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The Influence of Non-Neuronal Cells on Catecholamine and Acetylcholine Synthesis and Accumulation in Cultures of Dissociated Sympathetic Neurons

(cell culture/cell–cell interaction)

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ABSTRACT **The effects of several non-neuronal cell types on neurotransmitter synthesis in cultures of dissociated sympathetic neurons from the new-born rat were studied. Acetylcholine synthesis from radioactive choline was increased 100- to 1000-fold in the presence of non-neuronal cells from sympathetic ganglia. This increase was roughly dependent on the number of ganglionic non-neuronal cells present. The effect did not appear to be due to an increased plating efficiency of neurons, since the non-neuronal cells were capable of increasing acetylcholine synthesis after only 48-hr contact with neurons that had been previously grown without non-neuronal cells for 2 weeks. C6 rat glioma cells were also able to stimulate acetylcholine synthesis, but 3T3 mouse fibroblast cells had little or no effect. None of the non-neuronal cell types synthesized detectable acetylcholine in the absence of the neurons. The ganglionic non-neuronal cells had no significant effect on catecholamine synthesis (which occurs in the absence of non-neuronal cells).**

Previous studies on primary cultures of dissociated sympathetic neurons have documented some of the basic biochemical properties of the neurons grown in the virtual absence of non-neuronal cells (1–3). Information was obtained on the kinetic parameters, nutritional requirements, and developmental time course of catecholamine synthesis and accumulation with radioactive tyrosine as the precursor. The cultures were also capable of synthesizing small amounts of [^{3}H]acetylcholine (AcCh) from the precursor [^{3}H]choline. It was not clear whether a very small population of postganglionic cholinergic cells was present or if the catecholamine-synthesizing neurons were also capable of producing very low levels of AcCh. The cultures did not synthesize and accumulate detectable levels of the neurotransmitter candidates histamine, serotonin, or gamma-aminobutyric acid from the respective radioactive precursors.

One of the objectives of this earlier work was to determine some of the biochemical properties of the neurons in the absence of influences from other cells. Addition of various types of non-neuronal cells to the cultures might then yield information concerning the role of non-neuronal cells in the development and function of sympathetic neurons. In this report preliminary findings are presented on the influence of several types of non-neuronal cells on neurotransmitter synthesis in cultures of dissociated sympathetic neurons. The accompanying paper reports electrophysiological and morphological data obtained on similar cultures (4).

Abbreviations: AcCh, acetylcholine; CA, catecholamine; NGF, nerve growth factor.

METHODS

Sympathetic neurons were obtained from superior cervical ganglia of newborn (1–2 days postnatal) rats and were dissociated by mechanical agitation (5). The dissociation medium contained glucose (final concentration 1.2% w/v) 2-fold higher than previously employed. The growth medium was either "L-15-Air" or "L-15-CO_2," both of which were based on Leibowitz's L-15 with modified osmolarity and pH and to which were added Methocel, penicillin–streptomycin, glucose, glutamine, nerve growth factor (NGF), adult rat serum, bovine-serum albumin, and a mixture of vitamins and cofactors (1). L-15-Air contained added imidazole to stabilize the pH, and L-15-CO_2 had added bicarbonate (25 mM final concentration). Dishes containing L-15-Air were kept in incubators without added CO_2, while L-15-CO_2 dishes were incubated in an atmosphere containing 8% CO_2.

The C6 rat glioma and 3T3 mouse fibroblast cell lines were obtained from the American Type Culture Collection. Ganglionic non-neuronal cells were grown in large batches by plating small chunks of superior cervical ganglia in L-15-CO_2 minus NGF; in the absence of NGF the neurons die. After 1–2 weeks growth, these cells were removed by trypsinization for plating on neuronal cultures. The non-neuronal cells were grown in either L-15-CO_2 or L-15-Air. However, before being added to neuronal cultures in L-15-Air, the non-neuronal cells were adapted to this medium by pre-incubation for about 1 week. The number of non-neuronal cells added to the neuronal cultures on day 1 (the day the neurons were plated) was adjusted so that there would be about 5 to 10 $\times$ 10^4 on days 12–17, when transmitter production was assayed.

Four-hour isotopic incubations were carried out with [*2,3*-^{3}H]tyrosine (Amersham Searle), [*methyl*-^{3}H]choline (New England Nuclear Corp.), and eserine sulfate (15 μg/ml, Sigma) in L-15-CO_2 as previously described (1). The final specific activities of the isotopes were 3.67 Ci/mmole for tyrosine and 2.34–11.1 Ci/mmole for choline. The concentrations were saturating for these mixed nerve, non-neuronal cell cultures (200 μM tyrosine and 70 μM choline), and total incorporation was linear for 4 hr (P. H. Patterson, L. L. Y. Chun, and R. E. Mains, in preparation). The radioactivity in norepinephrine, dopamine, and AcCh was determined after electrophoresis and chromatography of the culture extracts as previously described (1). The data shown are means of quadruplicate dishes of 12- to 17-day-old neuronal cultures, and are from representative cell platings. All results were repeated at least four times.

TABLE 1. *The effect of non-neuronal cells on neurotransmitter synthesis and accumulation*

Expt	Medium	Cell Types Present	pmole CA*	pmole AcCh*	AcCh/CA†
I.	L-15-Air	Neurons	8.82 ± 0.44	<0.003	<0.001
	L-15-CO_2	Neurons + satellite cells‡	8.71 ± 1.10	1.091 ± 0.160	0.128 ± 0.018
	L-15-CO_2	Satellite cells alone‡	<0.02	<0.003	
II.	L-15-CO_2	Neurons + satellite cells	5.58 ± 2.03	2.40 ± 0.52	0.53 ± 0.15
	L-15-CO_2	Neurons + extra added satellite cells§	4.77 ± 1.91	15.76 ± 7.52	2.77 ± 0.62
	L-15-CO_2	Neurons + satellite + 3T3 cells	4.91 ± 0.70	1.12 ± 0.21	0.23 ± 0.12
III.	L-15-Air	Neurons	1.35 ± 0.12	<0.003	<0.002
	L-15-Air	Neurons + C6 cells	2.08 ± 0.58	0.903 ± 0.494	0.353 ± 0.125
	L-15-Air	Neurons + 3T3 cells	1.77 ± 0.27	0.007 ± 0.002	0.004 ± 0.001
	L-15-Air	C6 cells alone	<0.03	<0.003	
IV.	L-15-CO_2	Neurons + satellite cells	0.542 ± 0.044	0.380 ± 0.001	0.720 ± 0.006
	L-15-CO_2	Neurons + cytosine arabinoside¶	1.381 ± 0.510	0.035 ± 0.002	0.028 ± 0.007
	L-15-CO_2	Neurons + FdU and uridine¶	0.951 ± 0.188	0.029 ± 0.007	0.029 ± 0.006
V.	L-15-Air	Neurons	8.82 ± 0.44	<0.003	<0.001
	L-15-Air	Neurons + satellite cells for 48 hr‖	5.05 ± 1.17	0.042 ± 0.005	0.011 ± 0.003

* Apparent pmole per culture values were derived from the specific activity of the added isotopes, assuming full and rapid equilibration of the intracellular pools with the medium and subject to the qualifications outlined previously (1, 2). Expressed as mean ± SEM for quadruplicate cultures.

† The ratios of the individual cultures were determined and then the mean ± SEM of those ratios was calculated.

‡ As previously described (1), cultures grown in L-15-CO_2 with NGF contain both neurons and ganglionic non-neuronal cells. The latter appear by phase microscopy to be of several morphological types not characterized as yet. The term satellite cell is used here for convenience. As noted in *Methods*, cultures of ganglionic non-neuronal cells without neurons were grown in L-15-CO_2 by omitting NGF.

§ More satellite cells were added in addition to those satellite cells normally present in L-15-CO_2 cultures, as described in *Methods*.

¶ Cytosine arabinoside (Sigma) was added at a final concentration of 10 μM on the third day *in vitro* and removed on day 7. The isotopic incubations were carried out on day 15. Fluorodeoxyuridine (FdU) and uridine (Sigma) were also added at the same concentrations and with the same schedule.

‖ Neuronal cultures were grown for 13 days in L-15-Air and then satellite cells were added at a concentration high enough to achieve confluency in 24 hr (about 75,000 cells per well). Isotopic incubations were then carried out on these cultures 48 hr later.

RESULTS

Catecholamine Synthesis. It was previously reported (1) that cultured sympathetic neurons develop the ability to synthesize and accumulate norepinephrine and dopamine to the same extent when grown in the virtual absence (L-15-Air) or in the presence (L-15-CO_2) of ganglionic non-neuronal cells. This apparent insensitivity of catecholamine (CA) metabolism to the presence or absence of ganglionic non-neuronal cells was confirmed in the present study (Table 1, Exp. I) and extended to several other types of non-neuronal cells. As shown in Table 1, Exps. I, II, and III, the presence of several different non-neuronal cell types, in the two different media, had no significant effect on catecholamine synthesis and accumulation with radioactive tyrosine as the precursor. This result, which is in contrast to that reported below for AcCh metabolism, is useful in that it enables us to use CA synthesis and accumulation as an assay for neuronal viability and growth under a variety of culture conditions.

Acetylcholine Synthesis. It was previously found that older neuronal cultures (4 weeks) grown in L-15-Air made very small amounts of radioactive AcCh, that could be detected only in the presence of eserine (1). The levels were variable and were 100- to 1200-fold less (in moles) than the amounts of radioactive catecholamines synthesized in the same incubations (in the absence of CA breakdown inhibitors). In contrast, neuronal cultures grown in L-15-CO_2, where the ganglionic non-neuronal cells proliferate, synthesized and accumulated over 120-fold more AcCh than their sister cultures grown in L-15-Air (Table 1, Exp. I). This difference in AcCh synthesis between the two media varied from 30- to 1000-fold and appeared to be correlated with the age of the cultures assayed; the older cultures, with larger numbers of non-neuronal cells, showed the higher levels of AcCh synthesis. The identity of the radioactive AcCh from mixed cultures was verified by two-dimensional paper chromatography and susceptibility to hydrolysis by acetylcholinesterase (P. H. Patterson, L. L. Y. Chun, and R. E. Mains, in preparation).

One obvious question arising from this result is whether the increase in AcCh synthesis in the L-15-CO_2 cultures was due to the presence of bicarbonate or to the growth of the ganglionic non-neuronal cells, which was in turn caused by bicarbonate. Three lines of evidence suggest that the non-neuronal cells have an effect on AcCh synthesis: (*a*) the type of non-neuronal cell is critical, (*b*) increases in AcCh synthesis can be induced by adding non-neuronal cells to L-15-Air cultures, and (*c*) the number of ganglionic non-neuronal cells affects the level of AcCh synthesis; adding more cells increases AcCh and reducing their proliferation with mitotic poisons reduces AcCh synthesis markedly.

The effect on AcCh synthesis of the number and type of non-neuronal cells is shown in Table 1, Exp. II. The presence of 5 to 10 $\times$ 10^4 ganglionic non-neuronal cells significantly increased the AcCh/CA ratio, while the presence of a similar number of 3T3 cells had no stimulatory effect over the L-15-CO_2 control. The AcCh synthesis in the latter two cases presumably was due to the presence of the normally occurring ganglionic non-neuronal cells in these cultures. This hypothesis is supported by the results shown in Table 1, Exp. III. In this case, all of the neuronal cultures were grown in L-15-Air, in which the ganglionic non-neuronal cells do not proliferate, thereby giving lower "control" AcCh levels. It can be seen

that 3T3 cells had only a slight effect on AcCh synthesis in L-15-Air. However, the rat glioma cell line, C6, employed because it might have some properties in common with the ganglionic non-neuronal cells, greatly increased the AcCh/CA ratio of the neuronal cultures. It should be noted that the C6 cells alone do not synthesize detectable AcCh or CA. The C6 cells also stimulated AcCh synthesis when added to L-15-CO_2 neuronal cultures (data not shown).

The importance of the non-neuronal cells is further demonstrated when mitotic inhibitors were employed to substantially reduce the proliferation of ganglionic non-neuronal cells (Table 1, Exp. IV). These drugs blocked markedly the usual increase in AcCh synthesis seen in L-15-CO_2 cultures, but had no detectable deleterious effect on CA synthesis and, in fact, appeared to stimulate it somewhat.

It was possible that non-neuronal cells exerted their effect by allowing a population of cholinergic neurons, otherwise non-viable in L-15-Air, to survive, perhaps by increasing the plating efficiency of a particular population of neurons. Therefore, non-neuronal cells were added after the neurons had had a chance to grow up (or presumably die) but just before the incubation with isotope. In the experiment illustrated in Table 1, Exp. V, the neuronal cultures were grown in L-15-Air for 13 days and the non-neuronal cells were added 48 hr before the incubation. Even after this relatively short time together, the mixed cultures showed significantly increased AcCh synthesis.

DISCUSSION

Our data show that certain types of non-neuronal cells have profound and selective effects on neurotransmitter synthesis and accumulation in cultures of dissociated sympathetic ganglia. The AcCh production caused by non-neuronal cells from the ganglion itself or by a glioma cell line is specific in that CA synthesis and accumulation is not affected by these cells. The increase in labeled AcCh found in mixed cultures could be due to increased intracellular choline concentration or specific activity, higher choline acetyltransferase activity, or decreased AcCh breakdown. We do not yet have enough information to distinguish between these possibilities. However, explanations involving changes in the specific activities or sizes of the intracellular choline pools appear unlikely because (*a*) incubations were done under conditions where the choline concentration in the medium is saturating (P. H. Patterson, L. L. Y. Chun, and R. E. Mains, in preparation), and (*b*) the presence of a 10- to 100-fold excess of non-neuronal cells over the neurons should, if anything, dilute the specific activity of the radioactive choline and its product AcCh.

There is, as yet, no direct evidence that the large increase in AcCh synthesis seen in the mixed cultures occurs in the neurons rather than in the added non-neuronal cells. Although there was no radioactivity above background in AcCh in incubations of various non-neuronal cell types in the absence of sympathetic neurons, it is possible that the neurons stimulate the non-neuronal cells to synthesize AcCh rather than vice versa. A preliminary finding that may suggest a change in AcCh metabolism in the neurons themselves is the formation of cholinergic synapses between the neurons in the presence, but not in the absence, of the ganglionic non-neuronal cells (4).

The difference in effectiveness in stimulating AcCh production between C6 and ganglionic non-neuronal cells on one hand, and 3T3 cells on the other, indicates some cell specificity in the interaction with the sympathetic neurons. However, the difference may be quantitative rather than qualitative and evaluation of the effectiveness of a wider range of non-neuronal cell types will be necessary before conclusions as to cell specificity can be made. Preliminary experiments indicate that, in fact, other non-glial cell types are also capable of increasing AcCh production in the sympathetic cultures. Perhaps relevant to the present findings is the report of Giller *et al.* (6), who demonstrated a 4-fold increase in choline acetyltransferase activity in combined spinal cord–skeletal muscle cultures over combined spinal cord–fibroblast cultures and a 10-fold increase over spinal cord cultures alone. The fact that the fibroblasts were not as effective as skeletal muscle cells in increasing the choline acetyltransferase activity suggests some cell specificity in the interaction with the spinal cord cells. It should be emphasized that it is not yet known whether the increased AcCh synthesis and accumulation seen in the sympathetic cultures reflects increased acetyltransferase activity. Therefore, comparisons with the results of Giller *et al.* (6) are speculative at this point.

Part of the evidence for the influence of non-neuronal cells on AcCh synthesis in the present report was the effect of antimitotic agents. If the neurons produce the AcCh, then an important qualification on this experiment is the effect of these drugs on neuronal health or growth. The only assay for neuronal condition used here is CA production. This is a relevant marker only if the same sympathetic neurons are synthesizing both AcCh and catecholamines (see ref. 4 for discussion of this possibility). Other observations on the effect of anti-mitotic poisons are those of Werner *et al.* (7) and Schrier and Shapiro (8) who found that 5-fluorouracil and fluorodeoxyuridine considerably reduce choline acetyltransferase activity in cultures of dissociated chick and rat brain. One interpretation of their results is that the loss in acetyltransferase activity is due to death of the non-neuronal cells.

The effect of non-neuronal cells on AcCh synthesis reported here is probably not due to an increase in plating efficiency or initial growth because the non-neuronal cells stimulate AcCh production after as little as 48 hr on neuronal cultures grown for two weeks in the absence of non-neuronal cells. This apparent lack of effect of non-neuronal cells on the plating efficiency of the neurons (seen also with CA production as the assay) is consistent with previous reports of survival and differentiation of dissociated sympathetic (9, 5, 1) and dorsal-root (10) neurons in the absence of non-neuronal cells. The difference between these reports and the suggestion by Varon and Raiborn (11, 12) that non-neuronal cells play an essential role in the survival and initial growth of sensory and sympathetic neurons *in vitro* may be due to differences in media.

The observation that C6 glioma cells are effective in increasing AcCh synthesis in the sympathetic cultures is not the first finding of an effect of C6 cells on neuronal properties. Monard *et al.* (13) found that medium conditioned by C6 cells increased process formation by neuroblastoma cells in culture. Medium from other non-neuronal cells also increased process formation, but not as well as the C6 medium. Obviously, there need not be a correspondence between the increased process formation in neuroblastoma and increased AcCh synthesis in sympathetic cultures, but it will be of interest to test the factor active in the former system (a purified protein, D. Monard and F. Solomon, personal communication) on the sympathetic cultures.

Note Added in Proof. Preliminary choline acetyltransferase assays using minor modifications of the method of Fonnum (14) indicate that L-15-CO_2 sympathetic cultures have more than 100-fold higher activity than L-15-Air sister cultures. This may furnish at least a partial explanation for the difference in AcCh synthesis seen in the two media.

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