Exogenous basement-membrane-like matrix stimulates adrenergic development in avian neural crest cultures

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Summary

The development of quail trunk neural crest cultures was dramatically altered when the cultures were overlaid with a gel of reconstituted basement membrane (RBM) components derived from the Engelbreth-Holm-Swarm sarcoma. In the presence of the RBM gel overlay, the number of catecholamine-positive (CA+) cells that developed was increased 50-fold, while the final number of melanocytes and total cells was only half that seen in the control cultures. The presence of the RBM gel overlay did not alter the time of onset of differentiation of the CA+ cells or melanocytes. The stimulation of CA+ cell number was not observed with type IV collagen substrates, laminin substrates or type I collagen gel overlays with or without added laminin. The stimulation of CA+ cell development was dependent on initial plating density. The number of CA+ cells that developed in the presence of the RBM gel was proportional to the initial plating density at 80–320 cells mm\(^{-2}\), whereas no CA+ cells were observed below 20 cells mm\(^{-2}\) and only a few CA+ cells were detected at 40 cells mm\(^{-2}\). There was, however, extensive cell division and differentiation of melanocytes and unpigmented cells at the lower initial plating densities. When the RBM gel was used as a substrate, rather than as an overlay, a striking rearrangement of cells into interconnected strands was observed. After several days in culture, melanocytes, CA+ cells and unpigmented cells were present in these strands. These results indicate that molecules associated with a reconstituted basement-membrane-like matrix are a potent stimulatory influence on adrenergic development and also act to inhibit the production of other cell types in neural crest cultures.

Key words: neural crest, adrenergic differentiation, extracellular matrix, basement membrane components, quail.

Introduction

The vertebrate neural crest is an embryonic cell population that first forms on the dorsal portion of the neural tube. Subsequently, neural crest cells migrate along defined pathways to many specific sites where they differentiate into a diverse array of adult cell types including most of the neurones of the peripheral nervous system, pigment cells of the skin and irides, the chromaffin cells of the adrenal medulla, Schwann cells, skeletal and connective tissue of the head and face, and cells that form part of the aorticopulmonary septum of the heart (Weston, 1970; Noden, 1978; LeDouarin, 1982; Kirby, Gale & Stewart, 1983).

We have been studying the differentiation of neural crest cells into cells that possess an adrenergic phenotype. After neural crest cells cease migration and coalesce to form the primordia of the sympathetic ganglia, they begin to express adrenergic traits including the uptake, biosynthesis and storage of catecholamines (CA) (Enemar, Falck & Hakanson, 1965; Kirby & Gilmore, 1976; Allan & Newgreen, 1977; Rothman, Gershon & Holtzer, 1978; Cochard, Goldstein & Black, 1978; Teitelman, Baker, Joh & Reis, 1979). Adrenergic differentiation similar to that seen in vivo also occurs when neural crest cells are grown in tissue culture under the appropriate conditions. The adrenergic cells that differentiate in tissue culture are able to synthesize, store and release CA (Cohen, 1972, 1977; Norr, 1973; Kahn, Coyle & Cohen, 1980;
Fauquet, Smith, Ziller & LeDouarin, 1981; Maxwell, Sietz & Rafford, 1982; Maxwell & Sietz, 1983). Ultrastructural and light microscopic data indicate that in several respects the CA+ cells that differentiate in tissue culture resemble small intensely fluorescent cells observed in autonomic ganglia and extra-adrenal chromaffin tissue in many species (Christie, Forbes & Maxwell, 1987).

During the course of their in vivo development, neural crest cells migrate through a complex extracellular matrix composed of collagens, proteoglycans and glycoproteins, which is closely associated with many embryonic cell populations (Mayer, Hay & Hynes, 1981; Weston, 1982; Newgreen & Erickson, 1986; Pintar, 1978; Derby, 1978; Thiery, Duband & Tucker, 1985; Tosney, 1978). Neural crest cells in the developing embryo also encounter basement membranes that occur at the boundaries of several structures in the embryo, including the neural tube, somites and the ectoderm (Weston, 1970; LeDouarin, 1982; Noden, 1978; Erickson, 1987). Basement membranes, as well as individual extracellular matrix components, can influence a wide variety of developmental processes (Hay, 1981; Sanes, 1983; Yamada, 1983).

In particular, considerable evidence indicates that the migration and differentiation of neural crest cells both in vivo and in vitro can be influenced by their interaction with elements of the embryonic environment (LeDouarin, 1982; Noden, 1978; Weston, 1970; Runyan, Maxwell & Shur, 1986; Bronner-Fraser, 1985). Previous studies have reported that the incidence of CA+ cell development in neural crest cultures can be increased by a variety of factors including coculture with somitic mesenchyme, culture on substrate-attached extracellular material produced by embryonic cells, exogenous fibronectin and high concentrations of chick embryo extract (Cohen, 1972; Norr, 1973; Sieber-Blum & Cohen, 1980; Sieber-Blum, Sieber & Yamada, 1981; Loring, Glimeilus & Weston, 1982; Howard & Bronner-Fraser, 1986). The mechanisms by which these different tissue culture conditions result in increased adrenergic development are not understood.

Here we have examined the biological effect of a reconstituted basement membrane (RBM) gel on the adrenergic differentiation of neural crest cells. Recently Kleinman et al. (1986) have described a high salt–urea extract of basement membrane components prepared from the mouse Engelbreth–Holm–Swarm tumour. This preparation, which forms a gel at 35°C, has been shown to affect differentiation in several systems including melanoma cells, Sertoli cells, Schwann cells and mammary epithelial cells (Hadley et al. 1985; Kleinman et al. 1986; Carey, Todd & Rafferty, 1986; Li et al. 1987). This RBM gel also serves as a favourable substrate for neural crest cell migration (Runyan et al. 1986; Bilozur & Hay, 1986). We have examined the effect of the RBM gel on neural crest cell development, with particular attention to the differentiation of the adrenergic phenotype. Our results indicate that the RBM gel acts as a potent stimulatory influence on the differentiation of the adrenergic phenotype in cultured quail trunk neural crest cells, while the number of melanocytes and total cells that develop in the presence of the RBM gel is about half that seen under control conditions.

**Materials and methods**

**Neural crest cultures**

Primary neural crest cultures were prepared from the trunk region of stage-13 (Zacchei, 1961) Japanese quail embryos (Coturnix coturnix) and grown as described previously (Maxwell et al. 1982). Briefly, neural tubes containing the neural crest were isolated free of surrounding tissues and grown in a medium containing 37-5ml Dulbecco’s modified Eagle’s medium with 4-5g l-1 glucose, pH 7-4 (Gibco). 37-5ml F-12, pH 7-4 (Gibco), 15ml horse serum (Gibco), 10ml 9-day chick embryo extract (Cahn, Coon & Cahn, 1967), 10mg gentamicin sulphate (Schering), 1ml of 0-2mg glutamine (Sigma), and 1ml of 100× stock vitamin mix. Vitamin mix stock contained 1mg dimethyltetrahydropterin (Calbiochem), 100mg ascorbic acid (Sigma), 10mg glutathione (Sigma) and 20ml distilled water. final pH 6-0 (Mans & Patterson, 1973). Cultures were grown at 36-5°C in a 5% CO2 atmosphere in 60mm tissue culture dishes (Lux) coated with a thin film of rat tail collagen polymerized by ammonia vapours (Ehrmann & Gey, 1956). The collagen was washed once with Hanks’ balanced salt solution with 15mm-Hepes (HBSS–Hepes) pH 7-4 prior to the addition of medium and the neural tubes containing the neural crest.

Secondary cultures were prepared from primary neural crest outgrowths after 41–45 h in vitro. The neural tubes were removed from the cultures with tungsten needles and the cells were detached using 0-05% trypsin (Gibco) in calcium- and magnesium-free Dulbecco’s phosphate-buffered saline (Gibco) at room temperature while being monitored on a phase-contrast microscope. Trypsinization was stopped by the addition of an equal volume of growth medium. The cells were then centrifuged at 600 g for 10 min and resuspended in growth medium. The cells were plated at the densities described in either 35 mm or 16 mm diameter tissue culture plates (IBT Technologies) coated with bovine endothelial extracellular matrix. Cultures were fed every other day with growth medium. Similar results were observed if collagen-coated dishes were used, but the efficiency of initial cell attachment was somewhat better on the extracellular-matrix-coated plates so they were used in all the experiments reported here.

Secondary neural crest cultures prepared from pooled primary neural crest outgrowths were used in the experiments reported here because although initial experiments
demonstrated that the RBM gel stimulated CA+ cell development in primary neural crest outgrowths. There was considerable variability with respect to the number of CA+ cells that developed in each outgrowth.

Application of reconstituted basement membrane and extracellular matrix components

RBM material (Matrigel, Collaborative Research) was applied as an overlay after the cells were attached, usually 1h after plating. Prior to application of the RBM, the plating medium was removed, followed by addition of 0.4ml of RBM to a 35mm dish or 0.08ml to a 16mm well. After application of the RBM the dishes were returned to the incubator for 30min to allow the gel to solidify. Cultures were then fed with growth medium. In some experiments, the RBM gel was first applied to the dish, allowed to gel, and then the cells were plated onto the RBM gel substrate.

Type I collagen gels were prepared using bovine dermal collagen (Vitrogen 100, Collagen Corporation). Gels with a final collagen concentration of 0.8, 1.5 and 2.0mgml⁻¹ were tested. The gels were prepared by mixing the Vitrogen 100 with 10×F-12 (Gibco) and 0.142N-NaOH in the ratio 8:1:1 together with a volume of 1×F-12 sufficient to give the appropriate final collagen concentration (Elsdale & Bard, 1972; Tucker & Erickson, 1984). When laminin was added to the type I gels, it replaced a portion of the 1×F-12. Laminin was from the mouse EHS sarcoma. Laminin purchased from Collaborative Research and material that was the generous gift of Drs Marvin Tanzer & S. Chandrasekaran both gave the same results.

When laminin was used as substrate 45μg in 1ml were added to a 35mm dish and allowed to incubate for 45min at room temperature to give 5μgcm⁻². Laminin was added to the feeding medium at 12μgml⁻¹.

Type IV collagen (Collaborative Research) was prepared as a substrate by spreading 75μg on a 35mm dish. This material was allowed to air dry overnight and was washed twice with HBSS–Hepes before use. When we attempted to make type IV collagen gels using a protocol similar to that used for the type I gels, we found that we could not generate stable type IV collagen gels.

Histochemical detection and quantification of catecholamine-containing cells

Cells containing catecholamines were visualized using the procedure of Furness, Costa & Wilson (1977). Cultures were incubated in 4% paraformaldehyde (Baker) and 0.2% glutaraldehyde (Polysciences) in 0.1M-sodium phosphate buffer pH 7.0 for 2h at room temperature in the dark. This solution was removed and the cultures then washed once with Dulbecco's phosphate-buffered saline (PBS) pH 7.4 (Gibco), followed by mounting in a few drops of glycerol:PBS (3:1, v/v) and application of a coverslip. The catecholamine fluorophore was observed on either a Zeiss upright or inverted microscope equipped for fluorescence optics using a violet excitation quinacrine mustard filter cube (Zeiss catalog number 48 77 05).

The number of CA+ cells was determined in the culture dish after visualization of the CA fluorophore as described above. In cases where the total number of CA+ cells was less than 3000 the cells were counted directly. In the RBM-treated cultures the number of CA+ cells was too large to count directly. We determined that the density of CA+ cells per unit area was quite uniform in the RBM-treated cultures. Therefore the number of CA+ cells was estimated by multiplying the average density of CA+ cells per microscopic field by the total number of microscopic fields containing CA+ cells.

Determination of total cell number

Total cell number was determined using a Coulter ZM electronic particle counter (Coulter Electronics). The cells were removed from the plate using Dispase (Collaborative Research) and resuspended in 1ml of Dulbecco's PBS. pH 7.4. Triplicate counts of each sample were averaged. The standard deviation of counts from an individual sample was less than 15% of the mean value. In some cases, haemacytometer counts were performed to corroborate the cell number obtained by electronic particle counting and to establish the percentage of melanocytes present in the population.

Results

Development of CA+ cells

When secondary cultures of neural crest cells were assayed after 7 days in vitro for the presence of CA+ cells, a dramatic increase in the number of CA+ cells was observed in the RBM gel-treated cultures compared to control cultures (Fig. 1). The morphology of the CA+ cells was similar in both the presence and the absence of the RBM gel overlay (Fig. 1). The cells exhibited bright CA fluorescence in the cell body and in occasional short processes, consistent with previous reports of the morphology of young CA+ cells that differentiate in neural crest cultures (Cohen, 1972; Norr, 1973; Cohen, 1977; Maxwell et al. 1982; Christie et al. 1987). Quantification of the number of CA+ cells indicated that, after 7 days in vitro, 50-fold more CA+ cells had developed in cultures grown in the presence of the RBM gel overlay than in the control cultures (Fig. 2). In contrast, total cell number after 7 days in vitro in the RBM-treated cultures was about half that seen in the controls (Fig. 2). These data demonstrated that the stimulatory effect of the RBM gel was selective for the CA+ phenotype since the presence of the RBM gel did not result in a general increase in cell number of all phenotypic classes of cells that differentiate in the cultures.

The onset of the appearance of CA+ cells was similar in the presence and absence of the RBM gel (Fig. 2). No CA+ cells were observed at 3 days in vitro under either condition. By 5 days in vitro a small number of CA+ cells were observed in the control cultures and an increased number of CA+ cells in the RBM gel-treated cultures was already discernible at
this time (Fig. 2). This difference became even more pronounced by 7 days in vitro.

When total cell number is examined as a function of time in culture, a quite different pattern is observed. After 3 days in vitro, the total cell number had increased about 13-fold and was the same in both the control and RBM gel conditions. However, by 7 days in vitro the total number of cells was about twice as great in the control case as in the RBM gel case (Fig. 2). Thus, the difference in total cell number between the RBM gel overlay cultures and the controls manifested itself after 3 days in vitro, as was the case for the effect on the CA+ cells. However, in the case of the total cell population the effect was inhibitory, in contrast to the marked stimulatory effect on CA+ cell development.

**Pigment cell differentiation**

Melanocytes and nonpigmented cells were present under both growth conditions (Fig. 1). However, there was a decrease in the extent of pigmentation observed in the presence of the RBM gel. This difference in the extent of pigmentation was reflected in the number of melanocytes seen in microscopic fields (Fig. 1B,D) and also was observable macroscopically. Densitometry scans of the cultures demonstrated that there was on average about two thirds as much pigmentation in the presence of the RBM gel overlay as there was in the control condition. The fraction of the total cell population that were melanocytes after 7 days in vitro was essentially the same in the presence (15 ± 2% melanocytes, mean ± s.d. (n = 4)) and absence (18 ± 2% melanocytes, (n = 4)) of the RBM gel. Thus, pigmentation was reduced in the presence of the RBM gel because the total cell number was about half that seen in controls, while the proportion of cells that were melanocytes remained quite constant. This is in contrast to the situation of the CA+ cells, in which there was an increase in both absolute number of CA+ cells and the fraction of...
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Table 1. Specificity of the stimulation of the adrenergic phenotype

<table>
<thead>
<tr>
<th>Condition</th>
<th>CA+ cell number</th>
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<tr>
<td>RBM gel overlay</td>
<td>43,900 ± 3010 (6)</td>
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<tr>
<td>Type I collagen gel overlay</td>
<td></td>
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<tr>
<td>0.8 mg ml⁻¹</td>
<td>727 ± 301 (8)</td>
</tr>
<tr>
<td>1.5 mg ml⁻¹</td>
<td>555 ± 212 (3)</td>
</tr>
<tr>
<td>2.0 mg ml⁻¹</td>
<td>680 (2)</td>
</tr>
<tr>
<td>Type I collagen gel overlay plus</td>
<td></td>
</tr>
<tr>
<td>100 μg ml⁻¹ laminin</td>
<td>925 ± 297 (5)</td>
</tr>
<tr>
<td>Laminin</td>
<td>494 ± 375 (5)</td>
</tr>
<tr>
<td>Type IV collagen substrate</td>
<td>228 ± 113 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>519 ± 185 (7)</td>
</tr>
</tbody>
</table>

Values expressed as mean ± s.d. (n). Neural crest secondary cultures were prepared as described in the Materials and Methods. After 7 days in vitro under the conditions indicated, the cultures were processed to reveal CA+ cells.

CA+ cells in the total population in the presence of the RBM gel (see above).

Specificity of the RBM gel overlay

In order to ascertain the specificity of the RBM components for the observed stimulation of CA+ cell number, several control experiments were performed. In none of these conditions did we observe any increase in CA+ cell number that resembled the increase mediated by the RBM gel overlay. Neither type I collagen gel overlays alone nor type I collagen gel overlays polymerized with 100 μg ml⁻¹ of laminin had a stimulatory effect on CA+ cell number (Table 1). These data suggested that mere presence of a gel overlay is not sufficient to induce the stimulation of CA+ cell number. In addition, two of the major components found in the RBM gels were ineffective in stimulating CA+ cell number when added individually to the cultures as substrates or as soluble molecules. Laminin or type IV collagen as substrate were ineffective in promoting CA+ cell development (Table 1). Laminin added to culture medium also failed to promote CA+ cell development.

Cell density and the response to the RBM gel

In order to gain further insight into the nature of cellular response to the RBM gel, the effect of initial plating density on the differentiation of the CA+ cells in the presence of the RBM gel was examined. As shown in Fig. 3, at initial plating densities of 80–320 cells mm⁻² the number of CA+ cells was proportional to the number of cells plated, while below 20 cells mm⁻² no CA+ cells were seen in spite of the presence of the RBM gel. At 40 cells mm⁻² an average of only 12 CA+ cells was observed. Shortly after plating at 320 cells mm⁻² some cells were in contact, but the cells were not confluent. However, after 7 days in vitro, cells in cultures at all plating densities were confluent and at the higher initial densities they were multilayered (Christie et al. 1987).

One possible explanation for our findings of the density dependence of CA+ cell development was that cell growth and division at these lower initial densities was not robust. However, when final total cell number was examined as a function of initial cell number, it was observed that there were proportionally more cell divisions on average at lower cell densities (Fig. 3). That is, the ratio of final total cell number to initial cell number (or density) was greater at the low densities than at the higher densities. Thus, the inability to observe CA+ cell development in cultures initiated at low density in the presence of the RBM gel was not a result of a general inability of cells to undergo growth and cell division under these conditions. However, a selective adverse effect on
Initial plating density (cells mm$^{-2}$)

**Fig. 3.** Final CA+ and total cell number in secondary neural crest cultures in the presence of RBM gel overlay as a function of initial plating density. Cells were plated at the densities indicated in 16 mm diameter wells and RBM gel was applied after the cells had attached. The 320 cells mm$^{-2}$ point corresponded to 64000 cells per 16 mm well. After 7 days in vitro one set of cultures was processed to reveal CA+ cells (•) while the other was processed for counting of total cell number (◦). The ratio of the mean of the final total cell number to initial cell number was 26 at 320 cells mm$^{-2}$, 42 at 160 cells mm$^{-2}$, 56 at 80 cells mm$^{-2}$, 57 at 40 cells mm$^{-2}$, 63 at 20 cells mm$^{-2}$, 79 at 5 cells mm$^{-2}$, and 343 at 0.5 cells mm$^{-2}$. Values in the figure are expressed as the mean ± s.d. of triplicate determinations.

CA+ progenitors at low density cannot be excluded as a possibility by these data.

In light of the dependence of CA+ cell number on initial plating density in the presence of the RBM gel, we investigated whether or not cell density alone was responsible for the activation of the CA+ phenotype. Although we had determined (Fig. 2) that the presence of the RBM gel actually resulted in the production of fewer total cells than in the control case at an initial plating density of 320 cells mm$^{-2}$, it was still possible that the RBM gel overlay was acting to increase the local cell density above some critical threshold value that permitted some other event(s) to occur which then stimulated activation of the CA+ phenotype. If this were the case, increasing the density of cells grown under control conditions, i.e. in the absence of RBM gel, might substitute for the presence of the RBM gel and result in an increase in the number of CA+ cells that develop. However, our results showed that increasing the initial plating density in the absence of the RBM gel did not mimic the effect of the RBM gel. Cultures initiated with 1280 cells mm$^{-2}$ (four times the standard control density) and grown in the absence of RBM gel gave rise to 647 ± 171 (n = 6) CA+ cells per culture, which was similar to the control value observed from cultures plated in the absence of RBM gel at 320 cells mm$^{-2}$ (see Table 1). Final total cell density was 50% greater than at 320 cells mm$^{-2}$. Thus, increased cell density alone did not mimic the effect of the RBM gel.

**RBM gel as a substrate**

We have also examined the effect of the RBM gel when used as a substrate for the growth and differentiation of neural crest cultures. When cells from neural crest outgrowths were subcultured onto RBM gel substrates they behaved somewhat differently to when the RBM gel was used as an overlay. Although the cells were initially uniformly distributed on the RBM gel substrate, they soon became rearranged into interconnected networks on the surface of the gel. These networks became macroscopically visible as pigment cell differentiation proceeded in the cultures. After several days in vitro, CA+ cells also differentiated in these cultures. Based on qualitative assessment of CA+ cell fluorescence, it appeared that there were more CA+ cells on the RBM gel substrate than under control conditions. However, quantification of the number of CA+ cells was not feasible due to the aggregation of the cells in the cultures (Fig. 4) which did not permit the optical resolution necessary to count individual cells.

**Discussion**

Previous reports have provided evidence for increased adrenergic development by neural crest cells via interaction with cells of the somitic mesenchyme, substrate-attached material produced by embryonic cells, fibronectin, medium conditioned by neural tubes, and avian tissue extracts (Sieber-Blum & Cohen, 1980; Sieber-Blum et al. 1981; Loring et al. 1982; Howard & Bronner-Fraser, 1986). Although precise comparisons of these reports to the data presented here are complicated by differences in experimental methodology and data collection, it seems that the magnitude of the stimulation of adrenergic development we have observed using the RBM gel overlay is as great or greater than that reported previously.

In our present experiments, the assay for the differentiation into CA+ cells depends on the production of CA from tyrosine by a pathway that involves several enzymatic steps with tyrosine hydroxylase (TH) being the first and rate-limiting enzyme in the pathway (Blaschko, 1973). Our data might be explained, in part, by the maturation of a cell population that is TH+CA− into a TH+CA+...
Fig. 4. Melanocytes and CA+ cells in the strands of neural crest cells which differentiate on RBM gel substrate. Secondary neural crest cultures were prepared and plated on top of an RBM gel substrate. (A) Phase-contrast view of the strands of cells showing darkly pigmented melanocytes as well as some unpigmented cells. (B) Same field as shown in A after processing to reveal CA+ cells. Numerous CA+ cells are evident intermingled with the other cell's types present in the strands. Scale bar, 50 μm.

cell population. However, recent experiments from our laboratory have provided evidence against the presence of a large population of TH+CA− cells (Christie et al. 1987). Thus, it seems unlikely that the increase in the number of CA+ cells in the presence of the RBM gel is the result of the further development of a pre-existing but incompletely differentiated population of TH+ cells.

The source of the biological activity in the RBM gel
With regard to the nature of the cue in the RBM gel that mediates the observed biological effects there are several alternative models. One possibility is that a specific component or molecular complex in the RBM gel binds to the neural crest cells and acts as an instructive signal. A second possibility is that the presence of the RBM gel stimulates the neural crest cells to produce a factor which then acts in an autocrine fashion. A third model is that there is no signal per se in the RBM gel, but the gel acts as a receptor for a molecule that is produced by the neural crest cells in a constitutive manner and which is lost to the medium in the absence of the RBM gel and becomes inactive (see Vlodavsky et al. 1987; Gordon, Riley, Watt & Greaves, 1987). A fourth possibility is that the activity could be the result of the physical properties of the gel that are permissive for certain key developmental events.

The RBM gel contains laminin, type IV collagen, heparan sulphate proteoglycan and entactin (nidogen) as its major components (Kleinman et al. 1986). The stimulation of the development of the CA+ cell population by the RBM gel was specific in that other extracellular matrix components, including two of the major components of the RBM gel, failed to elicit a similar effect. The fact that overlays of type I collagen gels were tested and found to be without effect indicated that the mere physical presence of a gel on top of the cells did not promote adrenergic development.

Our results on the specificity of stimulation of CA+ cell development in neural crest cultures are in agreement with the studies of Carey et al. (1986) who showed that the RBM-mediated stimulation of myelin formation was not reproduced by adding type I collagen gels or laminin. In contrast, Li et al. (1987) have reported that either laminin or heparan sulphate proteoglycan can substitute, at least partly, for the RBM gel in stimulating accumulation of increased mRNA for β casein by mouse mammary epithelial cells.

The cellular response to the RBM gel
Two main responses of neural crest cell cultures to the RBM gel overlays were observed. First, the number of CA+ cells was dramatically increased. Second, total cell number and the number of melanocytes that differentiate were reduced to about one half of the control value.

One model which could explain these results is that, in the presence of the RBM gel, the proliferation of the precursors of CA+ cells or CA+ cells themselves is increased while the proliferation of non-CA+ precursors is reduced. It has been shown previously that the CA+ cells in neural crest cultures arise from a mitotically active cell population and that cell division is probably required for the expression of the adrenergic phenotype (Kahn & Sieber-Blum, 1983; Maxwell & Sietz, 1985). In addition, both in vivo and in vitro some cells that have become CA+ retain the ability to synthesize DNA and presumably
to divide (Cohen, 1974; Rothman et al. 1978; Maxwell 
Sietz, 1985).

A second possible explanation is that the RBM gel 
acts to alter the developmental fate of a cell. It is 
well documented that developmentally older neural-
crest-derived neurones and neuroendocrine cells can 
exhibit considerable phenotypic plasticity in response 
to environmental cues (Bunge, Johnson & Ross, 
1978; Patterson, 1978; Unsicker, Krisch, Otten & 
Thoennen, 1978; Potter, Landis & Fursihan, 1981; 
Coulombe & Bronner-Fraser. 1986; Doupe, Landis & 
Patterson, 1985; Doupe, Patterson & Landis, 1985; 
Anderson & Axel, 1986).

One way to distinguish between the two possi-
bilities cited above would be to conduct a clonal 
analysis of the size and incidence of adrenergic and 
nonadrenergic neural-crest-derived cells in the pres-
ence and absence of the RBM gel. However, our data 
on the production of CA+ cells as a function of initial 
plating density in the presence of the RBM gel 
indicated that, while CA+ cell number was pro-
portional to the plating density at 80–320 cells mm⁻², 
below 20 cells mm⁻² no CA+ cells were observed 
although the RBM gel was present. Thus, under these 
growth conditions, we cannot perform the required 
clonal analysis. Previous experiments, using different 
culture conditions, have reported the differentiation 
of CA+ cells in neural crest cultures initiated at 
clonal density (Sieber-Blum & Cohen, 1980).

While cell density may play a role in the expression 
of the adrenergic phenotype, it is clear that other 
actors are involved. Our experiments in which cell 
density was increased in the absence of the RBM gel 
did not result in a stimulation of CA+ cell develop-
ment, indicating that, although cell density may be a 
necessary condition for the expression of the CA+ 
phenotype, it is not by itself sufficient.

A third possibility is that the RBM gel does not act 
directly on CA+ cells or their progenitors, but rather 
acts by inhibiting the growth of other neural crest 
cells and allowing the CA+ progenitors to interact so 
that there is a synergistic stimulation of the CA+ 
phenotype in the culture (see Acheson & Thoenen, 
1983). This type of phenomenon has been observed 
to operate in the developing limb to stimulate carti-
lage differentiation (Gay & Kosher, 1984; Kulyk & 
Kosher, personal communication).

The RBM gel also affects neural crest cell develop-
ment when used as a substrate. There is a morpho-
genetic component to the response in that the cells 
reorganize into strands. This observation is consistent 
with findings using primary neural crest outgrowths 
that demonstrated that the RBM gel can act as a 
 favourable substrate for neural crest cell movement 
either when used as a planar substrate or as a three-
dimensional gel (Runyan et al. 1986; Bilozur & Hay, 
1986). Based on qualitative evaluations, using the 
RBM gel as substrate also increased the number of 
CA+ cells that developed. However, for purely 
technical reasons, it is difficult to compare the effect 
on CA+ cell development quantitatively in the two 
cases.

Conclusions

Our present results indicate that extracellular matrix 
molecules contained in a reconstituted basement 
membrane matrix or molecules that are bound to this 
matrix are a potent stimulatory influence on adren-
ergic neural crest development and also act to inhibit 
the proliferation of other cell types in the population. 
 Constituents present in the RBM gel may be involved 
in either the commitment of individual cells to a given 
phenotype and/or the regulation of the proliferation 
of the progenitors of specific cell classes.

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