduplication</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-reduplication</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>no reduplication</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Polarizing activity (%)</td>
<td>85</td>
<td>90</td>
<td>77</td>
<td>57</td>
<td>61</td>
<td>33</td>
<td>8</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spreading</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Effects of ultraviolet on avian limb polarizing activity

from the mesenchymes, placed in 250 μl of 0·25 % trypsin in PBScmf, and incubated for ~ 12 min at 38°, interrupted three times by gentle vortexing. After dissociation into single cells, 1 ml was added of cold Minimal Essential Medium (Gibco) containing Hank’s Salts, 25 mM HEPES, 2 mM L-glutamine, 10% foetal calf serum, and 1% antibiotic-antimycotic (HMEM). The cell suspension was mixed, counted, and centrifuged down at 50g for 10 min. The cells were resuspended in 300 μl of HMEM containing 3 mg/ml soybean trypsin inhibitor, recentrifuged and resuspended in PBS. The cell suspension was kept at 0 °C and divided equally into 1 ml aliquots (ca. 2 × 10⁶ cells each) placed in 35 mm diameter open tissue-culture Petri dishes which were then irradiated at 4 °C at a rate of 35 μW/cm² at a distance of 28 cm from a 12 in., 15 W bactericidal low-pressure mercury vapour lamp with ozone-suppressing liquid filter (Hanovia Model 16, Slough, U.K.). The u.v. radiation was 95% composed of the 253·7 nm mercury line. The cells were then removed into tubes, HMEM was added, and they were centrifuged, resuspended in HMEM, re-centrifuged and incubated at 38° for 1–3 h. In some cases 50 μg/ml concanavalin A (Sigma, London) was present in the reaggregation medium to promote cell–cell adhesion. Reaggregated cell pellets were cut into pieces ~ 300 μm square by 100 μm thick, consisting of about 10⁴ cells, and transplanted into stage-19 to -22 host chick wings (average stage 20 for each dose point), using the method of Saunders, Gasseling & Cairns (1959). The test tissue was positioned between uplifted apical ectodermal ridge and subjacent limb mesenchyme in an anterior position opposite somites 16/17.

Operated chick embryos were sacrificed after an additional 6–8 days incubation and the limbs were fixed and stained with Alcian Green 2GX (Summerbell, 1979). Polarizing activity was quantified (Honig et al. 1981) by assigning 3 points to limbs with duplicated digits 4 (Fig. 1a), 2 points to those containing duplicated digits 3 (but not 4; e.g. Fig. 1b), 1 point to limbs showing only duplicated digits 2 (Fig. 1c) and 0 points to limbs having no extra digits (Fig. 1d). Even if a digit was not fully or well-formed it was counted providing positive...
identification could be made (e.g. the 4 in Fig. 1a). The summed total is expressed as a percentage of the maximal possible total (all 4's, i.e. three times the total number of operated limbs in a set) to yield a value of 0% if there are no reduplications and 100% if all operations result in 4-reduplications. This percentage is a measure of the strength of polarizing activity. It has been shown that attenuation of the polarizing region by a variety of means results in the progressive loss of extra digits 4, 3, and 2 next to the graft (Smith et al. 1978; Honig et al. 1981; Tickle, 1981; Honig & Hornbruch, 1982). While the exact digit pattern obtained depends on the site and stage of the host limb, the digit adjacent to the graft on the anterior margin will depend only, for grafts opposite somites 16/17 in stage 19–22 embryos, on the ‘strength’ of the polarizing region.

Cell viability was monitored by the cell spreading assay (Honig et al. 1981) and by dye exclusion (Paul, 1975) using the inverted compound microscope. For spreading assays, five to ten pieces of cell pellet were incubated at 38 °C in HMEM for 24–48 h and the amount of cell spreading was scored with 0–3 plusses. In some cases, portions of treated cell suspensions were incubated as well. In these cases spreading was the same as that observed for the pieces of cell pellet. Control untreated cells scored +++. For dye exclusion, cell
suspensions were examined and counted in a haemocytometer after 5-10 min at room temperature in 0.01% trypan blue (Gibco).

Ultraviolet dose rates were measured using the Blak-Ray detector model J-225 (Ultra-violet Products Inc., San Gabriel, California) one of which was kindly loaned by Dr W. Tampion of the Royal Free Hospital Medical School.

RESULTS

Six experiments were performed consisting of a total of 22 separate irradiations and 119 embryonic operations. Each experiment had its own sham-irradiated control cells. The pooled data showing the effects of ultraviolet radiation on the polarizing region cells are presented in Table 1 and Figs. 1 and 2. Increasing doses of u.v. radiation caused attenuation of signalling ability. The effect on polarizing activity is not all-or-nothing but is graded: at doses higher than 14 J/m², no 4-reduplications occur and ability to specify extra digit 3 and extra digit 2 is progressively lost. Since control polarizing activity was 85%, the attenuation became significant ($P(t) < 0.01; P(\chi^2) < 0.001$) at a dose
Fig. 3. Cultured limb polarizing region cells following irradiation with u.v. light. Control (A) or irradiated quail cells were plated at $2 \times 10^6$ cells per 35 mm tissue-culture dish and incubated 2 days. Doses were (B) 7 J/m², (C) 21 J/m² and (D and E) 42 J/m² (D is a rare group of spread cells, E is a representative field). Magnifications are 430×.
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of 28 J/m² and severe at 60–70 J/m². At still higher doses the u.v.-irradiated cells did not signal. The semi-log plot in the inset to Fig. 2 has a shoulder and in form is typical of multi-target or multi-hit irradiation effects (e.g. see Casarett, 1968). There appears to be a threshold dose of \( \sim 20–30 \) J/m² followed by exponential decline of activity. The \( D_{57} \), or dose required for reduction by a factor of \( e \), is \( \sim 18 \) J/m² and the extrapolation number, \( n \), is 3·6. A figure of \( n > 1 \) implies some sort of cumulative radiation damage is necessary for complete inactivation of polarizing activity. The \( n \) value may indicate that with these small grafts of reaggregated cell pellet there is still a 3·6-fold excess of polarizing signal (number of cells?) over the minimum necessary to produce a 4-duplication. (Alternatively one may assume that most grafts have only that minimum strength, noting the presence of less than 100% activity in the 0–4 J/m² columns of Table 1. In this case the \( n \) value would suggest that 3 or 4 hits, at one or more targets, per cell are necessary for loss of polarizing activity.)

Spreading falls off in a similar manner to polarizing activity, although perhaps slightly more rapidly as can be seen in Table 1 and Fig. 3. When examined for trypan blue uptake 1 h after irradiation, at least 95% of cells exclude dye even after doses as high as 130 J/m² which completely abolishes polarizing activity. At the very highest dose used (2700 J/m²), 54% of cells excluded trypan blue. However, the lower doses of u.v. are ultimately lethal. After culture for 20 h, fewer cells are able to exclude trypan blue after doses of 28 J/m² (61%), 59 J/m² (49%), 130 J/m² (30%) or 2700 J/m² (0%).

DISCUSSION

Ultraviolet radiation, like \( \gamma \)- or X-radiation causes a progressive graded loss of polarizing activity. There is no evidence for an all-or-nothing effect, for which behaviour a tendency was observed in the chemical inhibitor study of Honig et al. (1981) in which 4-reduplications and non-reduplications exceed in number those of 3- and 2-reduplications. The graded nature of the u.v. results does not seem to depend on the disaggregated cell assay since the u.v. results are more graded than were chemical inhibitor grafts done by the same assay (Honig & Hornbruch, 1982). And the results of Smith et al. (1978) were graded despite using the conventional polarizing region assay. It is not certain whether the graded quality in the u.v. and \( \gamma \)-radiation experiments might be due to reduction of each cell to a fraction of its full signal strength (or signalling lifetime, e.g. Smith, 1980), or whether it might be due to reduction in the number of cells capable of signalling (as per Tickle, 1981). Spreading and long-term viability are affected in parallel with polarized activity following u.v. treatment, but during the initial time period in which signalling occurs (\( \sim 18 \) h; Smith, 1980), at least 30–50% of cells are ‘viable’ by the dye exclusion test over the dose range where there is negligible (0–10%) polarizing activity (59–130 J/m²). Reference
to the numbers of cells required for signalling (Tickle, 1981), shows that the u.v. inhibition of polarizing activity is not due simply to cell killing.

The effects of u.v. radiation on signalling occur over the dose range of 20–100 J/m². These amounts of radiation are similar to those lethal for cultured mammalian cells for which clonable cell survival is reduced 90% at doses of 20–50 J/m² (Erickson & Szybalski, 1963). However, the effective u.v. doses in the chick limb are considerably lower than those doses required to destroy ‘anterior determinants’ in insect eggs or dorsal organization in amphibian eggs. To produce 90% double abdomens in *Smittia* eggs requires about 2000 J/m² (Kalthoff, 1977). While u.v. opacity of the insect chorion is not sufficient to account for this difference, deep localization of the anterior determinants might be responsible. Alternatively, the sensitivities may differ because the insect phenomenon is nucleus-independent (cytoplasmic ribonucleoprotein is the presumed target) while the 20-fold more sensitive chick target might be nuclear. Similarly, the ultraviolet doses required to impair dorsalization in *Rana* or *Xenopus* eggs are 1000–2000 J/m² (Malacinski, Allis & Chung, 1974; Scharf & Gerhart, 1980); the probable targets are superficial but proteinaceous.

The effects of u.v. radiation may be compared to those of ionizing irradiation (Smith *et al.* 1978; Smith, 1982). Both agencies affect cell spreading and polarizing activity in roughly coordinated fashion. Comparison of the effective amounts of radiation of the two types may yield information about the cell target and can be performed in several ways. On the basis of pure energy absorption, disregarding differences in quality of energy, it is readily calculable from nucleic acid absorption coefficients, cell DNA content and volume that 1 J/m² of u.v. irradiation delivers about 10 Gy (= 1000 rad = 10⁵ erg/cm³) of energy to DNA and 50 Gy to the cell. (Considering the number of DNA lesions empirically observed by Paterson, 1978, i.e. ~ 1 hit/pg/rad X-rays and ~ 6000 hit/pg/J/m² u.v., one may calculate that 1 J/m² is equivalent to ~ 6000 rad = 60 Gy ionizing radiation which figure is similar to the immediately previous figure.) An alternative basis for comparison of u.v. and γ-radiation is provided by study of the biological effects such as those effects on cell proliferation. For example with human D98/AG cells, survival curves of colony-forming capacity (Erickson & Szybalski, 1963) show that 1 J/m² is equivalent to about 0.3 Gy which contrasts greatly with the values above. The two orders of magnitude discrepancy in equivalent γ-radiation doses reflects the potent lethality of ionizing radiation. Examination of u.v. sensitivity curves of quail polarizing activity shows that the D₃₇ is about 18 J/m² u.v. while for X-rays it is approximately 580 Gy (Smith, 1982). Hence, for polarizing activity, 1 J/m² is equivalent to about 32 Gy. Thus u.v. and X-radiation abolish polarizing activity at equivalent nucleic acid dosages, not at equivalent lethal doses.

For ionizing radiation, much greater doses are needed to eliminate positional signalling than to abolish cell survival while for u.v. radiation comparable doses are needed. The above analysis suggests a nuclear role in positional signalling,
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which may be contrasted with observations that cell proliferation (Smith, 1982) and DNA synthesis (Honig et al. 1981) are not required. Recent experiments designed to elucidate this matter have used cytochalasin B-induced enucleation of polarizing region cells: but neither cytoplasts nor karyoplasts appeared capable of signalling (Honig, 1981b). If the polarizing region signal was a cytoplasm-localized metabolic function or a cell surface function, the cytoplasts were expected to signal. They did not but neither did the karyoplasts which essentially contained only cell nuclei. These data, which suggest a requirement for the cell nucleus, the data from biochemical inhibition studies (Honig et al. 1981; Honig & Hornbruch, 1982) and the u.v. studies presented here are consistent with the possibility that nuclear transcription is an essential element of chick limb positional signalling.

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REFERENCES


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