In Vitro Cell. Dev. Biol. 27A:55–62, January 1991

0883-8364/91 $01.50+0.00

ENHANCED NEURONAL REGENERATION BY RETINOIC ACID OF MURINE DORSAL ROOT GANGLIA AND OF FETAL MURINE AND HUMAN SPINAL CORD IN VITRO

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(Received 3 January 1990; accepted 5 October 1990)

SUMMARY

This study demonstrates that retinoic acid (RA), an active metabolite of vitamin A, can act to enhance regeneration of neurites, at physiologic concentrations, in vitro. Explanted fragments of mouse dorsal root ganglia (DRG) and mouse and human spinal cord (SC) were maintained, in vitro, for periods up to 11 d. Murine DRG neurons were exposed to RA concentrations ranging from 100 μM to 1 nM, whereas neurons within murine and human SC explants were exposed to 10 μM to 10 nM RA. Results show that RA significantly ($P < 0.001$) increases mean neurite length but not neurite number. Specifically, murine DRG neurons showed increases in mean neurite length of 30.7% with individual explants showing increases of up to 133.5%. Murine and human SC showed mean enhancements of 43.4 and 58.1%, respectively, but did so at lower concentrations of RA. The results indicate that RA may play a potentially critical role in neuronal regeneration.

Key words: in vitro; neuronal regeneration; retinoic acid; dorsal root ganglia; spinal cord.

INTRODUCTION

Previous work from our laboratory (De Boni and Anderchek, 1986) employing external electric fields, applied to neurons regenerating in culture, showed some enhancement of regeneration; an enhancement probably related to changes in transmembrane ion currents. In view of the established role of membrane cation transport in regulation of cell growth and differentiation (Rosoff and Cantley, 1983; Felber and Brand, 1983; Kanje et al., 1988), it was decided to alter neuronal membrane currents in regenerating neurons, in vitro, by retinoic acid (RA). Sidell and Schlichter (1986) showed that RA, an active metabolite of vitamin A, acts on mammalian cell types by altering membrane ion-channel activity. RA decreased the potassium current in human T lymphocytes and natural killer cells in a dose-dependent manner. Moreover, Haskell et al. (1987) demonstrated that RA added to chick dorsal root ganglia (DRGs) in vitro resulted in an insulin-dependent enhancement of neurite extension and a significant increase in the number of nerve growth factor (NGF) receptors on neuroblastoma cells. RA has also been shown to modify synthesis of cell surface glycoproteins (Bonanni et al., 1973); to regulate cell growth, differentiation, and/or immune function (Lotan et al., 1980); and to stimulate neurite outgrowth and to inhibit cell proliferation in cultured human neuroblastoma cells (Sidell, 1982). Although some of these effects have been recognized for almost half a century, the exact mechanism of action of RA remains unknown.

Retinoic acid is ingested in the form of vitamin A (7000 IU or 2.1 mg/d) (Diem and Lentner, 1981) or as its precursor, beta-carotene; however, RA is not stored in the body (Nolen, 1986). Unlike alternative metabolites of vitamin A, such as retinol, RA does not have an exclusive carrier protein for transport to the cell, forming instead a complex with serum albumin (Smith et al., 1973). Cellular retinoic acid binding protein (CRABP) binds intracellular RA acting as a transport receptor and is thought to shuttle RA to nuclear compartments (Shubeita et al., 1987). More recently, two cDNA sites encoding a protein that binds RA have been cloned (Petkovich et al., 1987; Brand et al., 1988). Upon entering the nucleus, RA may combine with putative chromatin receptor sites (Petkovich et al., 1987; Brand et al., 1988) and may act to modify transcription. In fact it has been proposed that RA is a morphogen (Slack, 1987) due to its ability to induce differentiation programs during development (Thaller and Eichele, 1987; Hardy, 1989) and in melanoma cells (Niles and Loewy, 1989). Although retinoids occur at relatively low concentrations in some brain areas, compared to their concentration in liver or cells of the visual system (Phillips et al., 1989), little evidence is available regarding effects of RA on differentiated cells of the nervous system.

The experiments described here were undertaken to test the hypothesis that RA at physiologic (serum) concentrations will alter rates of neuronal regeneration of both peripheral nervous system (PNS) neurons and central nervous system (CNS) neurons of mammals, in vitro. Specifically, rates of neurite extension were measured on explants from neonate murine DRGs and fetal spinal cords, as well as on fetal human spinal cord explants.

MATERIALS AND METHODS

Tissue

Dorsal root ganglia cultures were derived from neonate CD-1 mice, of either sex, aged 1 to 3 d. Fetal spinal cord explants were derived either from mice of 16 to 17 days gestation or from human

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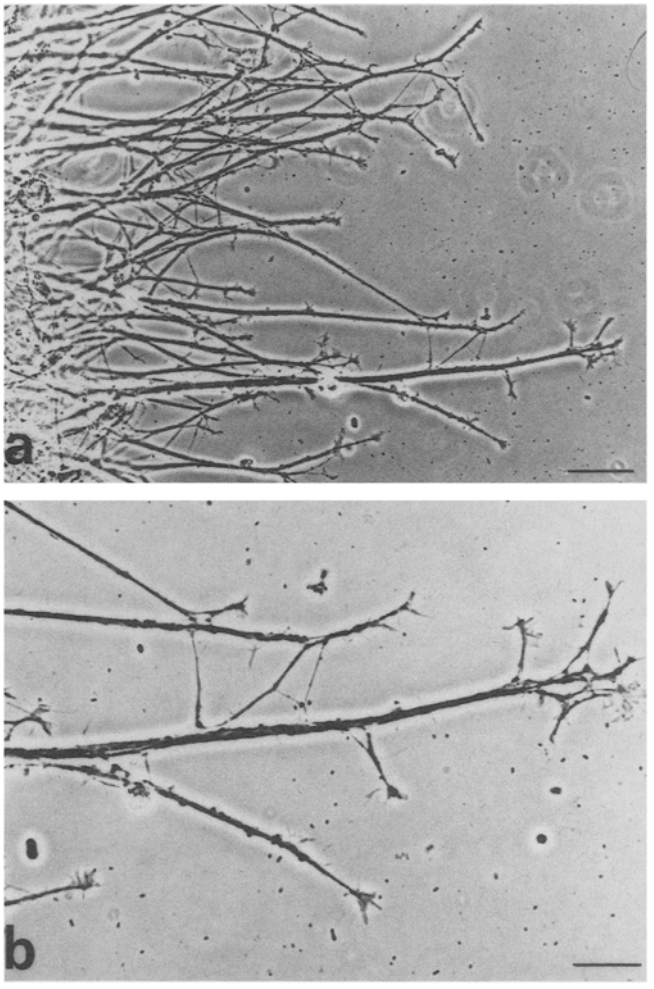

FIG. 1. Photomicrographs of edge of representative DRG explant, as digitized. *Note* resolution of individual growth cones especially evident in (*b*), fasciculation of neurites near explant and absence of non-neuronal cells. Magnification: *A*, *bar* = 100 μm; *B*, *bar* = 50 μm.

fetuses, obtained following therapeutic abortions at 6 to 9 wk gestation. Aseptic procedures were employed at all steps.

In general, procedures for maintaining neurons in vitro were identical with methods previously employed (De Boni and Crapper McLachlan, 1985; De Boni and Mintz, 1986; Fung and De Boni, 1988). It is to be noted that cultures were routinely maintained in an atmosphere of humidified air, without CO_2, for reasons given below.

Dorsal root ganglionic neurons. DRGs, usually 25 per mouse, were excised with the use of a dissecting microscope from their invertebral fossae and placed into calcium- and magnesium-free Hanks' balanced salt solution (HBSS) at room temperature. They were pelleted by centrifugation (1500 rpm, 2 min), through 5 ml HBSS. The supernatant was discarded and 2 ml of trypsin (0.25%, GIBCO, Grand Island, NY, 610–5050), prewarmed to 37° C, was added and the tissue incubated at 37° C for 20 min. Trypsin was inactivated by 1 ml fetal bovine serum (FBS), and the tissue aggregated by centrifugation (1500 rpm, 2 min). Supernatant was discarded, the aggregate suspended in complete culture medium (*see* below), and the now softened tissue mechanically divided into fragments which were placed into "Bionique" culture chambers (*see* below). These chambers were previously prepared with a collagen substrate and a small amount of medium. To permit contact and attachment of tissue to the substrate, only a maximum of 0.5 ml medium was present in the chambers at this time, with four to five explants placed into each chamber. The number of chambers employed at each concentration ranged from 2 to 19 for controls and from 2 to 24 for RA-treated cultures.

For an initial 24-h period, all chambers received identical control medium, containing the alcohol vehicle only, while the remainder received medium supplemented with RA diluted with culture medium from a RA stock solution in 100% ethanol (*see* below). Cultures were fed with 1 ml of the appropriate medium every 48 h thereafter.

Murine and human spinal cord. Spinal cord (SC) fragments were derived from fetal mice and human fetuses of gestational ages 16 to 17 wk and 6 to 9 wk, respectively. SCs, with meninges removed, were transversely cut with a razor blade into slices measuring approximately 0.5 × 0.5 × 0.5 mm. They were transferred to previously prepared culture chambers containing a minimum of culture medium. Upon attachment to the substrate, occurring within the initial 24 h, 1 ml of appropriate culture medium was added.

Preparation of culture chambers. To permit accurate quantification of neurite growth at high resolution, "Bionique" culture chambers (Bionique Laboratories) were used. These chambers are of the Rose chamber design, consisting of a Teflon body with a 25-mm diameter well; 45 × 50 mm no. 1 glass cover slips are held in place by an aluminum base plate and inert rubber O-ring, a complex that forms the base of the chamber. The tops consist of standard 35-mm diameter lids from Corning plastic culture dishes (no. 25000).

After steam sterilization of assembled chambers (without plastic lid), the glass substrate was prepared to either accept DRGs or SC fragments. Poly-D-lysine was used for DRG explants, whereas rat-tail collagen was used for SC fragments.

A stock solution of poly-D-lysine (Sigma, St. Louis, MO, molecular weight = 22 000) was prepared by dissolving 10 mg/ml in minimum essential medium (MEM) with Hanks' salts (GIBCO, 230–1575). This solution was filter sterilized (0.22 μm), diluted to a final concentration of 100 μg/ml with MEM, and stored as 0.5-ml aliquots at −20° C. The glass cover slip forming the chamber bottom was exposed to a small volume of this solution for 1 h at ambient temperature. Subsequently, excess solution was quantitatively aspirated and the chambers washed by two changes of culture medium.

Rat-tail collagen (Sigma, type VII) was prepared by dissolving 50 mg in 100 ml of 1:1000 glacial acetic acid, filter sterilized (0.22 μm), and stored at 4° C. For use, 0.5 ml of this solution was transferred to the culture chambers and permitted to dry onto the glass cover slips by exposure to the airflow in a laminar flow hood, usually overnight.

Culture medium. It is important to note that Hanks' salts, rather than the more common Earles' salts, formed the buffer system in the medium employed in this study. This system was chosen to circumvent the use of CO_2, which minimizes changes in pH dur-

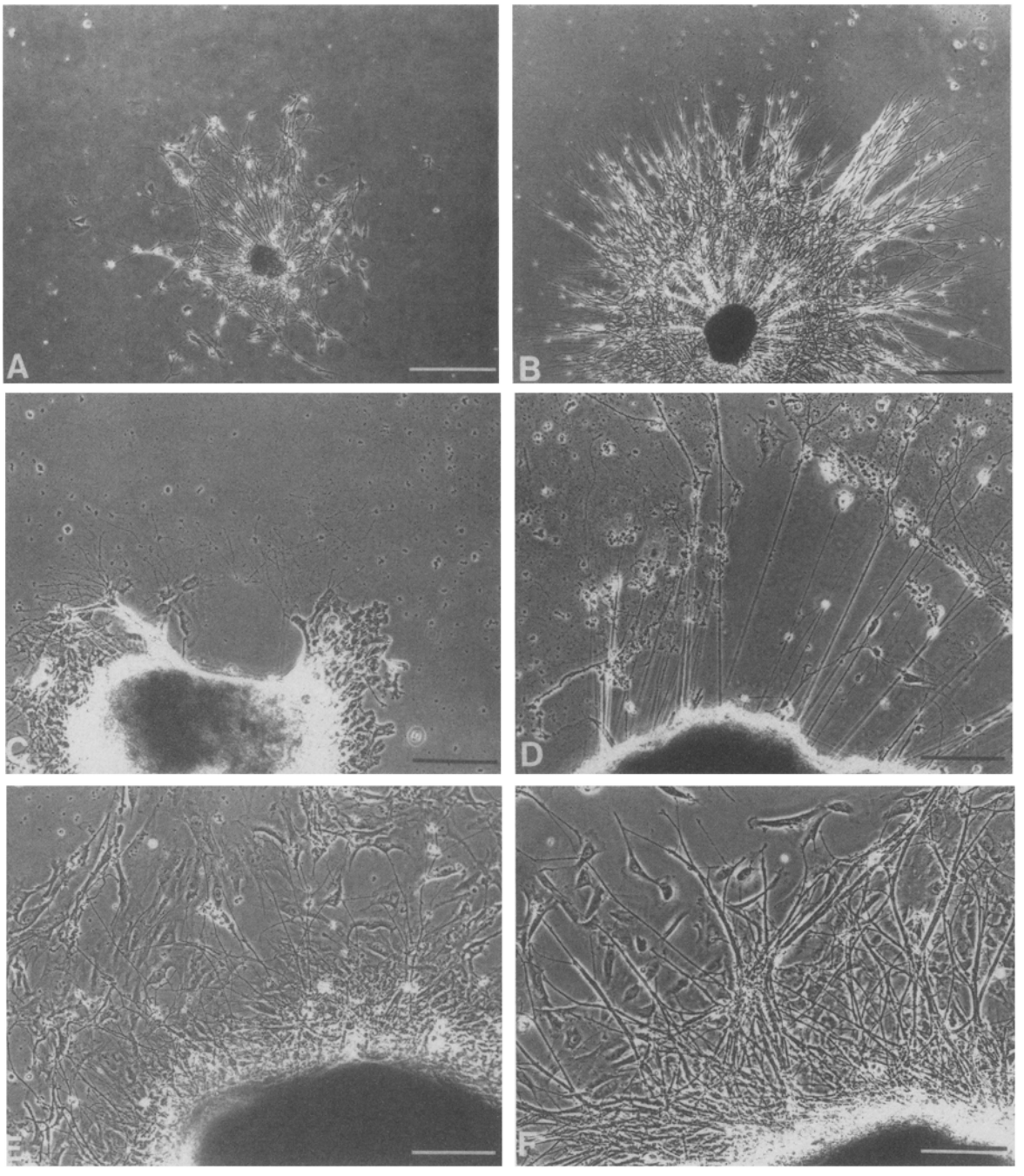

FIG. 2. Photomicrographs of murine dorsal root ganglia (*A,B*), murine spinal cord (*C,D*), and human spinal cord explants (*E,F*). *A, C, E* controls. *B*, murine DRG, 6 d, 10 μM RA. *D*, murine SC 3 d, 0.01 μM RA. *F*, human SC 5 d, 0.1 μM RA. *Note* dramatic effect of RA on neurite length. Magnification: *A,B: Bar* = 50 μm; *C–F: Bar* = 200 μm.

ing frequent transfers to the heated incubator stage of an inverted Nikon microscope, for photographic recording of neurite growth.

Culture media for PNS and CNS tissue consisted of 90% MEM, 10% FBS, 100 ng/ml 7-S NGF (Collaborative Research, Waltham, MA) and glucose, raised by use of an aqueous concentrate to 600 mg/100 ml. To inhibit proliferation of non-neuronal cells, potentially obscuring growth of very fine neurites, cytosine-*1*-beta-D-arabinofuranoside (Sigma), was added to the culture medium at a final concentration of 10 μM/L.

Preparation of RA solution. A 40-m*M* all-trans RA (Sigma, R2625) stock solution was prepared by dissolving 24 mg in 2 ml of 100% ethanol. To prevent isomerization the concentrate was stored in light proof aliquots, at −20° C for no more than 2 wk. Just before preparing culture medium, RA stock was thawed and appropriately

TABLE 1

MEAN NEURITE LENGTH (μm), MURINE DRG, AS A FUNCTION OF RA CONCENTRATION, OVER 11 D OF EXPOSURE (MEAN ± SD)

	Days of Exposure									
	1	2	3	4	5	6	7	8	9	11
Control	—	—	—	—	—	1067.3 ± 125.8 (121)	—	—	1207.6 ± 172.9 (118)	1355.3 ± 122.9 (114)
100 μM	—	—	—	—	—	1183.1 ± 231.4 (231)	—	—	1327.1 ± 327.6 (230)[a]	1474.7 ± 303.2 (226)[a]
Differential percent						+10.8			+9.9	+8.8
Control	406.7 ± 51.5 (89)	884.2 ± 224.5 (157)	—	953.8 ± 125.7 (48)	948.4 ± 212.2 (386)	1059.6 ± 277.2 (586)	—	1034.6 ± 244.5 (138)	1207.6 ± 172.9 (118)	1157.4 ± 308.2 (106)
10 μM	548.9 ± 144.8 (93)[a]	1044.2 ± 466.8 (216)[a]	—	1235.9 ± 156.5 (90)[a]	1120.4 ± 192.8 (438)[a]	1346.4 ± 312.5 (841)[a]	—	1526.1 ± 67.2 (181)[a]	1598.3 ± 340.4 (46)[a]	1598.3 ± 275.7 (148)[a]
Differential percent	+34.9	+18.1		+29.6	+18.1	+27.1		+47.5	+32.4	+38.1
Control	—	—	837.6 ± 134.3 (136)	803.6 ± 154.4 (39)	1016.3 ± 281.3 (201)	968.2 ± 32.2 (139)	1004.3 ± 270.2 (158)	1300.0 ± 206.9 (58)	1247.6 ± 194.7 (114)	—
1.0 μM	—	—	947.1 ± 72.4 (159)[a]	933.2 ± 60.0 (33)[a]	972.4 ± 218.9 (139) NS	1052.9± 138.9 (104)[a]	937.9 ± 143.0 (74)[b]	1701.9 ± 309.4 (51)[a]	1290.2 ± 4.5 (56)[a]	—
Differential percent			+31.1	+16.1	−4.3	+8.7	−6.6	+30.9	−9.6	
Control	—	—	897.7 ± 165.8 (81)	891.6 ± 107.4 (128)	1022.9 ± 307.5 (67)	844.8 ± 169.1 (114)	—	—	1427.6 ± 194.7 (114)	—
0.1 μM	—	—	1037.6 ± 85.9 (84)[a]	958.2 ± 176.2 (330)[a]	946.7 ± 255.8 (23) NS	1208.1 ± 270.6 (298)[a]	—	—	1620.6 ± 375.2 (142)[a]	—
Differential percent			+15.6	+7.5	−7.4	+43.0			+13.5	
Control	625.1 ± 216.3 (42)	765.5 ± 279.5 (123)	833.8 ± 174.2 (170)	950.8 ± 175.6 (176)	—	—	—	—	—	—
0.01 μM	713.9 ± 12.1 (27)[b]	773.8 ± 235.8 (110) NS	948.7 ± 274.2 (196)[a]	993.3 ± 261.7 (184) NS	—	—	—	—	—	—
Differential percent	+14.2	+1.1	+13.8	+4.5						
Control	625.1 ± 216.3 (42)	765.5 ± 279.5 (123)	890.1 ± 245.6 (115)	1097.9 ± 196.7 (137)	—	—	—	—	—	—
0.001 μM	655.8 ± 171.9 (68) NS	794.5 ± 136.7 (169) NS	950.7 ± 155.8 (210)[b]	1214.8 ± 171.1 (227)[a]	—	—	—	—	—	—
Differential percent	+4.9	+3.8	+6.8	+10.6						

[a] $P < 0.001$; [b] $P < 0.05$. NS = not significant; numbers in parentheses are neurites measured.

diluted with culture medium to the final concentrations indicated in Results.

Recording and quantification of neurite growth. To monitor neurite extension, cultures were photographed, employing phase optics, at intervals of 24 to 48 h over a maximum of 12 d. Growth of DRGs was generally so extensive that even with the use of a 4× objective lens, several exposures were required to permit recording of all neurites radiating from explants. For recording of growth from spinal cord, a 10× objective lens was routinely employed.

For quantification of neurite growth, photographic negatives were projected onto paper and neurites and explants outlined and digitized. Neurites were identified by the presence at their growing tips of clearly defined growth cones (Fig. 1). Through use of the mitotic inhibitor cytosine-arabinoside (*see* above), growth cones and their parent neurites remained for the most part unobscured by processes from non-neuronal cells and were clearly resolved, despite the presence fasciculation (Fig. 1). Each growth cone was taken to represent a neurite, and its length measured back to the edge of the explant. A program, prepared in-house and directly linked to the digitizer pad, provided information on number and mean lengths of all neurites traced, as previously described (De Boni and Anderchek, 1986).

Results reported represent mean neurite length ± standard deviation unless otherwise stated. Statistical significance was determined by Student's *t* test.

Results

The results clearly indicate that RA, at final concentrations as low as 1 n*M*, significantly enhances neurite regeneration of neonate mouse DRG neurons and of fetal mouse and fetal human spinal cord neurons in vitro (Fig. 2). It is noteworthy that RA acts by increasing mean neurite length and not by increasing the number of neurites per explant. Measurements showed that no correlation exists beween explant size and neurite length. Moreover, it was found that human and mouse CNS neurons, in contrast to DRG (PNS) neurons, exhibit increased regeneration at lower RA concentrations and that the RA-induced increase in neurite length of CNS neurons was significantly larger than that induced in cells of the PNS.

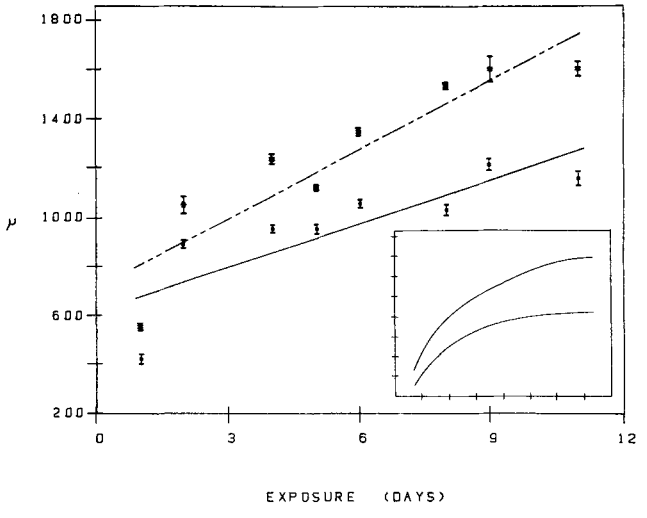

FIG. 3. Neurite growth, murine DRG. Regression analysis over 11 d exposure (mean length ± SEM). *Solid line*, control (1202 neurites, 66 explants). *Broken line*, 10 μ*M* RA (1424 neurites, 80 explants). *Inset* shows corresponding line plot of data. *Lower curve*; control, *upper*; RA.

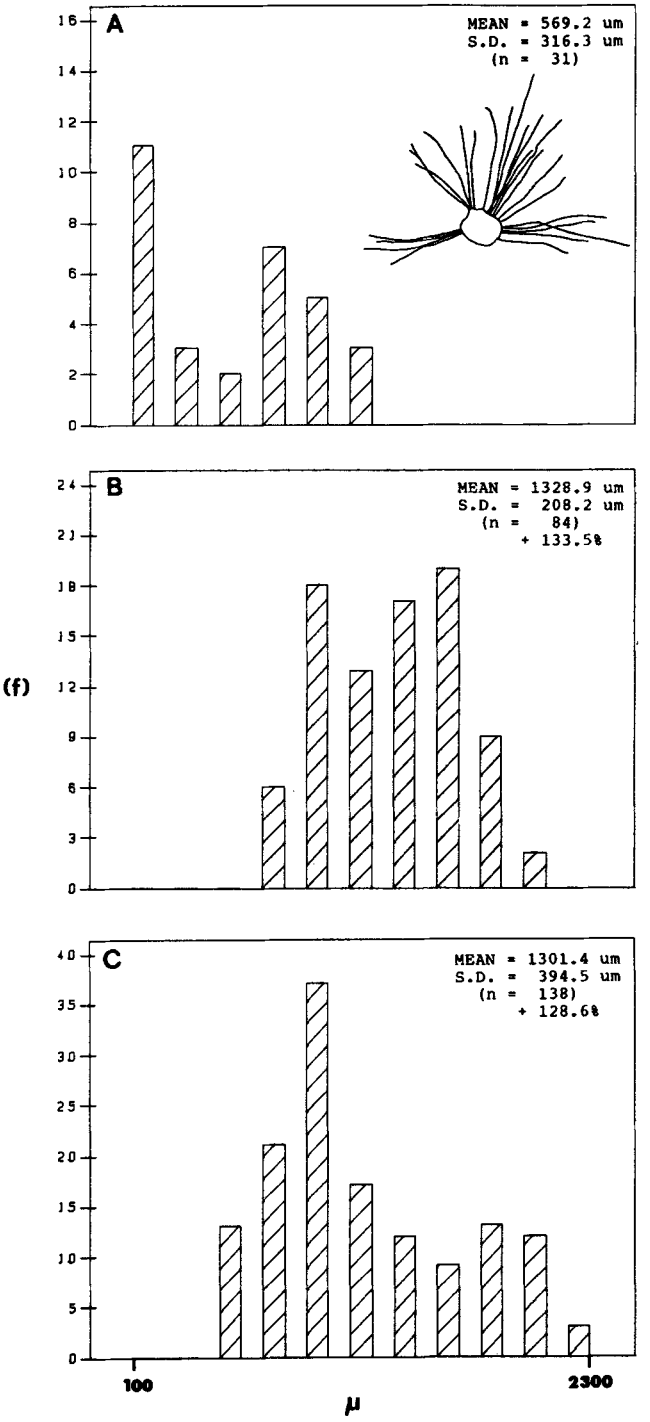

FIG. 4. Representative length distribution histograms of murine DRG at 6 d exposure. Neurite length ranges from 100 to 2300 μm, each bar representing a 200-μm bin. Vertical height represents frequency (*f*) of neurite length within bin. *A*, control; *B*, 10 μ*M* RA; *C*, 0.1 μ*M* RA. *Note* increase in mean neurite length of 133.5% (10 μ*M* RA) and 128.6% (0.1 μ*M* RA). *Inset* in *A* shows representative tracing of DRG explant as digitized.

TABLE 2

MEAN NEURITE LENGTH, MURINE SPINAL CORD, AS A FUNCTION OF RA CONCENTRATION[a]

[RA]	n	Length, μm	Change, %
Control	147	508.4 ± 168.1	—
1.0 μM	136	709.3 ± 313.3[b]	+39.5
0.01 μM	368	729.0 ± 208.3[b]	+43.4

[a] Three days exposure (mean ± SD); [b] $P < 0.001$.

TABLE 4

MEAN NEURITE LENGTH, MURINE SPINAL CORD, AS A FUNCTION OF EXPOSURE TIME[a]

Day	Control, μm	n	Treated, μm	n	Change, %
1	275.6 ± 112.3	311	364.8 ± 144.5[b]	265	+32.4
2	391.1 ± 84.3	90	389.3 ± 79.7	38	−0.5
4	952.8 ± 220.7	93	832.8 ± 35.3[c]	19	−12.6
5	847.9 ± 356.3	43	746.0 ± 108.9	33	−12.0

[a] 10 μM RA (Mean ± SD); [b] $P < 0.001$; [c] $P < 0.02$.

Dorsal root ganglionic neurons. Mouse DRG neurons were exposed to RA concentrations ranging from 100 μM to 1 nM, for periods of up to 11 d. Included in this study were 4541 individual neurites, emanating from 242 separate explants.

Of the concentrations employed, the effect was optimal at 10 μM RA, where a consistent and significant ($P < 0.001$) increase in mean neurite length was documented throughout all 11 d of exposure (Table 1). At 10 μM RA, the enhancement ranged from 18.1 to 47.5%, measuring 38.1% on Day 11 (Fig. 3), with an overall mean of 30.7% measured over all 11 d of exposure (Table 1). Although the response at concentrations higher and lower than 10 μM was less consistent and lower in magnitude, a significant response was evident for all concentrations (Table 1). Moreover, a line plot of data (Fig. 3, *inset*) indicates that the growth curves for controls and treated cultures exhibit similar profiles, although treated cultures respond with a steeper slope. Between Days 2 and 9 of RA exposure, neurites of treated cultures elongate at significantly higher rates ($P < 0.001$) than do those of controls (Table 1).

Mean length of neurites treated with 10 μM RA expressed a maximal 47.5% average increase over controls by Day 8 (Table 1). At this same concentration, enhancements of up to 133% were recorded for some explants (Fig. 4 *B*).

Representative length distribution histograms (Fig. 4) indicate that RA acts by significantly increasing the length of most neurites in the population rather than by acting on a subset of cells only. In the example shown (Fig. 4), controls display an average neurite length of 569.2 ± 316.3 μm compared to an average of 1328.9 ± 208.2 μm and 1301.4 ± 394.5 μm for 10 and 0.1 μM RA, respectively, representing increases of 133 and 128% over controls.

Fetal mouse spinal cord neurons. We employed 1264 neurites emanating from 114 explants to test the effects of RA on CNS tissue. Of these, 760 neurites (67 explants) were exposed to RA at 10.0, 1.0, and 0.01 μM. In contrast to DRG neurons, where an optimal response was induced at 10 μM RA (Table 1), this same concentration clearly inhibited neurite growth in murine CNS tissue at exposure times exceeding 24 h (Table 2). Specifically, data derived from 256 neurites (22 explants) showed an inhibition of up to 12.6% ($P < 0.02$) at 4 d.

In contrast, significant increases ($P < 0.001$) in mean neurite length were measured at 1.0 and 0.01 μM RA (Table 2); 1.0 μM RA induced an enhancement of 39.5% by Day 3 of exposure (136 neurites, 11 explants), whereas 0.01 μM RA (368 neurites, 34 explants) increased mean neurite length by 43.4%, after 3 d of exposure.

Fetal human spinal cord neurons. In this tissue, exposure to RA resulted in a very dramatic enhancement of regeneration (Table 3). A total of 111 neurites (20 explants) were analyzed. By Day 5 mean neurite length of controls measured 515.2 ± 186.9 μm whereas 10 and 0.1 μM RA induced a mean length of 728.3 ± 220.5 μm and 814.4 ± 41.3 μm, respectively, corresponding to average increases of 41.4 and 58.1%. RA concentrations of 10 and 0.1 μM not only resulted in significant increases in neurite length ($P < 0.001$) but also produced responses significantly different from each other ($P < 0.05$; Table 4), with the lower concentration resulting in a greater response.

TABLE 3

MEAN NEURITE LENGTH, HUMAN SPINAL CORD, 5 D EXPOSURE[a]

[RA]	n	Length, μm	Change, %
Control	54	515.2 ± 186.9	—
10.0 μM	27	728.3 ± 220.5[b]	+41.4
0.1 μM	30	814.4 ± 41.3[b]	+58.1

[a] Mean ± SD. Responses differ from each other ($P < 0.05$); [b] $P < 0.001$.

DISCUSSION

The results show that RA enhances rates of neuronal regeneration of both peripheral and CNS neurons of mammals in vitro. It is of potential clinical significance that this enhancement of neurite growth was induced at RA concentrations similar to those measured in normal serum. Specifically, human plasma has a RA concentration of 0.0163 μM, whereas rat serum has a concentration of 0.0067 μM (Napoli, 1986). The RA concentrations employed in the present study ranged from 0.001 to 100 μM.

Retinoic acid increased the mean length of neurites rather than neurite number, as has been previously documented for chick DRG using RA (Haskell et al., 1987) and for other neuronotrophic substances, including galactose, *N*-acetyl-glucosamine, and sialic acid (Liu et al., 1988). The observation (Fig. 4) that there exists a general, dose-dependent shift of the entire neurite population to increased mean neurite length indicates that RA has an affect on the majority of neurites and not a subpopulation thereof. This observation of increased neurite length but not neurite number excludes the possibility of enhancement of neuronal sprouting. Although DRG neurons in situ are nearly always bipolar, with only 7.4% displaying multipolar morphology (Parfianowicz et al., 1971), these same neu-

rons in vitro clearly have the capacity to become multipolar, an effect not evident from data presented herein.

Haskell et al. (1987) reported that neurite growth of chick DRG in vitro responded optimally at 1 to 0.1 μM RA, although this response was insulin-dependent. These results, nevertheless, compare favorably with the present study, wherein murine DRG respond optimally at 10 μM in the absence of insulin (Table 1).

Regression analysis (Fig. 3) illustrates that neurite lengths are consistently larger for RA-treated cells throughout the 11 d of exposure. It is noteworthy, moreover, that after the first 24 h of exposure mean neurite length of treated cultures already exhibits an initial enhancement of 34.9% over controls (Table 1). This indicates that RA acts within the very early stages of in vitro regeneration.

Contrary to observations on DRG neurons, where 10 μM RA produced an optimal response, this same concentration inhibited mean neurite length in murine SC tissue (Table 4). This inhibition may reflect an increased sensitivity of CNS neurons to RA. It is noteworthy that for murine SC a change in RA concentration by only one order of magnitude results in a shift from an inhibition at 10 μM RA to a dramatic enhancement of neurite length at 1 μM (Tables 2 and 4). Moreover, a further reduction in concentration to 0.1 μM does not decrease the dramatic enhancement recorded (Table 4). Comparison of the effects obtained at these three concentrations suggests that CNS tissue exhibits a greater sensitivity to RA, compared to neurons of the PNS.

Neurite growth of human CNS tissue exposed to 10 μM RA responded with a 41.4% ($P < 0.001$) enhancement over controls (Table 3), in contrast to murine CNS tissue where this concentration resulted in an inhibition. However, neurons of human SC, like murine SC, responded maximally at lower RA concentrations (Table 3), with the lowest concentration applied to human SC (0.1 μM), resulting in a maximal response.

The mechanisms by which RA enhances neurite growth remain obscure. It has been postulated that RA acts in a similar fashion to steroid hormones, binding directly to nuclear targets and altering gene expression (Green and Chambon, 1986). In support of this argument, more than 20 specific proteins inducible by RA have been identified (Strickland and Mahdavi, 1978; Chytil, 1984). Moreover, retinol, a metabolite of vitamin A, has been shown to bind to chromatin (Ferrari and Vidali, 1985), probably by interaction with specific chromatin acceptor sites. It is thus possible that the enhancement of neurite growth observed is the direct result of such interactions between RA and nuclear loci. Alternatively, as suggested by Haskell et al. (1987), RA may enhance regeneration indirectly by increasing the number of NGF receptors and resulting in an increase over the normal response to NGF present in the medium. It must be considered that the enhanced regeneration observed is the result of RA-induced changes in properties of the non-neuronal cells, which form the layer over which neurites usually elongate in vitro. In view, however, of significant RA-induced increases in mean neurite lengths, despite an obvious absence of such cells, induced by the cytostatic agent cytosine arabinoside, this is unlikely.

Although the present data do not permit definition of the mechanisms of the observed responses, it may be concluded, based on available evidence, that the enhancement of neurite growth described herein may be the result of RA-induced changes in mechanisms which govern differentiation and gene expression. This study represents a fist step designed to define the RA concentration required to enhance neuronal regeneration of mammalian neurons in vitro. In light of the observations presented and those referred to above, RA may be considered to play a potentially critical role in the process of neuronal regeneration.

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The authors acknowledge support by the Canadian Paraplegic Association and the Rick Hansen Man in Motion Legacy Fund.