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Retinoic Acid Enhances Neuronal Proliferation and Astroglial Differentiation in Cultures of CNS Stem Cell-Derived Precursors

by

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ABSTRACT

Stem cells isolated from the embryonic day 14 mouse striatum proliferate in response to epidermal growth factor (EGF). These stem cells produce clonally-derived clusters of undifferentiated cells (spheres) which, in turn, produce neurons, astrocytes and oligodendrocytes. Reverse transcription followed by polymerase chain reaction showed that EGF-generated spheres express several of the retinoid receptors. Thus, I asked whether all-*trans* retinoic acid (ATRA) could regulate the differentiation or proliferation of central nervous system stem cell-derived progeny or act on stem cells themselves. My results demonstrate that ATRA has three distinct effects on CNS stem cells and their related progeny: 1) ATRA promotes the survival of a dividing population of neuronal precursors isolated from cultures of dissociated spheres; 2) ATRA attenuates proliferation of dividing precursors while promoting astrocyte differentiation; 3) ATRA can direct stem cells to produce more neurons without apparent changes in expansion properties or multipotency. These results suggest that retinoids can regulate the proliferation of mitotically-active EGF-responsive stem cells and progeny to enhance neurogenesis and astrocyte differentiation.

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DEDICATION

To my parents and to Mike

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ABBREVIATIONS USED

ATRA	all-*trans* retinoic acid
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
DIV	days *in vitro*
DMSO	dimethyl sulphoxide
EGF	epidermal growth factor
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RARs	retinoic acid receptors
RT	reverse trascription
RXRs	retinoid X receptors
TGF-β	transforming growth factor beta

1 INTRODUCTION

Vitamin A (retinol), and its natural derivatives are required for a wide variety of physiological events, including embryonic development, maintenance of epidermal differentiation, testicular function, vision, and regulation of the proliferation and differentiation of many different cell types. Retinoids are potent teratogenic agents in both excess and deficiency. Adverse effects of excess retinoids on the nervous system have been demonstrated in *Xenopus* (Durston et al., 1989), mouse (Langman and Welch, 1967), and in human (Lammer et al., 1985). In the developing nervous system of *Xenopus* the administration of retinoic acid (RA) causes an anteroposterior transformation such that regions rostral to the midbrain are reduced or absent (Durston et al., 1989), and the segmentation pattern of homeobox gene expression is lost (Morris-Kay et al., 1991; Morris-Kay, 1993). In the human, maternal excess of RA (used as a treatment for severe cystic acne) causes a wide range of congenital malformations in addition to defects associated with the central nervous system (CNS): spina bifida, anancephaly, hydrocephallus, and eye malformation (Lammer et al., 1985). Although teratological experiments alone do not necessarily imply a role for endogenous RA during CNS formation, the presence of endogenous retinoids and their receptors in specific temporal and spatial patterns suggests that retinoids may play a functional role in the development of the nervous system.

1.1 General Mechanisms of Retinoid Action

1.1.1 Retinoid metabolism

The main sources of vitamin A in the diet are provitamin A (from carotenoids in vegetables) and retinyl esters (from animal tissues). Both are converted to retinol before interacting with long chain fatty acids within enterocytes to produce retinyl esters, which are then complexed to chylomicrons in liver and moved out into the general circulation (Blomhoff et al., 1990). Retinol is also present in the circulation bound to retinol binding protein (RBP) such that small amounts of free retinol in equilibrium with RBP-retinol complexes are found in plasma. The actual method of non-hepatic retinol uptake may involve any one of several processes, including specific RBP receptor-mediated retinol uptake, the non specific spontaneous transfer of retinol and retinoic acid, fluid phase endocytosis, and the uptake of chylomicron remnant retinyl esters (Blomhoff et al., 1990).

1.1.2 Retinoids

A number of retinoids in addition to retinol are present in plasma in nanomolar concentrations. These include all-trans retinoic acid, 13-cis retinoic acid, 13-cis-4-oxoretinoic acid (Eckhoff and Nau, 1990), and all trans retinoyl-b-glucuronide (Barua and Olson, 1986). Much of the recent focus on retinoid biology has been with regards to the effects of two specific retinoids: all-trans retinoic acid, and 9-cis retinoic acid. The metabolic pathway by which retinoic acid is synthesized in situ from retinol is poorly

understood. Although earlier studies suggested that retinol is metabolized via an alcohol dehydrogenase oxidation to retinoic acid, an alcohol dehydrogenase-negative strain of deermouse has been shown to synthesize retinoic acid (Posch et al., 1991).

1.1.3 Retinoid receptors and cellular binding proteins

The ultimate cellular interpretation of retinoid signals is determined by a number of factors, including the presence of cytosolic retinoid binding proteins, and specific nuclear receptor subtypes. There are two major classes of cellular retinoid binding proteins: the cellular retinol-binding proteins, or CRBPs, (I and II; Blomhoff et al., 1990; Giguere et al., 1990); and the cellular retinoic acid-binding proteins, or CRABPs (I and II). The CRBPs have been implicated in the storage and metabolism of retinol. CRBPI has been suggested to regulate the formation of retinyl esters from retinol, perhaps via lecithcin:acetyltransferase esterification (Blomhoff et al., 1990) and their subsequent conversion to retinoic acid. CRBPI expression may therefore indicate cells in which RA is synthesized. CRBPI localization in embryonic ventral spinal cord, where high levels of RA are detected, supports this theory.

CRABPs appear to exert a complimentary function: there is strong evidence to suggest that CRABPI serves to bind and retain cytoplasmic RA as well as to promote RA breakdown to polar metabolites (Boylan and Gudas, 1991; Napoli et al., 1991). CRABPI would therefore decrease the amount of RA available for binding by nuclear receptors. In the case of RA excess, saturation of the binding and metabolic activities of CRABPI would enable RA to reach the nuclear receptors and induce an abnormal program of gene

expression (Ruberte et al., 1993). The importance of CRABPI as a retinoid buffer is highlighted by its expression in tissues such as the neural tube and neural crest which are selectively vulnerable to excess retinoids (Dolle et al., 1990; Ruberte et al., 1991). The function of CRABPII is less clear; CRABPII may act as a further buffer when CRABPI binding is saturated (Bailey and Siu, 1988).

Many actions of the retinoids are mediated through high affinity nuclear retinoic acid receptors (RARs) and retinoid 'X' receptors (RXRs) which are members of the steroid/thyroid hormone receptor superfamily (Giguere et al., 1987; Petkovich et al., 1987; Mangelsdorf et al., 1990). The classification of RAR and RXR subfamilies is mainly based on differences in primary structure, sensitivity to synthetic and natural retinoid ligands, and their ability to regulate different target genes (Heyman et al, 1992). Both families have been divided into three subtypes, α, β, and γ, and within each subtype there are splice variants that give rise to multiple isoforms. In situ hybridization studies of mouse embryos show specific spatiotemporal patterns of expression during development and suggest that each receptor isoform may have a distinct set of target genes (Pfahl, 1994).

RA nuclear receptor function is further complicated by the fact that RARs and RXRs can bind with different affinities to specific endogenous retinoids. RARs can be efficiently activated by either ATRA or 9-cis RA at nanomolar concentrations of either ligand; RXRs, however, are only activated by 9-cis RA in this concentration range. Transfection studies which indicate that ATRA can activate RXRs have been explained

by the prohormone role of retinoic acid as a precursor for 9-cis RA; that is to say, that RXR activation in response to (apparently) ATRA is probably due to intracellular conversion of ATRA to 9-cis RA.

Both the existence of two ligand pathways and the multiplicity of RAR and RXR receptors may contribute to the pleiotropic effects of retinoids. However, additional complexity is introduced by the fact that steroid receptors bind as dimers to repeat half sites in DNA sequence elements with specific affinities and allosteric orientations. RXR/RAR heterodimers and RXR/RXR homodimers bind to direct repeats spaced by different numbers of nucleotides. The net result is that RAR in the form of a heterodimer can act as an activator of RAR or RXR induced transcription, or as an inhibitor of RXR-dependent transcription. In this respect, RAR-mediated transcriptional activation or inhibition depends on the nature of direct repeat spacing in any given promoter, and the relative numbers of RAR or RXR receptors present (Kurokawa et al.,1994).

Aside from their ability to dimerize with RARs, RXRs have been shown to heterodimerize with other members of the steroid family. In these cases, RXR promotes the binding of these nuclear proteins to their cognate response elements, including the thyroid hormone receptor (Kliewer et al., 1992), the vitamin D3 receptor (Zhang et al., 1992), the chicken ovalbumin upstream promoter (Bugge et al., 1992), peroxisome proliferator activated receptor (Parker,1993), and the AP-1 transcription factor (Schule et al., 1990a; 1990b).

1.2 Retinoids in the CNS

1.2.1 Endogenous retinoids are present in the CNS

Support for the presence of retinoids in the developing CNS comes from several lines of evidence. It is clear that neural tissue can convert retinol to more active biological metabolites. For instance, the neural tube can synthesize RA and 3,4-dihydroretinoic acid when incubated *in vitro* with retinol (Wagner et al., 1990). Cultured cells have this capacity as well; astrocytes and Muller glial cells (but not neurons) can synthesize RA from retinol (Wuarin et al., 1991; Barres et al., 1994). Other studies using grafting techniques suggest that the embryonic CNS contains retinoids. Henson's node in the chick embryo (Hornbruch and Wolpert, 1986) and the ventral floor plate of the rat neural tube (Wagner et al., 1990) produce retinoid-like homeotic transformations when grafted to anterior chick limb bud. Additional evidence for the presence of retinoids comes from studies of transgenic mice. For example, transgenics produced by the introduction of a vector consisting of the RAR-β RARE upstream of a minimal promoter-reporter (hsp68lacZ) gene show reporter being expressed in a specific neural tube AP domain in the embryo following the onset of neurulation (Rossant et. al., 1991). Similar studies by Zimmer and Zimmer (1992) implicate RA in neuromeric organization of not only more posterior structures (such as hindbrain and spinal cord), but mesencephalon and prosencephalon. Although reporter expression in these animals cannot unequivocally be attributed to the action of RA, no other known ligand-activator complex will stimulate

transcription via the RARE (de The et al., 1990; see also discussion in Durston et al., 1991).

1.2.2 Retinoid nuclear receptors and cytoplasmic binding proteins are found in distinct spatiotemporal patterns in the CNS

1.2.2.1 RXRs in the CNS

Using in situ hybridization, Ruberte and colleagues (1993) have shown that two of the three RXR subtypes are expressed in the embryonic and adult nervous system. RXRα is not expressed in the CNS, and is mainly localized to gut, skin, and liver. RXRβ mRNA is found in almost all tissues, with increased expression in the spinal cord and hindbrain. RXRγ has the most restricted distribution. Although weak RXRγ expression can be detected in skeletal muscle, intense hybridization signals in the corpus striatum are noted by E13.5 and in the pituitary anlage, as early as E11. Additional RXRγ expression is observed at these early stages (E10-E13) in the somites and ventral horns of the spinal cord. Ventral spinal cord and striatal RXRγ transcripts are co-localized with RARβ transcripts, suggesting a possible preferential heterodimerization between RXRγ and RARβ in these regions (Dolle et al., 1994).

1.2.2.2 RARs in the CNS

Of the three RAR subtypes, only RARs α and β (but not γ) are expressed in the

developing nervous system. Using in situ hybridization, Ruberte and colleagues (1993) have reported that RARα transcripts are most abundant in the proliferating neuroepithelium (rather than the differentiating mantle layer of neural tube) of the spinal cord, as well as in the forebrain, where they are most abundant in the developing corpus striatum. Like RARα in the early spinal cord, RARβ transcripts are also found in the proliferating neuroepithelial layer, but with the onset of differentiation of motor neurons, intense expression of RARβ was found associated with developing motor columns. The rostral boundary of RARβ expression extends into the columns of general somatic efferent neurons of the caudal medulla oblongata, and is maintained until the formation of motor columns. According to Ruberte et al. (1993), RARβ expression suggests a functional association between RARβ and organization of the CNS in relation to innervation of somite derivatives. In addition to expression in general and somatic motor nuclei of the medulla between E12.5 and E13.5, RARβ transcripts are found by E12.5 in the corpus striatum and olfactory tubercle, and persist through to E18.5.

1.2.2.3 CRBPS and CRABPS in the CNS

CRBPI localizes to an extremely precise region of the developing spinal cord - the ventral floor plate, which consists of radial glia. CRABPI transcripts are found on individual neuroepithelial cells in the mantle layer of the neural tube which subsequently become commissural neurons (Maden and Holder, 1991). The CRBP/CRABP distribution in the neural tube, in combination with floor plate induction of polarizing

activity in the limb bud grafting bioassay, suggests that RA may be a chemoattractant (secreted by the floor plate) which acts as a commissural axon guide. An analogous distribution is found during the formation of the olfactory system, where both differentiation and neurite guidance roles have been suggested for retinoids (LaMantia et al., 1993).

1.3 *In vitro* manipulations of several cell types support a role for RA as a differentiation factor and cell fate modifier

Retinoids have been shown to have actions on proliferation and differentiation of various cell types. For instance, RA will induce growth arrest and neurite formation in cultured neuroblastoma cells at micromolar concentrations (Sidell, 1982). Although keratinocytes (Gudas et al., 1994) will proliferate in response to retinoid treatment, retinoids are particularly well known for their inhibitory actions on proliferation and ability to enhance differentiation. Embryonal carcinoma cells also respond to RA, however the cell type produced by differentiated embryonal carcinoma cells is RA-concentration dependent: at nanomolar concentrations, cardiac and skeletal muscle form, and at micromolar concentrations, neurons and glia are formed (Edwards and McBurney, 1983). Additionally, RA has been shown to promote neurite outgrowth and branching in dissociated cultures of embryonic rat spinal cord (Wuarin et al., 1991) and in explants of Xenopus (Hunter et al., 1991), mouse and human spinal cord (Quinn et al., 1991). Aside from neurite promotion, RA treatment has been reported to selectively promote the survival or differentiation of neurons from several sources. In dissociated rat spinal cord

cultures, RA treatment increases overall neuron numbers, but the effects are selective to cholinergic and met-enkephalinergic neurons with no effect on GABAergic neurons (Wuarin and Sidell, 1991). Increases in neuron numbers have also been noted in studies of tissue derived from neural tube. A dramatic increase in the proportion of neurons produced by cultured neural crest cell populations is found in RA treated cultures (Henion and Weston, 1994).

More recent examples of reported retinoid effects include the proposed role for retinoic acid regulating the counting mechanisms of oligodendrocyte precursor divisions (Barres et al., 1994), and the promotion of rod photoreceptor differentiation as a result of progenitor cell fate switch (Kelly et al., 1994). In the retina, newly generated cells which would otherwise have differentiated into amacrine cells, are induced by RA to form photoreceptor cells instead (Kelly et al., 1994). A similar fate determination role for RA has been proposed in the blood where lymphohaemopoietic stem cell production of the granulocyte/monocyte lineage is switched to the monocyte lineage when retinoid signaling is altered (Göttgens and Green, 1995). These studies show that retinoids may act at a multitude of levels during cell genesis from primitive precursors – from actions on proliferation to a role in fate determination and maturation.

1.4 Identification of a central nervous system stem cell and manipulation of the stem cell *in vitro*.

In the mouse striatum, neurogenesis begins at embryonic day 12 (E12) and continues until the early post-natal period; gliogenesis begins after the onset of

neurogenesis and peaks during the first post-natal week (Fentress et al., 1981). Our laboratory has identified a primitive cell, isolated from the embryonic mouse striatum, that proliferates in response to epidermal growth factor (EGF) to form a clonally-derived cluster (sphere) of undifferentiated cells. When plated onto an adhesive substrate, spheres will generate the three major cell types of the mature CNS - neurons, astrocytes, and oligodendrocytes (Reynolds et al., 1992; Reynolds and Weiss, 1996). Further studies revealed that this proliferating EGF-responsive cell, by virtue of its multipotency and self-renewal/expansion *in vitro* is a stem cell (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992; Weiss et al., 1996). *In vivo*, EGF-responsive cells participate in the repopulation of the adult subependyma and can be induced to generate new neurons in the striatum (Morshead et al., 1994; Craig et al., 1996).

The *in vitro* culture of multipotent CNS (striatal) stem cells permits the investigation of factors that may influence their proliferation and differentiation. With respect to stem cell proliferation, Bjornson (1997) showed that a member of the bone morphogenetic protein family (BMP-2) was able to inhibit the proliferation of CNS stem cells cultured in the presence of EGF. BMP-2 was shown to have a second action restricted to stem cell-derived progeny, resulting in enhanced differentiation of post-mitotic neuronal precursors. Several studies have shown that neurogenesis from stem cell-derived progeny can be modified in the presence of other growth factors. For example, Vescovi et al. (1993) showed that bFGF can regulate the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. In a separate study, Ahmed et al. (1995) showed that brain-derived neurotrophic

factor (BDNF) can enhance the differentiation and morphological maturation of neurons from EGF-responsive stem cell progeny. The study by Ahmed et al. (1995) was complimented by a more recent study (Arsenijevic and Weiss, 1998) showing that insulin-like growth factor-I can also act as a differentiation factor for postmitotic CNS stem cell-derived neuronal precursors, but that its actions were distinct from those of BDNF. These studies provide evidence that various factors can influence the decision of a stem cell to divide, as well as alter the fate of stem cell-derived progeny.

Given the results of previous *in vitro* studies suggesting that ATRA can influence neural cell fate, and the presence *in vivo* of both EGF molecules (Lazar and Blum, 1992) and retinoids (LaMantia et al., 1993) in the developing mouse forebrain, I asked whether ATRA could affect the proliferation and/or differentiation of EGF-generated stem cells or stem cell-derived progeny *in vitro*.

1.5 Statement of hypothesis

Retinoids regulate the proliferation and/or differentiation of EGF-responsive embryonic murine CNS stem cells and progeny.

1.6 Specific questions

Specific Question 1: Do retinoids modulate neurogenesis in cultures of EGF-generated stem cell progeny?

To determine if ATRA can alter the production of neurons in cultures of EGF-

responsive stem cell-derived progeny, spheres were dissociated and plated onto poly-ornithine coated coverslips. The cells were cultured in the absence or presence of ATRA under conditions which promote the differentiation of stem cell progeny (1% fetal bovine serum; FBS). The numbers of neurons generated under these conditions was assessed after 7 DIV using indirect immunocytochemistry and cell-type specific antibodies. Delayed additions of ATRA were used in order to understand the time dependency of its actions. BrdU incorporation was also used as a tool to further investigate the mechanism of ATRA actions.

Specific Question 2: Do retinoids affect the proliferation of stem cell progeny?

Retinoids are well known for their ability to affect the proliferation of multiple cell types (Gudas et al., 1994). Single cell suspensions of dissociated spheres were plated onto poly-ornithine coated glass coverslips in the presence of EGF. Cells were left to proliferate for 7 DIV. After fixation and immunocytochemistry, total cell numbers as well as the numbers of mature cell types were observed in order to ascertain whether ATRA affects progenetor cell production under conditions which normally promote proliferation of EGF-responsive stem cells and stem cell progeny.

Specific Question 3: Can retinoids alter stem cell expansion/renewal or the types of cells it produces?

To answer this question, EGF-generated spheres were dissociated to form a single cell suspension. Cells were then plated at a clonal density (150 cells/mL in 35mm plates) in the presence of EGF. Under these conditions, it is reasonable to assume that each clone is produced by a single stem cell or mitotically active precursor (Ito and Morita, 1995). Cells were exposed to ATRA or solvent (DMSO) only for the first 24 hours in culture, before the first stem cell division, and subsequently rinsed free of ATRA using warmed culture media. It is important to note that the rinsing process was very thorough. After removing all media containing ATRA or DMSO from the well, cells were rinsed twice with warmed culture media to ensure complete removal of ATRA and DMSO. Media containing EGF only was added to the cultures, and cells were left to proliferate for a further 13 DIV. Each cluster of cells which was observed at the end of the culture period is presumably a clonally related sphere, in other words was derived from a single cell.

Clones were subsequently used for either single sphere dissociation experiments, or for phenotype analysis. Single sphere dissociations allow the inspection of the clone with respect to how many times the stem cell renewed, or expanded - a characteristic feature of stem cells. Phenotype analysis of each clone using cell-type specific antibodies allows the investigation of what types of cells were produced by the original parent cell after exposure to ATRA or solvent.

2 MATERIALS AND METHODS

2.1 Primary cell culture and passaging of EGF-responsive embryonic striatal stem cells

Striata were removed from 14-day-old-CD1 albino mouse embryos (Charles River) and mechanically dissociated with a fire-narrowed Pasteur pipette in culture medium containing DMEM/F12 (1:1) (Gibco), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), and HEPES buffer, supplemented with insulin (25 μg/mL), transferrin (100 mg/mL), progesterone (20 nM), putrescine (60 mM) and selenium chloride (30 nM) (all from Sigma except glutamine; Gibco). Cells were plated at a density of $0.2x10^6$ cells/mL in Corning T75 (Gibco/BRL) culture flasks in the presence of 20 ng/mL EGF (purified from mouse submaxillary gland; Collaborative Research). The free-floating clusters of cells (spheres) that form are referred to as "primary" spheres. To passage spheres, 7 DIV primary spheres were centrifuged, pelleted, dissociated, and plated in culture flasks at $0.05x10^6$ cells/mL in EGF-containing medium, and left to proliferate for 7 DIV; the resultant spheres are referred to as Pass 1 spheres.

2.2 Differentiation of EGF-responsive stem cell progeny under differentiation promoting versus proliferation-promoting conditions

(i) *For progenitor cell assays under conditions which promote differentiation* (Ahmed et al., 1995), Pass 1 spheres were rinsed free of EGF, dissociated and plated at

$0.1x10^6$ cells/mL onto poly-ornithine (Sigma) coated glass coverslips in the presence of 1% fetal bovine serum (FBS; UBI).

(ii) For progenitor cell assays under conditions which promote astroglial proliferation, EGF (20ng/mL) was included in the media (Kilpatrick and Bartlett, 1995) and cells were cultured in the absence of FBS. When proliferation was to be quantified, bromodeoxyuridine (BrdU; 0.5μM) was included throughout the culture period.

All-*trans* retinoic acid stock solution (ATRA; Sigma; 0.01M in DMSO) was stored at -80°C in the dark. Individual aliquots were thawed and diluted in culture medium directly prior to use. EGF and fetal bovine serum solutions were filtered through 0.2μm nylon sterile syringe filters (Corning) prior to use. All controls contained the highest concentration of solvent (DMSO) found within ATRA-containing samples.

2.3 Reverse Transcription and Polymerase Chain Reaction

Total RNA from EGF-generated spheres was prepared using the phenol-guanidine isothiocyanate reagent Trizol (BRL). Pass 1 spheres were pelleted at 1500 RPM for 10 minutes. After removing most of the media, cells were centrifuged again for another 10 minutes and the appropriate amount of Trizol was added (0.1 mL/10^6 cells) to the pellet and left at room temperature for 5 minutes. To separate the organic and aqueous phases, 0.2 volumes of chloroform were added, followed by centrifugation at 3600 RPM for 20 minutes. Total RNA was precipitated overnight at 20°C after adding one volume of isopropanol. Isopropanol was removed the following day after centrifugation (10 000

RPM) for 20 minutes at 4°C. The pellet was re-suspended in 4 M guanidine isothiocyanate and combined with 2 volumes of 95% ethanol. RNA was re-precipitated overnight at -20°C. Tubes were spun at 13 000 RPM for 20 minutes at 4°C. The resultant pellet was rinsed with 70% ethanol then re-suspended in water for use in reverse transcription reactions. Stock and diluted RNA samples were stored at -80°C until required.

To prepare first strand cDNA, 1μg total RNA and 0.5μg oligo (dT)12-18 (0.5μg/μL; Pharmacia) were combined with sterile water to 12μL and heated at 65°C for 10 minutes. The mixture was quenched on ice then combined with 5X first strand buffer (GIBCO-BRL), 0.2 mM DTT, and 0.5 mM dNTP's to a total volume of 19μL. After a 2 minute incubation at 42°C, reverse transcriptase (GIBCO-BRL) was added (200 units), and tubes were further incubated for 90 minutes at 42°C. The transcriptase was inactivated by incubating the tubes at 65°C for 10 minutes, then total volume was brought to 50μL with water. This reaction mix was used directly in PCR reactions.

PCR reactions were performed in a final volume of 50μL containing 25 pmol of each primer pair, 3μL of template (except for plasmid positive controls; 0.25μL), 10 mM Tris-HCL, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200μM each of the four dNTP's, and 1U Taq DNA polymerase (GIBCO-BRL). PCR was performed on cDNA prepared from 7 DIV Pass 1 spheres. Unless otherwise specified, in cases where more than one isoform may exist due to alternative splicing, primers were designed to hybridize to all isoforms. The following primer pairs directed against reported sequences for mouse retinoid

receptors were used: (1) RARα1-6 (accession #X56565-8, X56570-2), 5'-agagcagttccgaagagatag-3', 5'-cgactgtccgcttagagtgtccaa-3'; (2) RARβ1-4 (accession # X56569,73,74, S92180) 5'-gcaggaatgcacagagagctatgagatgac-3', 5'-ggtgactgactgactccactgttctccact-3'; (3) RARγ A,B (accession # M34475,6), 5'-tgtatgcaatgacaagtcttctgg-3', 5'-atgaggcagatagcactaagtagc-3' ; (4) RXRα (accession # X66223), 5'-cactgaggatatcaagccgccact-3', 5'-ggtgtcacaccagctctgctatgc-3'; (5) RXRγ 1 (accession #S62948), 5'-tcttcagaagcgcagcagag-3', 5'-ggaatcaacttggtggctcc-3'. All mouse RARs and RXRs cDNA plasmids (controls) were kindly provided by P. Chambon. I did not observe any PCR product when the template was the RNA mixture without reverse transcription.

2.4 Immunocytochemistry

For indirect immunocytochemistry, cells were fixed at 7 DIV with 4% paraformaldehyde for 20 minutes. Following three five minute rinses in phosphate buffered saline (PBS), primary antibodies were diluted in PBS containing 10% normal goat serum and 0.3% Triton X-100 (BDH, Toronto, Ontario) applied to coverslips, and incubated at 37°C for 2 hr. Monoclonal anti-ß-tubulin (TUJ1; Sigma) was used at 1:1000, and rabbit polyclonal anti-GFAP (Biomedical Technologies, Inc.) was used at 1:400. Following further rinses in PBS, appropriate secondary IgG antibodies (Jackson Immunochemicals, Westgrove, PA.) conjugated to rhodamine isothiocyanate (RITC, 1:200) or fluorescein isothiocyanate (FITC, 1:100) were applied at room temperature for

30 minutes. Anti-bromodeoxyuridine (BrdU) primary antibody (1:5) used in proliferation assays was obtained from Amersham (Oakville, Ontario). Secondary labeling of the BrdU antibody was achieved using a biotinylated goat anti-mouse antibody (1:1000; Jackson) for 2 hr at 37°C, followed by a 30 minute incubation with cy3-conjugated streptavidin (1:1000; Jackson). When cultures were double-labeled for BrdU and ß-tubulin, the antibody procedures were carried out in series. After removal of the secondary antibodies, Hoechst bis-benzimide 33258 (Sigma, 1 mg/mL in PBS), a nuclear stain, was applied for 5 minutes (total cell number counts). After three final washes in PBS, coverslips were rinsed with double distilled water and mounted with FluorSave on glass slides. Fluorescence was detected and photographed with a Nikon Optiphot photomicroscope.

2.5 Clonal cell culture

Pass 1 spheres were dissociated and plated in 35mm culture plates (Nunc) at clonal density (150 cells/mL) in the presence of EGF with or without ATRA (all controls contained equivalent concentrations of solvent). After 24 hours, cells were rinsed free of ATRA using warmed culture media which was subsequently replaced with media containing EGF only. For each rinsing procedure, all media containing ATRA or solvent was aspirated from the well, then replaced with warmed culture media. Each well was completely rinsed twice before adding media supplemented with EGF only. Clones were left to proliferate for a further 13 DIV. Clone numbers and diameters were observed after a total of 14 DIV. Clone diameters were measured using a set of concentric rings fitted

into the eyepiece of the microscope. Individual clones were subsequently used for phenotype analysis (section 2.5.1) or single sphere dissociations (section 2.5.2).

2.5.1 Differentiation of clones

After noting their diameter, individual clones were plated onto poly-ornithine coated glass coverslips in regular media which was devoid of growth factors in order to promote differentiation. Note that no serum was used under these conditions in the interest of determining the actions of ATRA in the absence of unknown factors (as would be found in serum). Clones were left to differentiate for 7 DIV. The cells were labeled with cell-type specific antibodies and Hoechst to determine the phenotype composition of the clones, as well as neuron numbers per clone.

2.5.2 Single sphere dissociation

Individual 300μm clones were placed in EGF-containing media in 96-well plates. Spheres were triturated 25 times each with a Pipettman. Each well was inspected before and after trituration to ensure that only one sphere per well was present, and that each sphere was completely dissociated.

2.6 Statistical Analysis

When comparisons were made between two conditions, Student's T-test was used to determine the level of significance. When multiple comparisons between conditions

were being performed, analysis of variance was followed by post hoc analysis to determine significance (Tukey test).

3 CHARACTERIZATION OF ATRA ACTIONS ON NEUROGENESIS FROM EGF-RESPONSIVE STEM CELL PROGENY

3.1 RESULTS

3.1.1 Retinoid Receptor Expression in EGF-Generated Spheres

ATRA receptors are members of the steroid receptor superfamily which include retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (reviewed in Chambon, 1994). The RAR and RXR families contain three subtypes (α,β,γ), each of which have been shown to have multiple isoforms. RT-PCR analysis, using primers designed against conserved regions of the receptor subtypes, was used to identify the presence of RAR and RXR mRNA in EGF-generated spheres. Messenger RNAs for all three subtypes of RARs (RARα, RARβ, and RARγ) as well as for RXRα were present in EGF-generated Pass 1 spheres (Fig. 1). RXRγ, a receptor normally found in the developing striatum (Mangelsdorf et al., 1992), was also detected at low levels in spheres and confirmed by sequence analysis (data not shown). It is interesting to note that Ruberte and colleagues (1993) reported that RXRα and RARγ are not expressed in the developing CNS. However, these observations were based on *in situ* hybridization results which may have been unable to detect low levels of receptor mRNA, or even high levels within single cells. The expression of mRNA for the retinoid receptors in EGF-generated spheres

Figure 1 Retinoid receptor expression in EGF-generated Pass 1 spheres

RT-PCR was used to detect several types of retinoid receptors in Pass 1 EGF-generated spheres: RARs α,β,γ, and RXRα are shown here using positive (+) plasmid controls, and cDNA produced from one week old Pass 1 spheres (s). For each of the receptors, primers were designed to allow detection of all isoforms. RXRγ1, a brain specific isoform of RXRγ was also detected in spheres, and subsequently confirmed by sequence analysis (not shown).

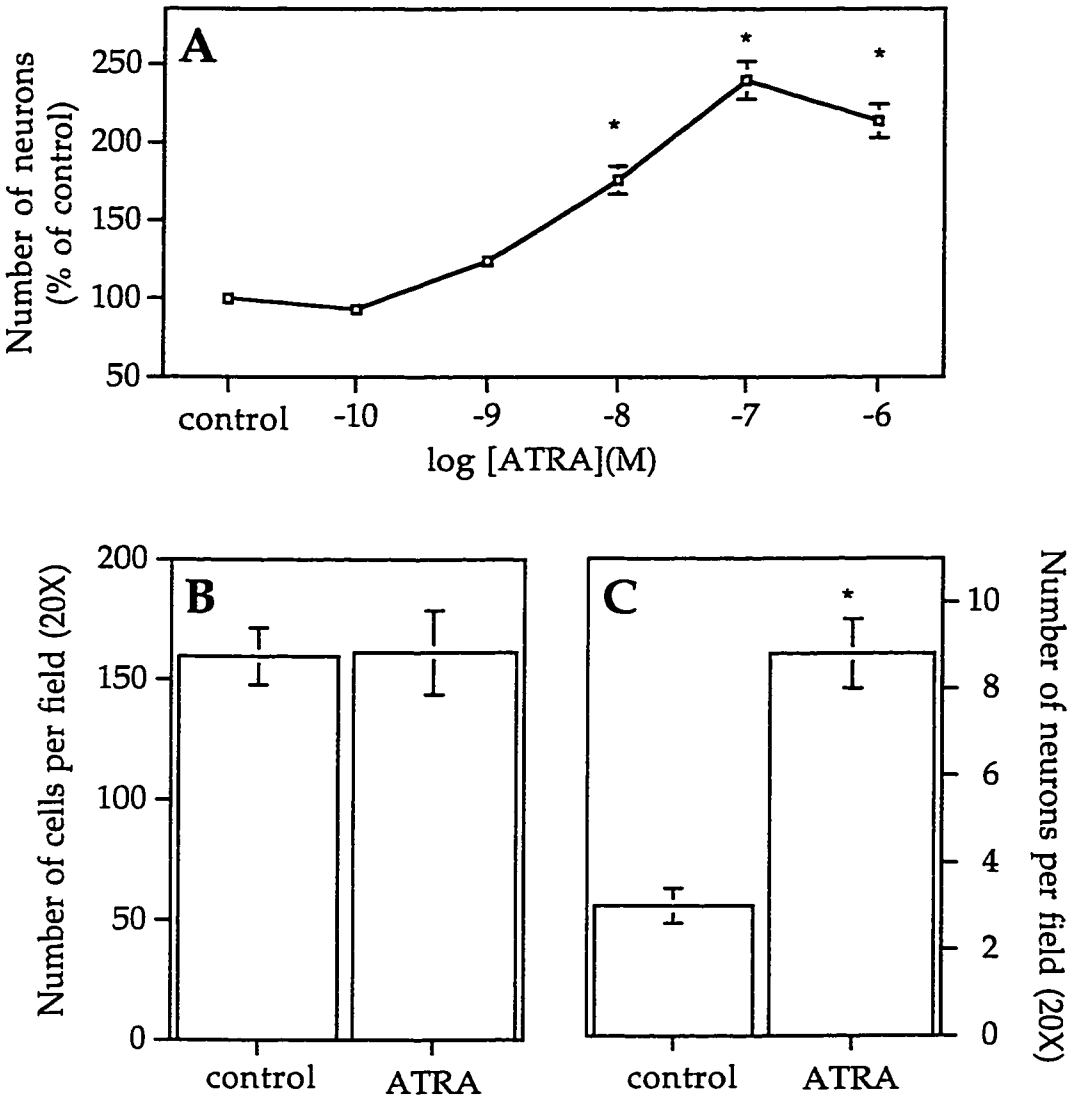
suggested that ATRA might induce a retinoid-specific effect on stem cell-derived progenitor proliferation or differentiation.

3.1.2 ATRA enhances neurogenesis but not gliogenesis from stem cell progeny

The differentiation of stem cell-derived progeny was studied by rinsing Pass 1 spheres free of EGF and culturing the cells in the presence of fetal bovine serum (FBS) to facilitate progenitor cell differentiation (Ahmed et al., 1995). Dissociated spheres were plated onto coverslips in the presence of 1% FBS and in the absence or presence of 0.1μM ATRA. ATRA treatment resulted in a dose-dependent increase in the number of neurons (ED_{50} ~0.01μM; Fig. 2A), with maximum neuronal production obtained at 0.1μM ATRA. The total numbers of cells produced after 7 days *in vitro* (DIV) in the presence of serum and ATRA did not differ from control (Fig. 2B), whereas numbers of neurons are approximately doubled (per 20X field, control cultures contained 3.0 ± 0.4 neurons, while ATRA-treated cultures contained 8.8 ± 0.8 neurons; n=3 independent culture preparations, $p<0.01$; Fig. 2C). No difference in astrocyte or oligodendrocyte numbers are observed under these conditions (per 40X field, control cultures contained 16.1 ± 7.2 astrocytes and 2.3 ± 1.9 oligodendrocytes, while ATRA-treated cultures contained 17.4 ± 7.9 astrocytes and 2.1 ± 2.4 oligodendrocytes; n=3 independent culture preparations; $p= 0.26$ and 0.33, respectively).

Figure 2. ATRA enhances neuronal production by EGF-generated precursors

Pass 1 EGF-generated spheres were dissociated and plated in the absence of EGF, in the presence of 1% FBS and with or without ATRA. (A) Increasing concentrations of ATRA induced a dose-dependent increase in ß-tubulin-immunoreactive neurons, expressed as a % of control. (B) The total numbers of cells produced after 7 DIV is not affected by treatment with ATRA (0.1μM), but neuron numbers (C) are significantly increased. (n=4 independent culture preparations. *$p<0.01$; **$p<0.05$ using a Student's T-test in both B and C, and post-hoc analysis in A.)


A
Number of neurons
(% of control)
250
200
150
100
50
control
-10
-9
-8
-7
-6
log [ATRA](M)
*
*
*
B
Number of cells per field (20X)
200
150
100
50
0
control
ATRA
C
Number of neurons per field (20X)
10
8
6
4
2
0
control
ATRA
*

3.1.3 ATRA increases neuronal production by acting as a survival factor for mitotically active neuronal precursors

To determine whether ATRA acts as a survival or differentiation factor for neural precursors, the administration of ATRA to stem cell progeny was delayed. Survival factors are required throughout the culture period. In the delayed addition experiments, ATRA was added at the time of plating, at 1 DIV, or at 4 DIV. The control under these conditions was vehicle added at the time of plating. Only cultures exposed to ATRA from the beginning of the culture period (0 DIV) had a significant increase in neuronal numbers relative to control (Fig. 3A). When ATRA addition was delayed for 24 hours (1 DIV) or for 96 hours (4 DIV), no significant increases in neuron counts resulted, indicating that ATRA acts early during the neurogenic process. These results suggest that ATRA is likely not a differentiation factor, but rather may act as a survival factor for neuronal precursors.

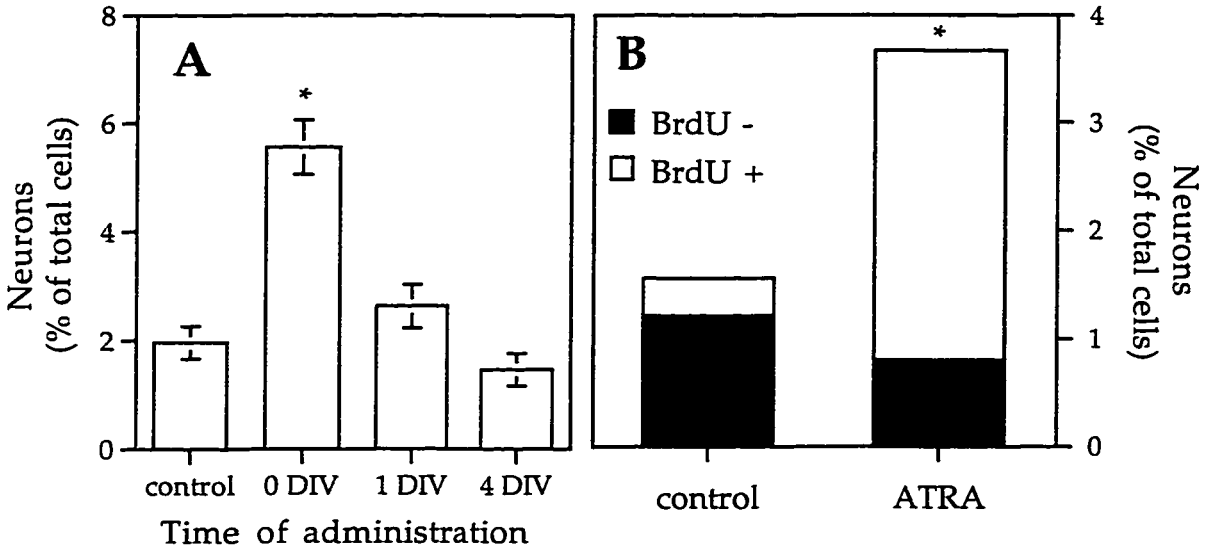
I next asked whether ATRA acts upon a mitotically active population of cells or on post-mitotic, committed neuronal precursors. In these experiments, bromodeoxyuridine (BrdU; 0.5μM) was included in the culture media to label dividing cells. BrdU is a thymidine analogue which is incorporated into the DNA of dividing cells during DNA synthesis. A monoclonal antibody can be used to visualize cells which have incorporated BrdU (Granzber, 1982). After 7 DIV, indirect immunocytochemistry was used simultaneously to visualize BrdU and β-tubulin double-labeled cells. The doubling in neuron numbers seen in cultures treated with ATRA was entirely accounted for by an

increase in BrdU-labeled neurons (Fig. 3B). These results suggest that ATRA acts on a newly generated population of neuronal precursors.

Figure 3. ATRA increases neuronal production by acting as a survival factor for mitotically active neuronal precursors

(A) ATRA (0.1μM) was added to cultures containing 1% FBS either at 0 DIV, or after 1 DIV or 4 DIV. After 7 DIV, the numbers of ß-tubulin-immunoreactive neurons was counted, and expressed as a % of total cells. Neuron production increased only in cultures which were exposed to ATRA from the time of plating (n=3 independent culture preparations; *$p<0.01$ Student's T-test).

(B) Pass 1 EGF-generated spheres were dissociated and plated onto coverslips in the presence of 1% FBS and 0.5μM BrdU, with or without 0.1μM ATRA. Virtually all new neurons incorporated BrdU. (n=2 independent culture preparations; *$p<0.01$ Student's T-test).


A
B
Neurons
(% of total cells)
8
6
4
2
0
*
control
0 DIV
1 DIV
4 DIV
Time of administration
BrdU -
BrdU +
4
3
2
1
0
control
ATRA

3.2 DISCUSSION

In this study I have shown that ATRA can enhance neurogenesis, but not gliogenesis in cultures of dissociated EGF-generated stem cell-derived spheres. If ATRA is added at the onset of culture (in the presence of 1% serum), a dose-dependent increase in neuron numbers is seen. BrdU incorporation by neurons in ATRA-treated cultures suggests that ATRA is acting on a mitotically active population of cells. Under these conditions, no difference in total cell numbers or other phenotypes was observed, relative to controls. The time dependence of the ATRA-mediated neuron increase is particularly striking. Even a short (24 hour) delay in ATRA exposure results in no increase in neuronal numbers, suggesting that a transient population is the target for ATRA actions. Similar results have been seen in neural crest cell populations (Dupin and Le Douarin, 1995) where it was proposed that precursors, which are sensitive to ATRA, lose their responsiveness as development progresses. Interestingly, one of the most sensitive gestational periods for ATRA teratogenesis in the developing mouse brain is between E11 and E13 (Holson et al., 1997), a period during which intense neurogenesis is occurring in the developing striatum (Fentress et al., 1981).

These results are in contrast to other studies from our lab in which BDNF or IGF-I enhance neurogenesis in stem cell-derived cultures by acting as differentiation factors for neuronal precursors rather than survival factors for a mitotically active population of cells (Ahmed et al., 1995; Arsenijevic and Weiss, 1998). The increase in neuron numbers in response to BDNF or IGF-I treatment can be achieved even when growth factor addition

is delayed up to 10 DIV for BDNF, or 5 DIV for IGF-I. Similar results with respect to delayed addition have been noted in perinatal spinal cord cultures in which ATRA treatment up to five days after seeding increased neuron production (Wuarin et al., 1990). Interestingly, in studies where delayed addition was effective, either neurite numbers or branching increased, providing further support for the roles of the growth factors as differentiation factors. Given that delayed additions of ATRA do not increase neuron numbers from EGF-generated precursors, and no increase in branching or neurite length was observed (C. Wohl, unpublished observations), my results support the contention that ATRA is likely a survival factor for neuronal precursors. Alternatively, ATRA may act in concert with another factor, which itself has a very short window of activity, and thus only promotes increased neurogenesis at the onset of culture.

That a relatively undifferentiated population is being targeted by ATRA in our cultures is supported by the fact that the additional neurons produced in response to ATRA are labeled with BrdU. ATRA also induces a dramatic increase in the proportion of newly-generated neurons in cultured neural crest cell precursor populations (Henion and Weston, 1994; Ito and Morita, 1995; Dupin and Le Douarin, 1995; Rockwood and Maxwell, 1996). It is unclear, however, at which stage of neurogenesis ATRA acts to elevate neuron production. ATRA could be acting: (1) at the stem cell level to promote enhanced neuron production; (2) as a survival factor for proliferating, committed neuroblasts; or (3) as a phenotype switch factor for uncommitted, mitotic precursors to push them to a neuronal fate. A study performed on developing mouse neural crest cells *in vitro* has shown that ATRA can act on both committed and multipotent cells to alter

the numbers and types of cells produced (Ito and Morita, 1995), suggesting that ATRA can act at multiple stages of neurogenesis. In our culture system however, it is unlikely that ATRA is acting as a phenotype switch factor, since the increase in neuron numbers is not accompanied by a comparable decrease in either astrocytes or oligodendrocytes.

Given that ATRA has a neurogenic effect *in vitro* on stem cell-derived progeny, it is possible that ATRA may play a role *in vivo* to promote neurogenesis from stem cell progeny. It has been established that the forebrain subependyma contains cells that migrate to the olfactory bulb both post-natally as well as in the adult (Luskin, 1993; Lois and Alvarez-Buylla, 1994). There has been speculation that ATRA, which is present along with all-trans retinol in the developing forebrain (Horton and Maden, 1995) may indeed be involved in the normal development of the mammalian olfactory bulb. Homozygous Pax6 (small eye:Pax6$^{Sey\text{-}Neu}$) mutant mice have a very specific loss of the olfactory bulb, whereas other forebrain subdivisions are still normal (Anchan et al., 1997). Interestingly, the loss of the entire olfactory bulb is accompanied by a failure of retinoid-mediated gene expression in the frontonasal region and forebrain. In these mutants, ATRA is not produced by the frontonasal mesenchyme, which normally provides local retinoid signals to the placode and forebrain (Anchan et al., 1997). It is not clear from these studies what causes the loss of the olfactory bulb. It is possible that migrating olfactory precursors die in the absence of ATRA as a survival factor. Alternatively, loss of ATRA signaling could cause a defect in their migration from the ventricular zone. In light of my data suggesting that ATRA promotes neurogenesis in cultures of CNS stem cell-derived progeny, it is tempting to speculate that the

proliferation of the migrating olfactory bulb precursors is promoted by ATRA produced by the frontonasal mesenchyme.

The downstream effectors of the retinoid response with respect to enhancing neurogenesis from CNS stem cell progeny are unkown. The RARs and RXRs are transcription factors which have been shown to influence the expression of growth factors and growth factor receptors, as well as other transcription factors. For example, in cell lines such as F9 embryonal carcinoma cells and pluripotential mesenchymal cell lines like the mouse C3H-10T1, ATRA treatment drastically alters expression of members of the BMP (BMP2,4) and TGFβ (TGFβ1,3) families, resulting in changes in osteogenesis and formation of parietal endoderm (Rogers et al., 1992; Gazit et al., 1993). The normal patterning activity of the polarizing region of the limb can be mimicked by exogenously applied ATRA, resulting in dramatic mirror-image duplications of the limb and changes in FGF-4, sonic hedgehog, BMP-2, and HOX gene expression (Tickle et al., 1982; Tickle, 1991; Francis et al., 1994; Niswander et al., 1994). These patterning changes in the limb indicate that ATRA can activate a number of gene families to alter the decision of a cell to die or live, and what phenotypes are ultimately produced. In the developing tooth, several of the Notch gene family members are upregulated in response to ATRA, resulting in an alteration of ameloblast cell fate (Mitsiadis et al., 1995). Future investigation coupling cell and molecular biology may provide more evidence as to which genes are necessary and sufficient to increase neurogenesis from CNS stem cell-derived progeny.

4 ATRA INHIBITS CELL PRODUCTION AND ENHANCES ASTROCYTE DIFFERENTIATION UNDER PROLIFERATION-PROMOTING CONDITIONS

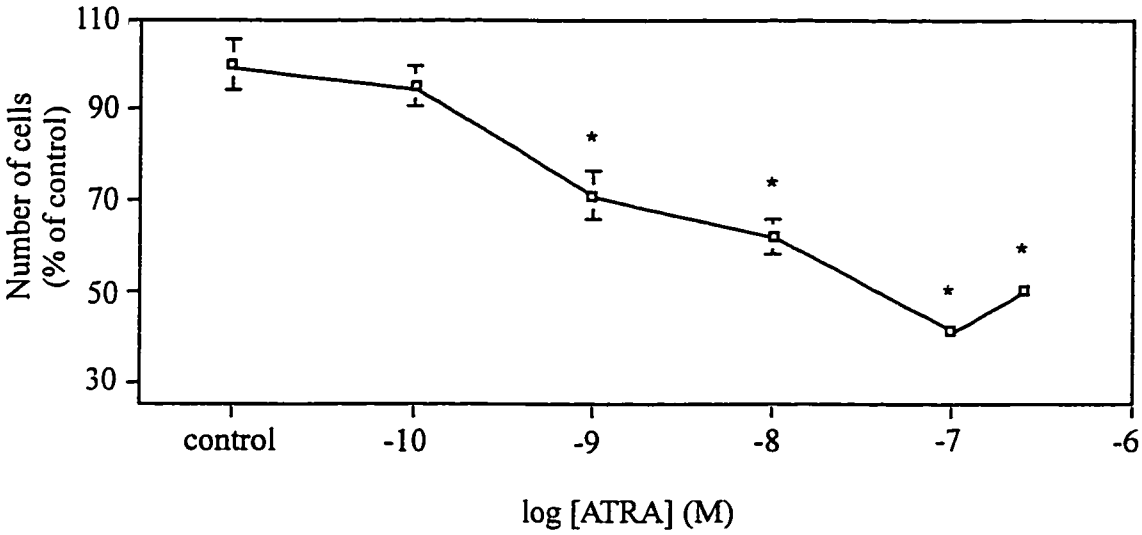
4.1 RESULTS

4.1.1 ATRA induces a dose-dependent decrease in cell production in cultures of dissociated spheres proliferating in the presence of EGF

Wuarin et al. (1990) reported that astrocyte differentiation was enhanced in perinatal spinal cord cultures treated with ATRA. Surprisingly, although neuron numbers are increased in cultures containing FBS and ATRA, no effect on glial cell numbers was noted. It is possible that serum promotes astrocyte differentiation and possibly masks the actions of ATRA. Therefore I examined the actions of ATRA under serum free conditions in the presence of EGF - a mitogen for astrocyte progenitor cells (Kilpatrick and Bartlett, 1995). Pass 1 spheres were dissociated into a single cell suspension, then plated onto poly-ornithine coated coverslips in media containing EGF. ATRA or solvent was added at the time of plating, and cultures were left to proliferate for 7 DIV. ATRA induced a dose-dependent decrease in the total number of cells produced after 7 DIV relative to vehicle controls (ED_{50}, ~0.2μm; Fig. 4). For subsequent analysis of the phenotypes of the resulting cells, the saturating concentration of 0.1μM ATRA was used.

Figure 4. ATRA induces a dose-dependent decrease in cell production in cultures of dissociated spheres proliferating in the presence of EGF

Pass 1 spheres were dissociated and plated onto poly-ornithine coated coverslips at $0.1x10^6$ cells/mL in the presence of 20ng/mL EGF and varying concentrations of ATRA. Cells were fixed after 7 DIV, and nuclei were counted after bis-benzimide dye incorporation (n=2 independent culture preparations; *$p<0.01$ post-hoc analysis).


110
90
70
50
30
Number of cells
(% of control)
control
-10
-9
-8
-7
-6
log [ATRA] (M)

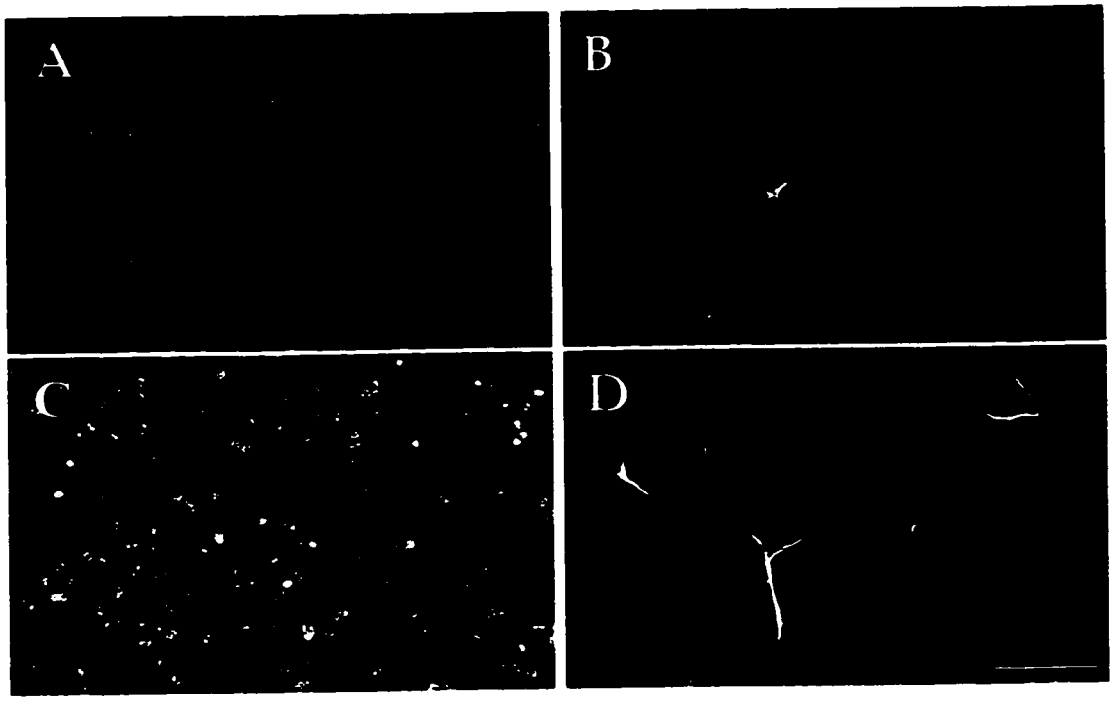
4.1.2 Decreased cell production in the presence of ATRA is associated with an increase in the numbers of GFAP-immunoreactive astrocytes

I next asked whether the decrease in cell production was accompanied by a change in the phenotypes of cells produced in response to ATRA treatment. When immunocytochemistry was used to detect the astrocyte specific antigen GFAP, a striking difference was noted between control and ATRA-treated cultures. Under control conditions, few strongly-immunoreactive, GFAP expressing cells are observed (Fig. 5 A,B). The lack of mature phenotype expression is not surprising however, as signals that promote proliferation are known to inhibit or delay differentiation *in vitro* (Laeng et al., 1994). GFAP-labeled cells counter-stained with bis-benzimide to visualize nuclei were counted in three separate experiments. Treatment with 0.1μM ATRA resulted in a three to four fold increase in the absolute number of astrocytes per field (Fig. 5 C,D; Fig. 6). Relative to the total numbers of cells, astrocytes comprise roughly 1.5% versus 7% of all cells in control and ATRA-treated cultures, respectively (n=3 independent culture preparations). Immunocytochemistry against the neuron-specific antigen type III β-tubulin and the oligodendrocyte-specific antigen O4 showed no difference in the absolute numbers of neurons or oligodendrocytes per field in ATRA-treated cultures relative to controls ($p=0.14$, 3 independent culture preparations).

Given that EGF is known as a mitogen for astrocytes, these results indicate that ATRA may be: (1) acting on proliferating precursors to inhibit proliferation, resulting in differentiation of astrocytes; (2) a specific differentiation factor for astrocytes, resulting

Figure 5. The decreased numbers of cells in the presence of ATRA is associated with an increase in the numbers of GFAP-immunoreactive astrocytes.

ATRA (0.1μM) treatment of EGF-containing cultures is compared to controls after 7 DIV. Total numbers of cells per field are determined using bis-benzimide staining (A, C) and astrocytes are counted using immunocytochemistry against the astrocyte-specific antigen GFAP (B, D). EGF control cultures (A) do not contain many GFAP-expressing cells (B) per field. ATRA-treated cultures however, show increased numbers of astrocytes (D) despite a marked reduction in cell production (C) relative to controls. See Fig. 6 for quantitative analysis and statistics. Bar, 50μm.


A
B
C
D

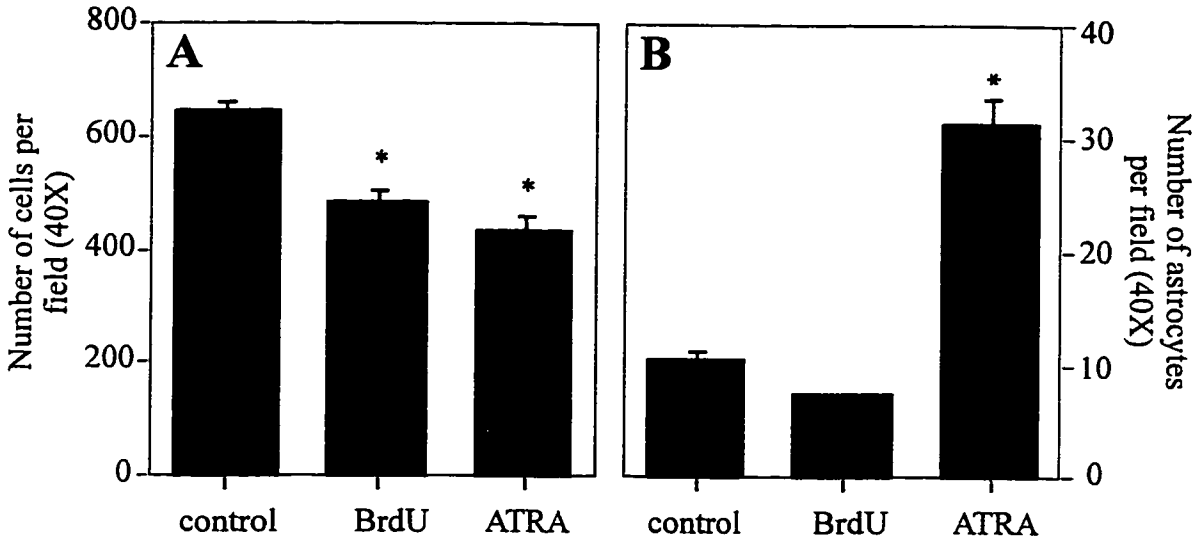
in the net reduction in the number of proliferating cells (hence the reduction in total cell number).

4.1.3 Inhibition of proliferation is not sufficient to cause ATRA-like increases in astrocyte numbers

To determine if ATRA increases the number of astrocytes as a result of inhibition of proliferation, I attenuated cell division using BrdU treatment. BrdU (1μM) inhibited the production of cells to the same degree as ATRA (Fig. 6A). The number of astrocytes per field in BrdU-treated cultures remained identical to controls (Fig. 6B) and comprised 1.5±0.1% and 1.6±0.2% of the total cells in BrdU-treated and control cultures, respectively, compared to 7% in ATRA-treated cultures. BrdU treatment did not alter the ability of astrocytes to differentiate, as in both control and BrdU treatment the same number of astrocytes differentiated during the 7 days *in vitro* (at plating virtually no GFAP-immunoreactive cells are present - data not shown). These results suggest that astrocyte differentiation in the presence of EGF does not necessarily occur in response to inhibition of proliferation, and that ATRA may provide a specific differentiation signal to astrocyte precursors.

Figure 6. Inhibition of proliferation is not sufficient to cause ATRA-like increases in astrocyte numbers.

(A) In the presence of EGF, both BrdU- and ATRA-treated cultures showed similar significant decreases (*$p<0.01$) in total cell numbers after 7 DIV. (B) However, only ATRA-treated cultures produce a three-fold increase in the absolute numbers of astrocytes per field, relative to both control and BrdU-treated cultures (*$p<0.01$). Data are the means ± S.E.M. of 3 independent culture preparations.


A
B
800
600
400
200
0
40
30
20
10
0
Number of cells per field (40X)
Number of astrocytes per field (40X)
*
*
*
control
BrdU
ATRA
control
BrdU
ATRA

4.2 DISCUSSION

ATRA has diverse effects on cell proliferation *in vitro* (see Sporn et al., 1994). In our culture system, EGF-stimulated proliferation is significantly reduced by ATRA. This decrease in cell production is accompanied by a four fold increase in the number of astrocytes. The morphology of the astrocytes is also markedly different from controls - an effect also noted by Wuarin et al. (1990) on perinatal primary cultures of rat spinal cord treated with ATRA.

The ATRA-mediated reduction in cell numbers after one week could be explained several ways. Given that EGF is a mitogen for astroglial precursors (Kilpatrick and Bartlett, 1995), inhibition of their proliferation could result in their subsequent differentiation - thus reducing the number of cells available to proliferate (hence the reduction in total cell numbers). In this case, glial cell differentiation may occur directly as a consequence of inhibition of proliferation. Alternatively, ATRA could specifically drive astrocyte precursors to differentiate, again resulting in fewer mitotically active cells and less overall cell production. Whether the decrease in cell production is directly related to glial cell differentiation is not obvious, given that decreased proliferation does not necessarily lead to differentiation (Hosoi et al., 1985; Edward et al., 1988; Sporn and Roberts, 1994). When our cultures were grown in the presence of 1μM BrdU to inhibit mitosis, astrocyte numbers were no different from controls. The possible "non-physiological" mechanism by which BrdU attenuates mitosis may make such direct

comparisons difficult. Moreover, these findings do not preclude that ATRA has two separate and distinct actions: inhibition of precursor cell proliferation, and promoting the differentiation of astrocytes.

If ATRA specifically drives astrocytes to differentiate, one is tempted to speculate which downstream genes may be the effectors. TGF-β1 is a cytokine which exerts multiple effects on growth and differentiation of different cell types. TGF-β1 has been shown to inhibit the proliferation of rat astrocytes derived from cerebellum and cortex, most notably in response of the astrocyte cultures to EGF-induced proliferation (Vergeli et al., 1995). Several studies (Mehler et al., 1995; Gross et al., 1996; Bjornson, 1997) have suggested that BMP-2 can enhance the production of astrocytes from CNS stem cell progeny. It is difficult to directly compare the actions of BMP-2 and ATRA actions on stem cell progeny however, given that culture conditions in each study were different. The fact that ATRA has been shown to upregulate both TGF-β1,2 and BMP-2 expression in several progenitor cell types (Rogers et al., 1992; Gazit et al., 1993; Francis et al., 1994) makes these cytokines possible downstream targets of ATRA actions.

It remains unclear whether ATRA is acting on a mitotically active population of glial precursors, or a committed, non-dividing set. It is possible that under control conditions, post-mitotic astrocytes simply do not express sufficient amounts of GFAP to be detected by traditional immunocytochemical labeling. It is possible that ATRA simply increases GFAP expression in these same cells, resulting in the apparent increase in astrocyte numbers. Finally, these experiments do not exclude the possibility that

ATRA-induced cell death underlies decreased cell production. The method of death, however, would presumably not be general toxicity, since astrocytes survive and differentiate (as do neurons under other conditions), and cell growth continues.

5 CLONAL ANALYSIS OF ATRA ACTIONS ON STEM CELLS

5.1 Introduction

The previous findings have shown that ATRA can act on stem cell-derived progeny to enhance neurogenesis and astrocyte differentiation under distinct culture conditions. However, a major question remaining unanswered by these results is whether ATRA acts directly on the EGF-responsive stem cell itself. Previous data from our lab (Bjornson, 1997) have suggested that BMP-2 can act at the level of the stem cell (to inhibit proliferation) as well as on post-mitotic stem cell progeny (resulting in enhanced neuronal differentiation). My bulk culture findings do not preclude the possibility that ATRA may be acting at the level of the stem cell as well. Microwell culture experiments with primary embryonic striatal cells found that ATRA did not inhibit stem cell proliferation per se; the overall numbers of spheres produced are the same, although ATRA-treated primary spheres are smaller than controls (data not shown).

In preliminary experiments, I found that dissociated spheres exposed only transiently to ATRA in the presence of EGF would produce large numbers of neurons. In these experiments, dissociated spheres were plated onto coverslips in the presence or absence of ATRA for only the first 24 hours in culture (before the first stem cell division) in the presence of EGF. After 1 DIV, coverslips were transferred to differentiation promoting media containing 1%FBS for the remaining 6 DIV. Under these conditions, ATRA induced dramatically enhanced neurogenesis (up to 19.3±1.2% neurons in ATRA-treated cultures, versus 4.9±2.2% neurons in control cultures; n=3 independent culture

preparations). This suggests that even transient exposure to ATRA can instruct the cells responsible for increases in neuron production - possibly stem cells themselves.

It is possible that progeny of stem cells (e.g. dividing neuroblasts) are simply instructed to divide several more times in the presence of ATRA before terminal differentiation, thus resulting in the increase in neurogenesis. Alternatively, stem cells could be directed by ATRA to become "less" multipotent - perhaps bi-potent, or neuron-containing only. Evidence from other studies suggests that ATRA has the ability to function in both capacities. Kelly et al. (1994) have reported that ATRA mediates a cell fate switch, such that rod photoreceptor cells are generated at the expense of amacrine cells. On the other hand, Barres et. al. (1994) have reported that ATRA plays a role in the number of times an oligodendrocyte precursor turns over - in other words, a counting mechanism. These possibilities, as regards EGF-responsive stem cells, were investigated with clonal analyses. Given that a transient (24 hour) exposure was shown to enhance neurogenesis in bulk culture conditions, I grew dissociated Pass 1 spheres under clonal conditions (Ito and Morita, 1995) with only a transient exposure to ATRA . Clonal culture conditions provide a reasonable level of certainty that each cluster of cells is derived from a single cell. Additionally, 24 hour transient exposure to ATRA ensures that each cell receives the ATRA signal *before it divides* (B. Reynolds and S. Weiss, unpublished observations). Using this approach, I asked whether ATRA could act at the level of the stem cell to change: (i) the types of cells produced from a single cell; (ii) the numbers of neurons produced from that cell; (iii) stem cell self-renewal/expansion.

5.2 RESULTS

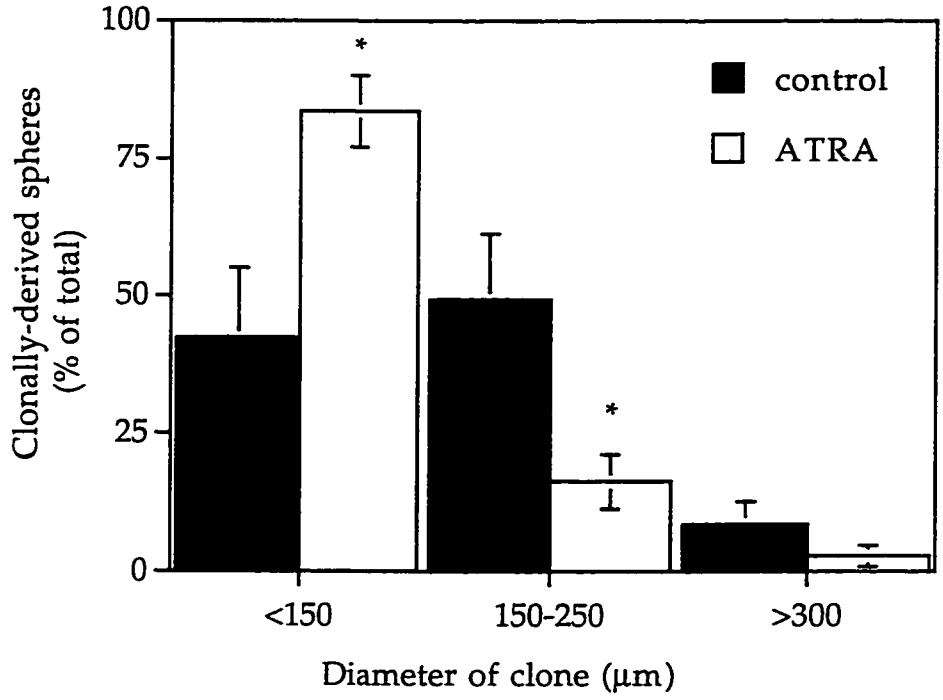
5.2.1 Clones generated from cells treated transiently with ATRA are smaller than controls

In these experiments, dissociated Pass 1 spheres were seeded at clonal density (150cells/mL) into 35mm culture dishes in the presence of EGF with ATRA (0.1μM) or solvent (0.001% dmso). After 24 hours (before the first stem cell division), cells were rinsed with media to remove ATRA or solvent. Clones were left to grow for a further 13 DIV in the presence of EGF only. The total numbers of all clones produced under control versus treated conditions were observed. Control cultures produced 12.7±9.3 clones per well, whereas ATRA-treated cultures produced 12.0±8.9 clones per well (7 independent cultures, 12 wells per experiment). Although there is no difference in the overall numbers of clones produced in control versus ATRA-treated cultures a difference in clone diameters was noted. All clone diameters were observed using a concentric ring set placed in the eyepiece of the microscope. These results are shown in two ways in Fig. 7. If clones are grouped into small (25-100μm), medium (150-250μm), or large (<300μm) size groups, the small and medium sized ATRA-treated clones were significantly smaller than controls (Fig. 7A). For example, 84% of all ATRA-treated clones are small, as opposed to only 42% of control clones. The majority of control clones (49%) are medium sized, in contrast to only 12% of ATRA clones in this size category. Fig. 7B

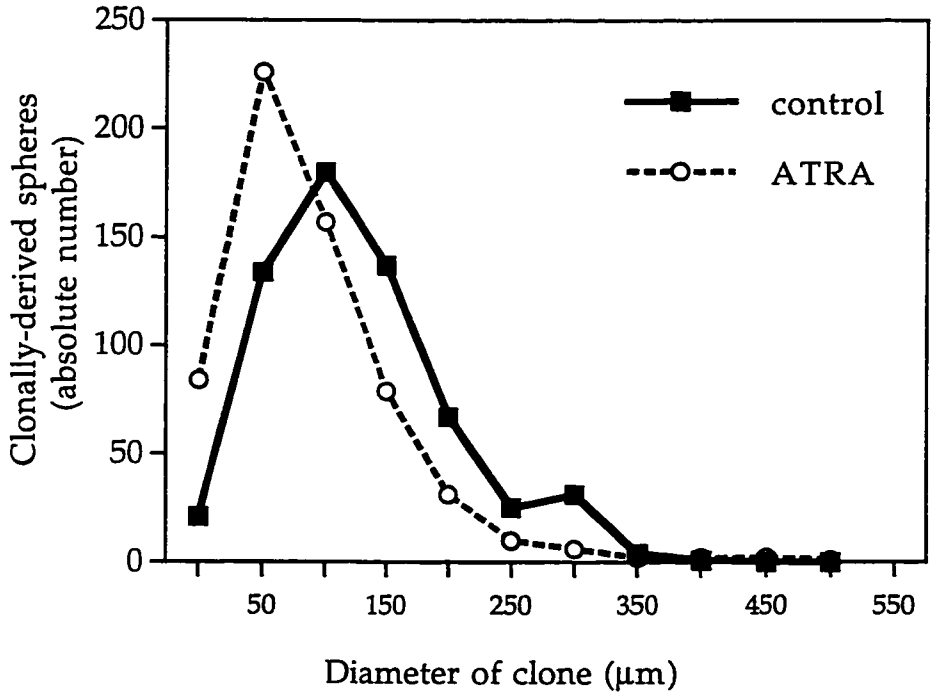
Figure 7. Clonal analysis indicates that spheres treated transiently with ATRA are smaller than controls

(A) 600 clones per condition from 7 independent culture preparations were sized. Post hoc analysis shows that a larger proportion of small (<150μm) and medium (150-250μm) sized clones are found in ATRA-treated cultures ($p<0.01$ using Student's t-test with all clones combined; post hoc analysis indicates a difference in only the small and medium sized population proportions). (B) A histogram representation of the same data shown in (A).

B A


100
75
50
25
0
*
*
control
ATRA
Clonally-derived spheres
(% of total)
<150
150-250
>300
Diameter of clone (μm)

A B


250
200
150
100
50
0
control
ATRA
Clonally-derived spheres
(absolute number)
50
150
250
350
450
550
Diameter of clone (μm)

shows this data as a histogram of the size distribution of 600 clones (100 clones observed from a total of 6 independent cultures) from ATRA versus control treated cells. These data echo the results shown in Fig. 4 under bulk culture conditions, reflecting decreased cell production in response to ATRA treatment.

5.2.2 Phenotype analysis of clones suggests that there is no significant effect of ATRA on multipotency

As mentioned above, there was no difference in the overall numbers of clones produced in control versus ATRA-treated cultures. In order to determine if the multipotency of the stem cells was altered by ATRA treatment, it was important to determine whether the phenotypes generated from each clone were similar. For instance, are clones generated under the two culture conditions equally multipotent with respect to the cell types produced? Is the survival or differentiation of a particular phenotype favored? To determine this, 14 DIV control and ATRA-treated clones (generated as described above) were picked up individually with a pipette and plated onto poly-ornithine coated glass coverslips. Clones were subsequently left to differentiate for 7 DIV in media devoid of growth factors or serum. Note that serum was omitted from the differentiation media in an effort to minimize possible unknown growth factor effects during the differentiation process. Cell type specific antibodies were used to assess the presence or absence of neurons, astrocytes, and oligodendrocytes in each clone. Although the cells in each tightly clustered clone tend to spread away from each other on the coverslip, the center of each clone often remained particularly dense with cells,

making quantitation of overall cell numbers difficult. Fortunately however, it *was* possible to accurately identify the numbers of neurons per clone because of their clear staining pattern when labeled with the β-tubulin antibody. Therefore, for each clone, it was possible to determine which phenotypes were generated, as well as the numbers of neurons per clone.

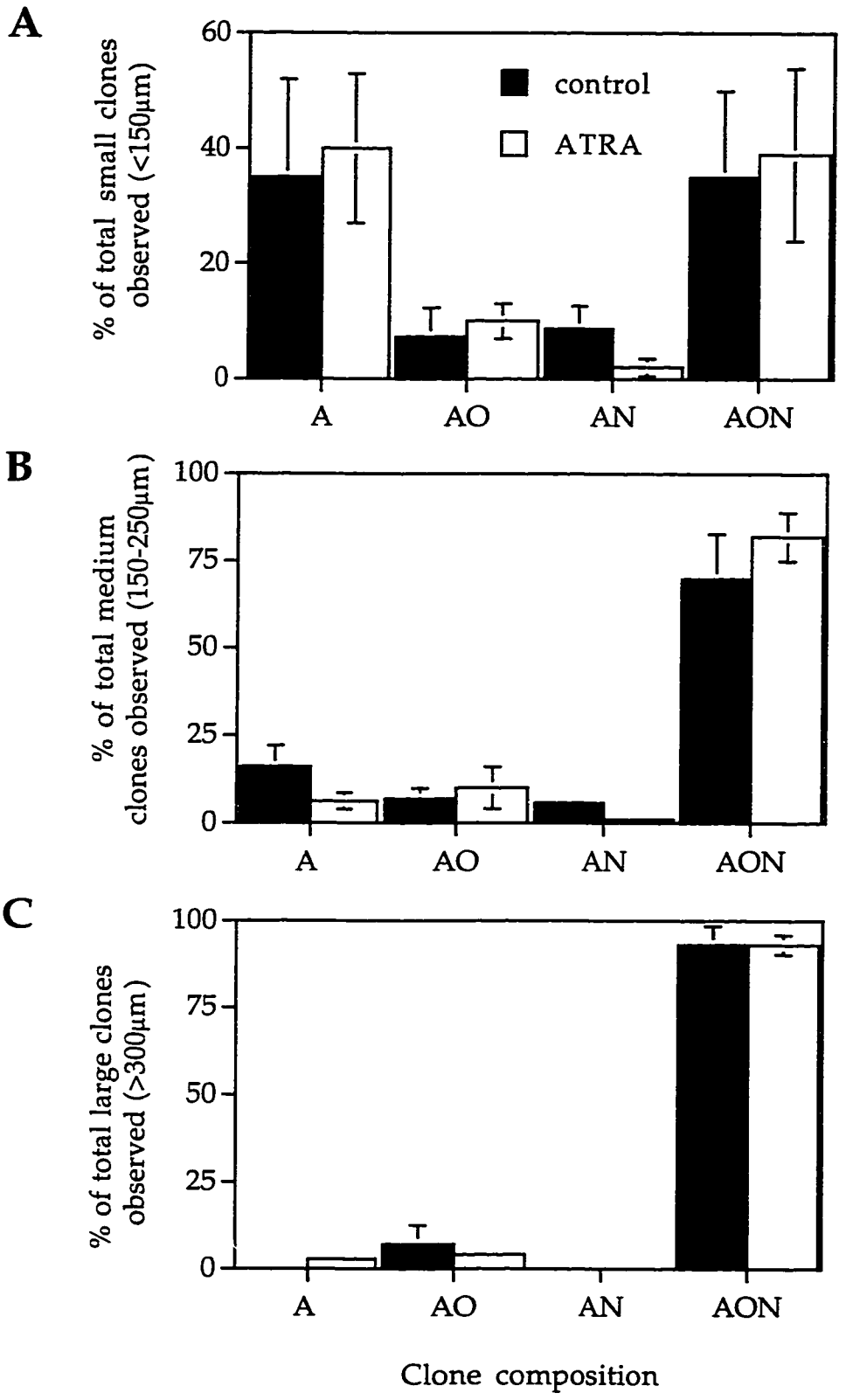
In a series of eight independent cultures, a total of 310 control and 307 ATRA-treated clones were observed. For small (<150μm in diameter) clones, 35±17% of controls and 40±13% of ATRA-treated clones contained astrocytes only. The majority of remaining clones in this size category contained all three cell types; 35±15% and 39±15% of control and ATRA-treated clones (respectively) contained neurons, astrocytes and oligodendrocytes (Fig. 8A). Medium sized clones (150-250μm) from control and ATRA-treated cultures were predominantly tri-potent (i.e. contained all three cell types; 69±13% controls, 82±7% for ATRA-treated clones; Fig. 8B). Over 90% of all clones from control (93±5.4%) and ATRA-treated (93±2.8%) cultures contained all three cell types (Fig. 8C). A small population of clones containing astrocytes only, astrocytes and oligodendrocytes only, or astrocytes and neurons only was observed in both large control and ATRA-treated clones. No clones containing oligodendrocytes only or neurons only were observed under either condition. According to this data, regardless of clone size, there were no significant differences in the proportion of uni-potent, bi-potent, or tri-potent clones in control versus ATRA-treated cultures. These data suggest that despite the propensity for ATRA-treated clones to be smaller on average, their phenotype production

Figure 8. Phenotype analysis of clones suggests that there is no significant effect on the degree to which sphere-derived cells are multipotent

Dissociated Pass 1 spheres were seeded at clonal density (150 cells/mL) in 35mm plates in the presence of media containing EGF and either ATRA or solvent. After 24 hours, the cells were rinsed with culture medium and clones were left to grow for an additional 13 DIV in the presence of EGF only. Individual clones were subsequently plated onto poly-ornithine coated glass coverslips and left to differentiate for 7 DIV in culture media devoid of growth factors or serum. Phenotypes were identified with the use of cell-type specific antibodies (A: astrocyte-containing clone; AO: astrocyte and oligodendrocyte-containing clone; AN: astrocyte-neuron containing clone; AON: astrocyte, oligodendrocyte, and neuron-containing clone).

(A) Small (under 150μm in diameter) clones (n=149 and 144 clones examined for controls and ATRA-treated clones, respectively). (B) Medium sized (150-250μm in diameter) clones (108 and 143 clones examined for controls and ATRA-treated clones, respectively). (C) Large (over 300μm in diameter) clones (53 and 20 clones examined for control and ATRA-treated cultures, respectively).

(n=8 independent cultures, n=310 clones for control, n=307 clones for ATRA-treated cultures; post hoc analysis indicates no significant effect).


A
% of total small clones observed (<150µm)
60
40
20
0
control
ATRA
A
AO
AN
AON
B
% of total medium clones observed (150-250µm)
100
75
50
25
0
A
AO
AN
AON
C
% of total large clones observed (>300µm)
100
75
50
25
0
A
AO
AN
AON
Clone composition

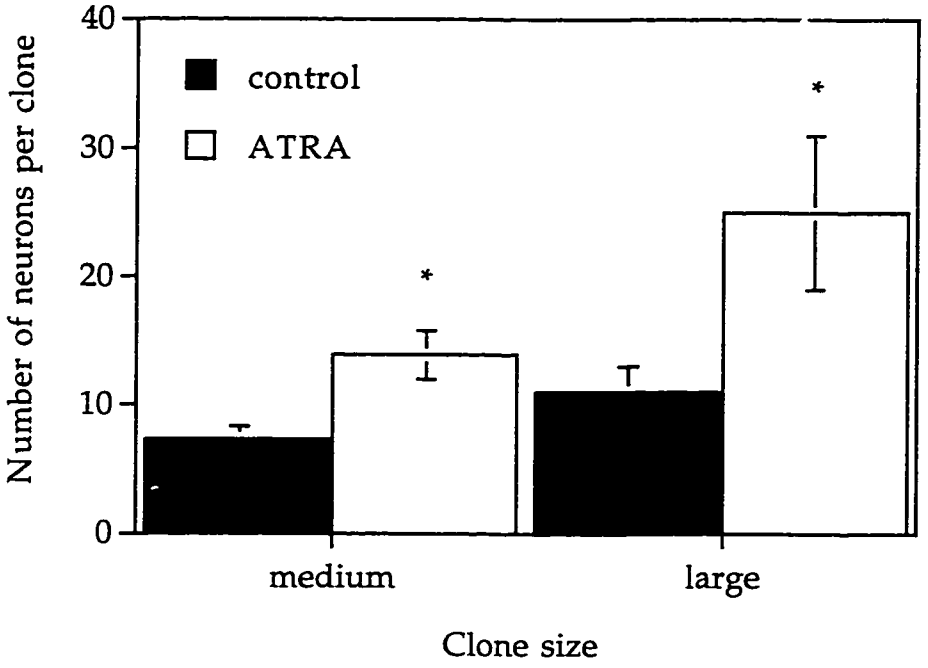
is unchanged relative to control.

5.2.3 Clones treated transiently with ATRA produce more neurons per clone than controls

Given that ATRA enhances neurogenesis under bulk culture conditions, I asked whether more neurons were generated under clonal culture conditions. Dissociated Pass 1 spheres were seeded at clonal density and transiently exposed to ATRA or solvent for the first 24 hours in culture in the presence of EGF. After rinsing, clones were grown and differentiated as described in section 5.1.2. Both medium and large sized clones containing all three cell types produced approximately twice as many neurons per clone in response to ATRA treatment. Medium sized ATRA-treated clones contained 13.9±1.9 neurons per clone, whereas controls contained 7.3±1. As clone size increased, more neurons per clone were generated, although the difference in ATRA to control numbers were maintained. Large clones treated with ATRA contained 25±6 neurons, whereas controls contained 11±2. Small clones containing all three cell types showed no significant difference in the numbers of neurons per clone ($p=0.2$). There was a population of clones which contained neurons and astrocytes only (no oligodendrocytes). Small clones containing astrocytes and neurons only comprised 8±4% of the control population, and 2±2% of the ATRA-treated clones. Medium-sized clones containing astrocytes and neurons only formed 5±2% of the control population and 1±1% of the ATRA-treated clones. No large clones containing astroctyes and neurons only were observed. No significant difference was noted with respect to neuron numbers per clone

Figure 9. Clones treated transiently with ATRA produce more neurons

The clones shown in Figure 8 were labeled with a β-tubulin antibody, allowing the numbers of neurons per clone to be observed. Only large and medium sized clones containing all three cell types showed an increase in neuron numbers in response to ATRA treatment ($p=0.004$ for medium clones; $p=0.006$ for large clones).


40
30
20
10
0
control
ATRA
*
*
Number of neurons per clone
medium
large
Clone size

in ATRA versus control cultures in clones containing only astrocytes and neurons (no oligodendrocytes). The increase in neuron numbers can therefore only be attributed to medium and large clones containing all three cell types. These data suggest that ATRA can instruct a presumptive stem cell *even before its first division* to increase the number of neurons which it ultimately produces without sacrificing the production of other cell types.

5.2.4 Single sphere dissociations of ATRA-treated clones suggest that stem cell self-renewal and expansion are not affected by ATRA

It is clear from my results that the stem cell's ability to produce neurons is altered after exposure to ATRA, even before the first stem cell division. I therefore asked whether stem cell maintenance or expansion was affected by ATRA. These experiments require single sphere dissociations. After a sphere is dissociated into its constituent cells, presumably a mixed population of cells is present, from stem cells to post-mitotic neural cells. If the cells from a dissociated primary sphere are exposed to EGF again, stem cells would proliferate to give rise to secondary spheres. If any spheres are produced from a single sphere dissociation, one can assume that self maintenance was not altered. If several spheres are formed after a single sphere dissociation, then the stem cell from the original sphere was displaying its expansion ability. The spheres dissociated in these experiments were clonally derived, and only same-sized spheres from control and ATRA-treated populations were compared. Each sphere was dissociated and the resultant cells were left to grow in the presence of EGF for 10 DIV. Under these conditions, control and

ATRA-treated clones produced identical numbers of secondary spheres (139±32 versus 156±33 for control and ATRA-treated cultures, respectively) after single clone dissociation ($p=0.1$) with each sphere producing at least one sphere after dissociation. These data support the contention that stem cell self renewal and expansion are not affected by ATRA treatment.

5.3 DISCUSSION

Clonal analysis of stem cell-derived progeny suggests that the stem cell itself may be induced by ATRA to increase neuron production even before its first division, with no effect on stem cell self renewal or expansion. The reason for smaller clone size after transient ATRA treatment is unclear. It is possible that ATRA triggers a "counting mechanism", as has been suggested by Barres et al. (1994) for oligodendrocyte precursors, resulting in less cell turnover by the cell exposed to the retinoid signal. Alternatively, ATRA may slow cell cycle progression in stem cell-derived progeny. Presumably cell cycle length of the stem cell is not altered, since the numbers of spheres produced from an ATRA-treated clone is identical to controls as indicated by single sphere dissociations. Based on the bulk culture data shown in Fig. 4 and Fig. 5, it is possible that the differentiation of mitotically active astrocyte precursors simply reduces the population of dividing cells, leading to smaller clone size in response to ATRA. It is possible that selective cell death may also play a roll in making ATRA-treated clones smaller, however the data presented here neither support nor refute that possibility.

It would have been interesting to know the exact numbers of cells representing all three phenotypes within the clones, in order to ascertain whether the stem cell is indeed producing more neurons at the expense of another cell type. There is evidence from studies on the neural crest that not only does ATRA promote the proliferation and survival of neuronal precursors (Henion and Weston, 1994), but that ATRA may affect the developmental options of mouse neural crest stem cells. Ito and Morita (1995)

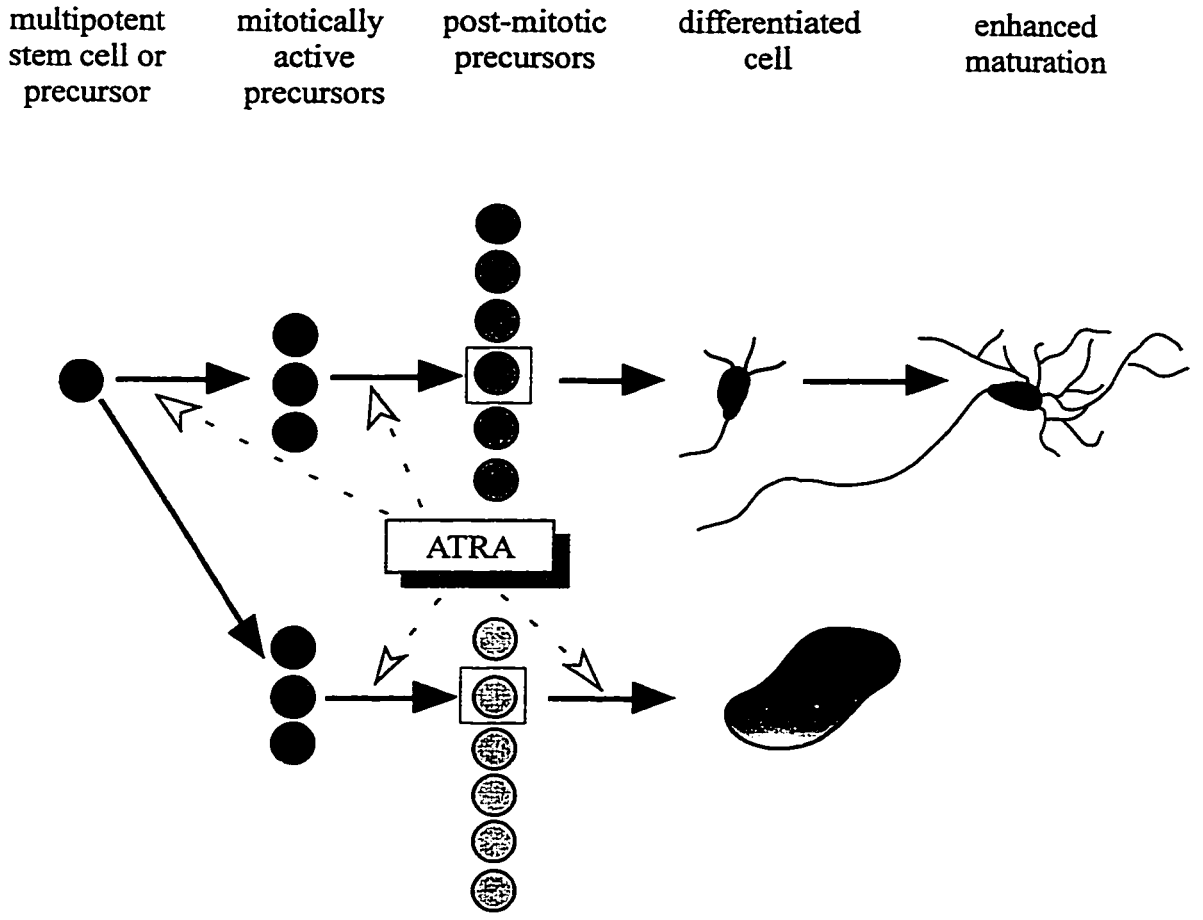
suggest that ATRA is likely to act upon multipotent mouse neural crest cells and influence the expression of developmental capacities. In other words, that ATRA can alter the developmental options of multipotent cells while also affecting the proliferation and/or survival of a particular lineage. In the study by Ito and Morita, proliferation of serotonergic neurogenesis from neural crest was accompanied by suppression of melanogenesis. Several studies from other tissues suggest that ATRA plays a role in cell fate specification. In the retina, rods are produced at the expense of amacrine cells in response to ATRA exposure (Kelly et al., 1994). In the tooth, ATRA mediated regulation of Notch gene expression in the overlying epithelium is thought to control the decision of a mesenchymal cell to adopt an ameloblast fate (Mitsiadis et al, 1995). It would have been very interesting to know if a similar phenomenon occurs in our cultures in response to ATRA in the clonal analysis study. *In vivo* retroviral tracing studies in the CNS have provided support for the existence of a precursor which can produce either oligodendrocytes or neurons (Price and Thurlow, 1988; Williams et al., 1991). Unfortunately, due to the limitations of getting an accurate count for all other cell types aside from neurons in the clonal study (most notably oligodendrocytes), it is impossible to say with any certainty that a "switch" factor is being activated upon ATRA exposure.

6 GENERAL CONCLUSIONS

These studies have shown that ATRA could act at the level of the EGF-responsive stem cell to enhance neurogenesis and may act at the level of more restricted EGF-generated precursors (Fig. 10). The mechanism by which a cell decides to adopt a certain fate either by differentiating into a unique cell type or choosing to proliferate or die is elusive. It is important to note that each clonally derived cluster of cells is a tightly clustered group of cells where cell-cell interactions could in part, mediate cell fate decisions. Cell-cell interactions play a crucial role in specifying cell fate during development. In this respect, it is interesting to ponder the development of the early nervous system, where equipotent cells can adopt different identities and behaviors (Vervoort et al., 1997). In the Drosophila embryo, a transmembrane receptor controls cell fate decisions in clusters of cells with equivalent developmental potential in many tissues, such as the nervous system and muscle (Artanvanis-Tsakonas et al., 1991; Corbin et al., 1991). In the nervous system, cells are specified to become neuronal or epidermal through interactions between neighboring cells. Loss-of-function alleles of the transmembrane protein Notch lead to production of excess neurons at the expense of epidermis. Several members of the mammalian Notch gene have been identified (Notch 1-4; reviewed in Weinmaster, 1997). In the developing retina, the numbers of ganglion cells produced is inversely related to the level of Notch 1 and 2 activity (Austin et al., 1995). During the process of haemopoesis, activated Notch 1 influences the choice between CD4 and CD8 T cell lineages. A study of the developing mouse tooth draws a

Figure 10. Possible sites of action of ATRA on EGF-responsive stem cells and their progeny

Bulk culture studies suggest that ATRA can act on a population of mitotically active precursors to enhance neurogenesis as well as astrocyte differentiation. Clonal culture data suggests that the stem cell itself can be influenced before its first division to enhance neurogenesis.


multipotent stem cell or precursor
mitotically active precursors
post-mitotic precursors
differentiated cell
enhanced maturation
ATRA

very interesting connection between ATRA treatment and Notch activation. ATRA upregulates Notch 1 and 2 expression, resulting in an alteration of the number of cells which adopt an ameloblast cell fate. Interestingly, Notch 1 and 3 (found in the developing striatum) mRNA expression was upregulated in spheres treated with ATRA after 12-18 hours in culture as indicated by PCR-Southern (C. Wohl, unpublished observations). Although preliminary antisense experiments against Notch 1 were inconclusive, a retrovirally mediated antisense approach would be a sensible and interesting study to further understand the downstream effectors of ATRA actions.

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IMAGE EVALUATION
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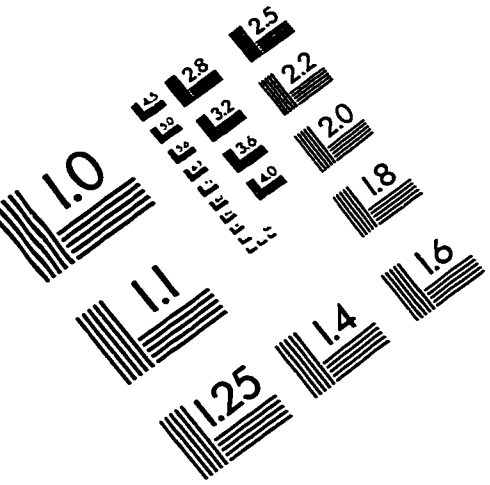

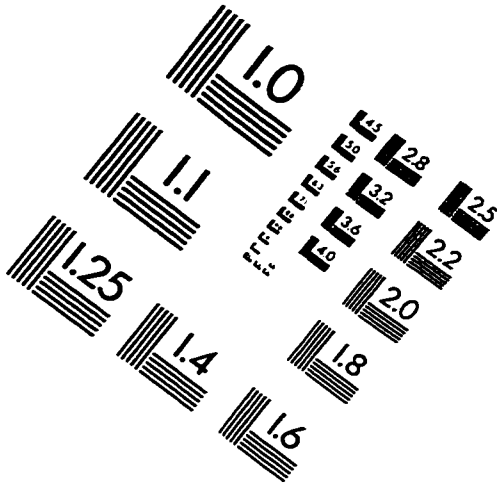

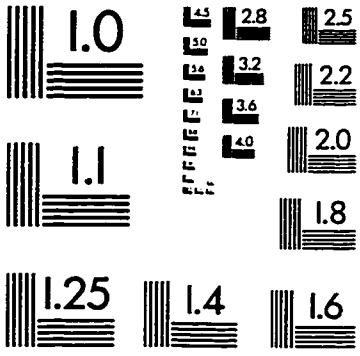

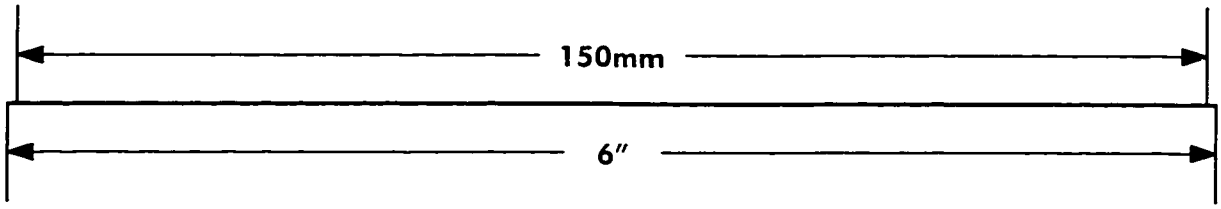

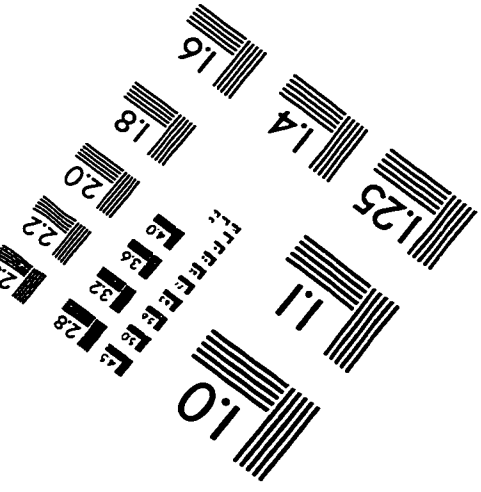

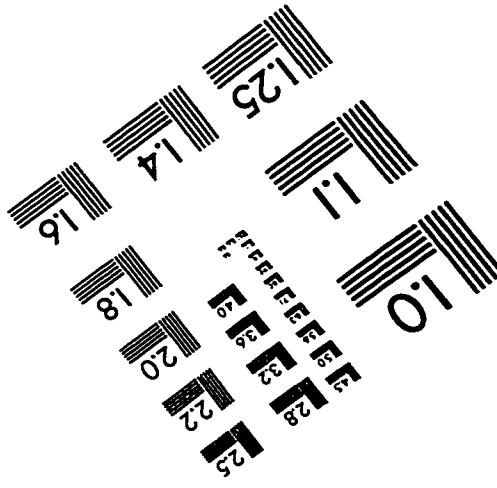

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