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Induction of neurite outgrowth by a conditioned-medium factor bound to the culture substratum

(nerve cell culture/substrate-associated material/parasympathetic neurons)

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ABSTRACT **Heart-cell conditioned medium (HCM) induces rapid neurite outgrowth from isolated neurons in culture. The following evidence indicates that this action of HCM is due to a trypsin-sensitive factor which attaches to the polyornithine-coated culture substratum: (*i*) Pretreatment of the culture substratum with HCM allows rapid neurite outgrowth to occur even in unconditioned media. The active factor remains bound to the substratum during the period of neurite outgrowth. (*ii*) The substratum-bound activity is destroyed by trypsin treatment, but is insensitive to collagenase, RNase, and DNase. (*iii*) The factor that binds to the substratum is essential for neurite outgrowth, because HCM is no longer active when the material that binds to the polyornithine substratum has been removed by passage of the HCM over a series of culture dishes. However, this "depleted" HCM is still able to support the growth of non-neuronal cells. (*iv*) Most significantly, when neurons are cultured in whole HCM, the extent of neurite outgrowth is proportional to the amount of substratum-bound activity and not to the amount in solution, indicating that the substratum-bound form of the factor is more active. Previous observations [Collins, F. (1978) *Dev. Biol.* 65, 50–57] suggest that HCM promotes neurite outgrowth by increasing the adhesion between nerve cell surface extensions and the polyornithine-coated culture substratum. It is possible, therefore, that the factor in HCM that binds to the substratum possesses sites to which nerve cell surface components adhere.**

Culture medium conditioned by the growth of embryonic heart cells contains a powerful inducer of neurite outgrowth (1–4). When individual parasympathetic neurons from the ciliary ganglion of chicken embryos are plated out in unconditioned medium, they attach to the polyornithine-coated culture substratum and exhibit cell surface movements for many hours, but they do not extend axons (1). Within 30 min after the addition of heart-cell conditioned medium (HCM) to such cultures, > 80% of the neurons have begun to extend axons (1).

HCM also induces neurite outgrowth from sympathetic neurons of the lumbosacral chain of chicken embryos (4). Present evidence suggests that the same HCM factor, which is different from nerve growth factor (4), is responsible for inducing neurite outgrowth from both sympathetic and parasympathetic neurons (3).

The results presented here indicate that whole HCM is not necessary for neurite outgrowth; rather a trypsin-sensitive component of HCM, which binds to the culture substratum, is sufficient to support outgrowth in unconditioned medium. The results also suggest that binding of this component to the substratum may be an essential part of its action in the present culture system.

Only the results of experiments on parasympathetic neurons are presented here, but the conclusions apply equally well to neurons of the sympathetic chain (3). A subsequent paper will describe the recovery from HCM-pretreated dishes of the factor that promotes neurite outgrowth from both neuron classes.

MATERIALS AND METHODS

Culture Conditions. Ciliary ganglia were dissected from 8- to 9-day White Leghorn chicken embryos and dissociated into single cells with trypsin as described (1). Unconditioned medium was modified Ham's F12 (5) containing 10% fetal calf serum (Gibco). HCM was prepared by culturing confluent monolayers of 8- to 9-day chicken embryo heart cells in unconditioned medium for 96 hr (1). HCM was titered by 1:2 serial dilutions and the titer was expressed as the greatest dilution that still gave 10% or more initiated neurons (1). Unless specified otherwise, only HCM of high titer (1:8) was used. Tissue culture plastic dishes (Falcon) with or without glass coverslip bottoms were coated with polyornithine (1).

To achieve rapid neurite outgrowth, neurons were allowed to recover from the dissociation procedure before use (1) by incubation in unconditioned medium for 4 hr in plastic Petri dishes (Falcon), to which the neurons do not adhere. To avoid neuronal aggregation during recovery, only *ca.* 15,000 neurons were incubated in 10 ml of medium in a 100 × 15-mm dish. After 4 hr the cells were pelleted and resuspended either in fresh unconditioned medium or in HCM and plated in 2.0 ml onto 35 × 10-mm polyornithine-coated culture dishes (*ca.* 2500 neurons and 2500 non-neuronal cells per dish). Cultures were incubated at 37°C in humidified 5% CO_2/95% air.

HCM Pretreated Dishes. The polyornithine-coated culture dishes were exposed to 2.0 ml of HCM at room temperature for various lengths of time. The HCM was removed and each dish washed three times with 2 ml of unconditioned medium. Half-pretreated dishes were prepared as above except that culture dishes were tilted at a 30° angle and HCM was added to cover only approximately one-half of the bottom surface. "Recovered" neurons were plated onto washed pretreated dishes in unconditioned medium.

Neurite Outgrowth. Neurons are easily distinguished from non-neuronal cells by size and morphology (1). Virtually all neurons attach to the polyornithine substratum within 15 min after plating; at the cell density used each neuron is widely separated from other cells.

Neurite outgrowth was assayed by one of two methods: (*i*) The percentage of initiated neurons (defined as those that bore at least one neurite longer than 15 μm) was determined out of 100 neurons examined per culture; or (*ii*) cultures were fixed in glutaraldehyde at a specified time after plating and the average total length of neurites per nerve cell was determined for 200 nerve cells per culture (including neurons that had not initiated). Data are presented as the average value and the range for duplicate cultures.

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Abbreviation: HCM, heart cell conditioned medium.

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Enzyme Treatments. Trypsin (2X crystallized, Worthington), DNase (Worthington), and collagenase (see below) were used in Hanks' basic salt solution. RNase (DNase-free, Sigma) was used in Ca^{2+}, Mg^{2+}-free Hanks' solution with 0.05% EDTA. The pH was maintained at 7.2–7.4 by using a bicarbonate buffer and a 5% CO_2 atmosphere. Soybean trypsin inhibitor (Sigma) was added to the trypsin in Hanks' solution 5 min before use. A highly purified preparation of collagenase was the generous gift of Lane Smith and Merton Bernfield (Stanford University). Collagenase was assayed by the method of Levenson (6). As used in Table 1, collagenase was able to digest 200 μg of rat tail collagen (the largest amount tried) deposited on a polyornithine-coated culture dish.

RESULTS AND DISCUSSION

HCM-pretreated Dishes Support Neurite Outgrowth. Dissociated ciliary ganglion neurons extended neurites in HCM, but not in unconditioned medium (Fig. 1). However, neurite outgrowth occurred in unconditioned medium on culture dishes that had been pretreated with HCM (Figs. 1 and 2). The extent of neurite outgrowth on HCM-pretreated dishes, as measured by the average neurite length, was proportional to the period of pretreatment (Fig. 3) and to the titer of HCM used (1).

Two different control experiments indicated that neurite outgrowth on HCM-pretreated dishes is not due to material that came back off the dish into the medium: (*i*) the unconditioned medium overlying pretreated dishes did not acquire the ability to support neurite outgrowth by neurons cultured in that medium in a fresh culture dish; and (*ii*) when only one-half of the surface of a culture dish was pretreated with HCM, neurons that attached to the untreated half did not extend neurites, even with repeated agitation of the culture dish, despite extensive outgrowth on the pretreated half.

Although culture dishes can be "conditioned" by pretreatment with HCM, nerve cells cannot. Dissociated neurons incubated in HCM for up to 6 hr in suspension did not extend neurites when subsequently plated out in unconditioned medium, although they would if plated out in HCM.

HCM Activity Is Trypsin-Sensitive. The ability of HCM-pretreated dishes to support neurite outgrowth in unconditioned medium was abolished by trypsin treatment, but it was insensitive to collagenase, RNase, and DNase as used here (Table 1). This effect of trypsin apparently depends upon its enzymatic activity, because excess soybean trypsin inhibitor prevented the inactivation of HCM-pretreated dishes by trypsin.

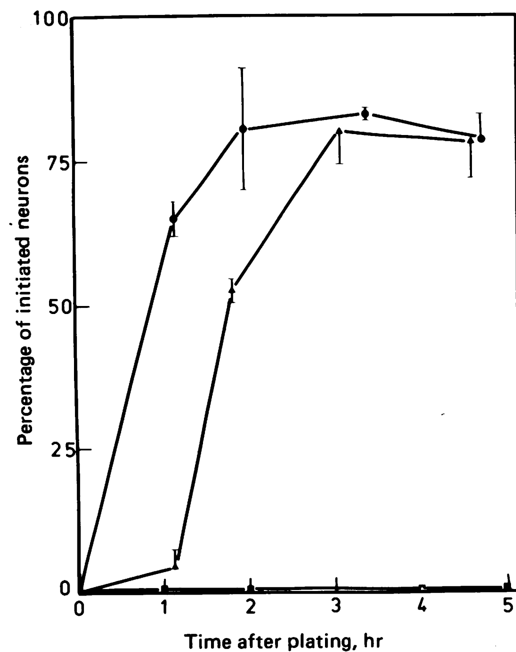

FIG. 1. Time course of neurite initiation on HCM-pretreated dishes compared to whole HCM. At time 0, dissociated and recovered ciliary ganglion cells were plated in: HCM (titer 1:16) on an untreated, polyornithine-coated culture dish (▲); or unconditioned medium on an untreated, polyornithine-coated culture dish (■); or unconditioned medium on a polyornithine-coated culture dish that had been pretreated with HCM (titer 1:16) for 12 hr and then washed in unconditioned medium (●). The percentage of initiated neurons in duplicate cultures was determined at various times after plating.

Two types of control experiments indicate that trypsin does not act simply by damaging the neurons or the polyornithine coating on culture dishes: (*i*) pretrypsinization of the polyornithine substratum did not prevent attachment of neurons or subsequent "conditioning" of the dish with HCM; and (*ii*) neurons that had failed to send out neurites on HCM-pretreated dishes inactivated by trypsin did so rapidly when HCM was added. Thus trypsin appears to inactivate or detach an HCM component bound to culture dishes.

Role of Bound Material in HCM-Induced Outgrowth. These and subsequent results indicate that HCM is fractionated

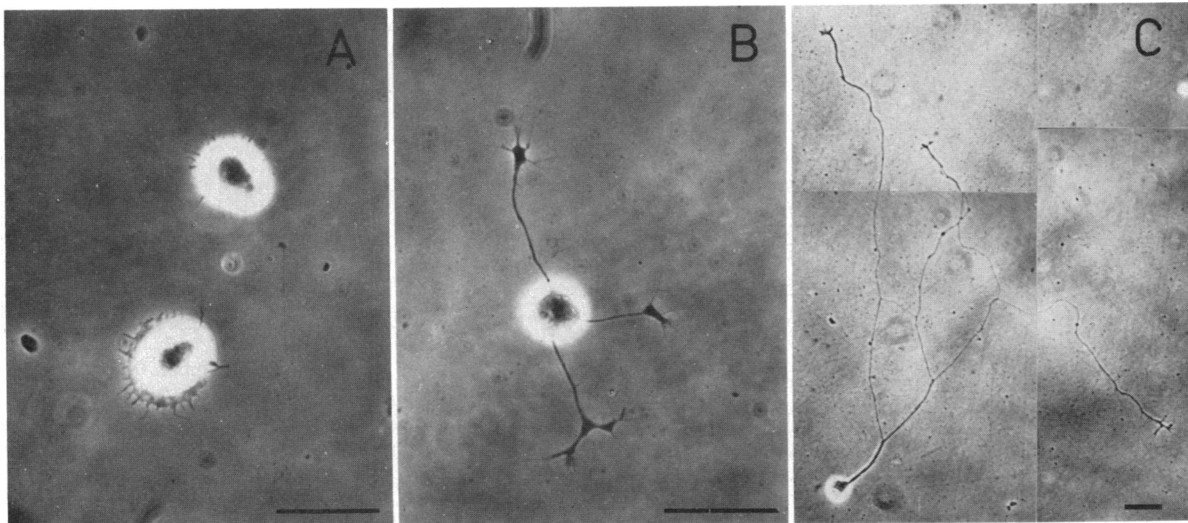

FIG. 2. Phase-contrast micrographs of neurons on untreated and HCM-pretreated dishes. Polyornithine-coated glass coverslip dishes (1) were either left untreated or exposed to HCM (titer 1:16) for 12 hr and then washed in unconditioned medium. Dissociated and recovered ciliary ganglion cells were then plated onto these dishes in unconditioned medium. (*A*) Neurons 12 hr after plating on an untreated dish. (*B*) and (*C*) Neurons 58 min and 12 hr, respectively, after plating on an HCM-pretreated dish. All bars represent 20 μm.

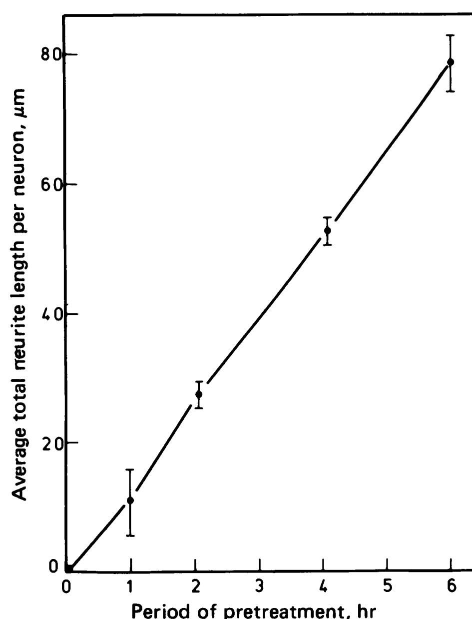

FIG. 3. The extent of neurite outgrowth in unconditioned medium as a function of the period of HCM pretreatment of dishes. Polyornithine-coated culture dishes were exposed to HCM (titer 1:8) for various lengths of time and then washed in unconditioned medium. Dissociated and recovered ciliary ganglion cells were plated onto the washed dishes in unconditioned medium. Pretreatment times were staggered so that all dishes were washed and received cells at the same time. After 90 min, fixative was added and the average total neurite length per neuron was measured. The values for duplicate dishes at each time point were averaged.

on a polyornithine substratum into bound and unbound components, the former containing a trypsin-sensitive factor that supports neurite outgrowth. The remainder of this paper attempts to assess the extent to which neurite outgrowth in whole HCM is due to the material deposited on the substratum.

The material that binds to the substratum seems to be essential for neurite outgrowth, because HCM can be depleted of its ability to support outgrowth by passage of the HCM over a sufficient number of culture dishes. In this experiment HCM was passed in sequence over 10 polyornithine-coated dishes for 30 min each. With sequential passage, HCM lost the ability to "condition" fresh dishes (Fig. 4). Concurrently, the passaged HCM decreased in titer and finally lost altogether the ability to support neurite outgrowth from neurons cultured directly in it (Fig. 4). However, non-neuronal ciliary ganglion cells and chicken embryo fibroblasts multiplied well in this "depleted" HCM, indicating that what was removed by the polyornithine substratum is required relatively specifically for neurite outgrowth.

Certain aspects of this depletion experiment require emphasis. Firstly, polyornithine is required for depletion of HCM, because HCM is not reduced in titer by passage over an equal number of uncoated tissue culture plastic dishes. Secondly, exposure to 10 dishes for 30 min each is much more effective in depleting HCM than exposure to 1 dish for 300 min. These results are consistent with the idea that depletion is due to binding of an essential component to the polyornithine substratum and not simply due to inactivation of HCM with time at room temperature. Indeed, HCM retains its titer after repeated cycles of refrigeration and heating to 37°C.

So far, polylysine or protamine are the only other substrata I have found, besides polyornithine, that (in the presence of HCM) produce rapid neurite outgrowth from a large percentage of ciliary ganglia neurons. These positively charged substrata also bind the HCM factor and can be used to deplete HCM.

Although the material that binds to the substratum appears to be essential for neurite outgrowth, an HCM-pretreated substratum does not substitute completely for whole HCM. HCM has two separable effects on neurons from the ciliary ganglion: (*i*) it induces neurite outgrowth and (*ii*) it supports long-term neuronal survival and thus continued neurite elongation. The material that binds to the substratum has the former effect of HCM, but not the latter. Although neurite outgrowth begins vigorously on HCM-pretreated dishes (Fig. 1), the rate of elongation decreases and degeneration of the nerve cells (1) is clearly visible 12–15 hr after plating in unconditioned medium, even when pretreatment times of several days are used. However, when cultured directly in HCM, neurons will remain intact for at least 4 days (also see refs. 2, 7, and 8). This observation suggests that there may be components of HCM that are involved in neuronal survival, in addition to the material that binds to the substratum and promotes neurite outgrowth.

Table 1. The effect of various hydrolytic enzymes on the activity of HCM-pretreated dishes

Enzyme treatment (µg/ml)	Percentage of control values
Trypsin (50)	0%
Trypsin (10)	48 ± 21%
Trypsin (50) + SBTI (100)	103 ± 4%
Collagenase (50)	98 ± 8%
DNase (50)	118 ± 14%
RNase (50)	100 ± 2%

Polyornithine-coated tissue culture plastic dishes (35 × 10 mm) were pretreated for 16 hr with 2.0 ml of HCM (titer 1:8) and then washed in the appropriate control buffer (Hanks' balanced salt solution for trypsin, collagenase, and DNase; Ca^{2+}, Mg^{2+}-free Hanks' solution/0.05% EDTA for RNase). Each dish then received 2.0 ml of control buffer or enzyme solution and was incubated at 37°C. After 60 min, dishes were washed with unconditioned medium and then received dissociated and recovered ciliary ganglion cells in unconditioned medium. The percentage of initiated neurons in enzyme-treated dishes at 12 hr after addition of neurons was divided by the percentage initiated in dishes treated with the appropriate control buffer. The absolute control buffer values were 73 ± 7% initiated neurons. The data presented here are the average and range for two experiments. SBTI = soybean trypsin inhibitor.

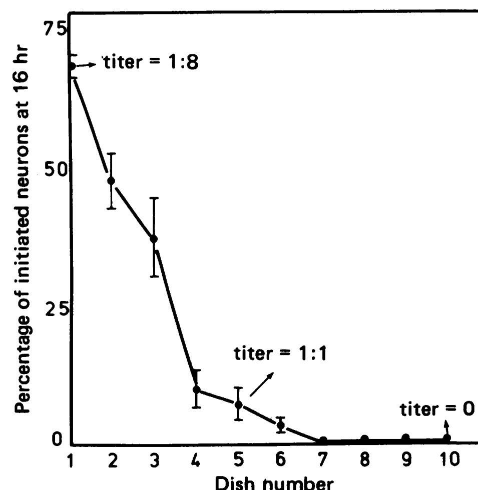

FIG. 4. Loss of activity of HCM passed over a series of polyornithine-coated dishes. HCM (titer 1:8) was incubated in sequence in 10 polyornithine-coated culture dishes for 30 min each. After HCM was removed, each dish was washed in unconditioned medium. After all 10 dishes had been treated, dissociated and recovered ciliary ganglion cells were cultured on these dishes in unconditioned medium for 16 hr, at which time the percentage of initiated neurons was measured. The values for three separate series of dishes were averaged. After passage over 1, 5, and 10 dishes, aliquots of the overlying HCM were titered by 1:2 serial dilution (1).

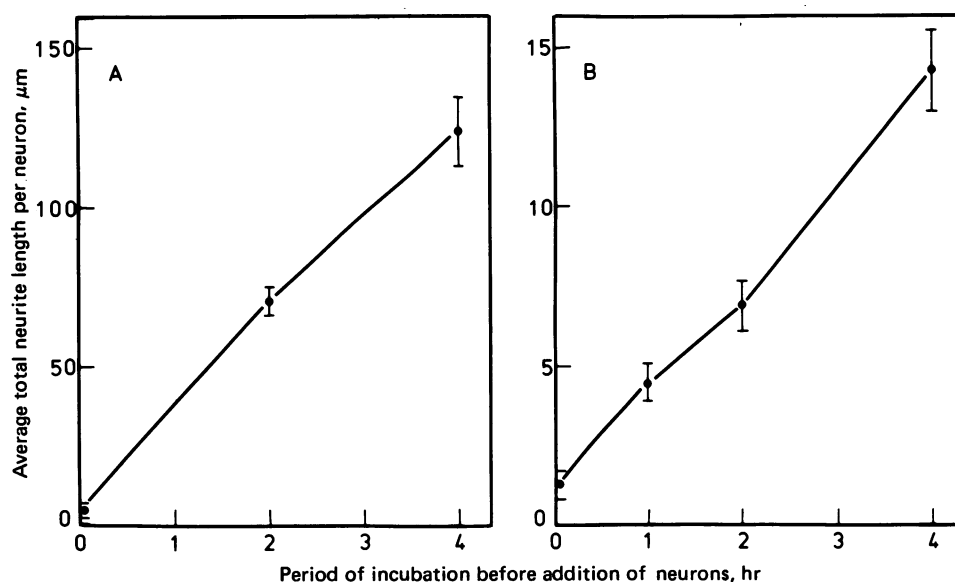

FIG. 5. The extent of neurite outgrowth in HCM as a function of the period of contact between HCM and the dish before addition of neurons. Polyornithine-coated culture dishes were incubated with 2.0 ml of HCM per dish for various lengths of time before dissociated and recovered ciliary ganglion cells were added (in 0.025 ml of unconditioned medium per dish) to the HCM in each dish. Incubation times were staggered so that all dishes received cells at the same time. After 90 min, fixative was added and the average total neurite length per neuron was measured in duplicate cultures for each time point. (*A*) HCM of titer 1:16. (*B*) HCM of titer 1:2.

Bound Material Is More Effective than the Same Material in Solution. It is typically the case, as shown in Fig. 1, that neurite outgrowth begins sooner on a 12-hr HCM-pretreated dish than in whole HCM. This suggests that the bound activity may be more effective in promoting rapid outgrowth than the same material when it is still largely in solution. In order to test this hypothesis, I have used the observation that the activity deposited onto the substratum increases with duration of contact between HCM and the dish (Fig. 3). A series of polyornithine-coated dishes was incubated with HCM for various lengths of time; this series then consisted of dishes each of which contained the same total amount of HCM, but which differed in the proportion of HCM that had become bound to the substratum. The same number of dissociated neurons in a very small volume of unconditioned medium was then added to the HCM in each dish and the average neurite length was determined after a brief period of outgrowth in the HCM. If outgrowth is independent of the ratio of bound to still unbound material, one might expect the same extent of outgrowth in all dishes. However, the results indicate that the extent of neurite outgrowth in HCM of high or low titer is proportional to the amount of activity bound to the substratum and independent of the amount still in solution (Fig. 5). The factor responsible for this activity thus appears to be much more effective when bound to the substratum than in solution.

The material that binds to the substratum could promote neurite outgrowth in any number of ways; however, it is difficult to see why the activity of this material should be so dependent on its attachment to the substratum, as indicated above, unless its presence changes the properties of that substratum. One property of the substratum that might be changed by HCM is its adhesiveness. In a recent time-lapse study of HCM-induced neurite initiation (1) I observed that the first visible change in neuronal behavior after addition of HCM was the attachment of formerly nonadhering filopodia to the substratum. It is therefore possible that the material in HCM that binds to the substratum provides sites for the adhesion of neuronal filopodia or other surface extensions and thereby promotes neurite outgrowth.

Changes in the physical nature of the substratum that increase its adhesiveness have been shown to greatly increase the percentage of neurons that extend nerve fibers (9). The present results indicate that molecules of biological origin can also modify the substratum to produce a similar effect. The HCM factor appears to be comparable to other substrate-associated materials that have been shown to affect cell attachment, spreading, and movement (10).

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