STUDIES ON DIFFERENTIATION OF NEUROBLASTOMA, USING NG108-15 CELLS AS A MODEL

By

© AZIZ GHAHARY

A Thesis submitted to the Faculty of Graduate studies of the University of Manitoba in partial fulfillment of the requirments for the Degree of

DOCTOR OF PHILOSOPHY

1988

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ISBN 0-315-47832-2

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TO

MY LOVELY SONS :

ALI, AMIR AND AHMAD

AND

MY SPECIAL WIFE

RUHI

Acknowledgements

I would like to express my sincere thanks to Dr. K.W. Cheng, my thesis supervisor, for his support of my research project, and his trust in allowing me to search for answers of the questions that I was searching for. I wish to thank the members of my advisory committee Drs. K. Dakshinamurti, R.P.C. Shiu, and P. K. Singal, for reviewing my thesis and for their advice and constructive comentaries during the course of my training. I am extremely grateful to Dr. S.K. Malhotra for his encouragement and for reviewing this thesis. Special thanks go to my best friend, Dr. R. Sanii, who facilitated my study at the University of Manitoba. I will always remember the kind co-operation of my colleague, P. McPherson. I also thank Mr. G. Queen for his assistance in the computer analyses of some of our data. The technical assistance of Mrs. E. Ling is gratefully acknowledged.

This work would not have been possible without the kind support and encouragement of my sister in law, Farahangiz Kilani-Salimi and her understanding husband, Mahmood.

I especially thank my very special sons : Ali, Amir and Ahmad for their patience, understanding and love.

Finally, I wish to thank my wife, Ruhi, for her constant support and encouragement before and during the course of my study.

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Abbreviations

QNB	Quinuclidinyl benzilate
DHA	Dihydroalprenolol
NE	Norepinephrine
ISO	Isoproterenol
DA	Dopamine
Dopac	Dihydroxyphenylacetic acid
L-dopa	3, 4- dihydroxyphenylalanine
5-HT	Serotonin
HVA	Homovanillic acid
5-HIAA	Hydroxyindolacetic acid
3-MT	Methoxytyromine
GABA	Gamma-aminobutyric acid
Ach	Acetylcholine
AchR	Acetylcholine receptor
CAT	Choline acetyltransferase
Achase	Acetylcholinesterase
DBH	Dopamine beta-hydroxylase
GAD	Glutamic acid decarboxylase
TH	Tyrosine hydroxylase
FCS	Fetal calf serum
DMEM	Dulbecco's modified Eagle's medium
CSF	Cerebral spinal fluid
BSA	Bovine serum albumin
Kd	Affinity constant at equilibrium
(K+1)	Association rate constant
(K-1)	Dissociation rate constant

Abbreviations

Bmax	Total binding at equilibrium
Ln	Natural log
Beq	Bound at equilibrium
Bt	Bound at indicated time
Ic50	Concentration of a ligand to inhibit 50% of total bindings
nH	Hill coefficient
MW	Molecular weight
Kda	Kilodalton
NGF	Nerve growth factor
NRF	Neurite retraction factor
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
PDGF	Platelet derived growth factor
TGF	Transforming growth factor
ECGF	Endothelial cell growth factor
NSILA	Non-supressible insulin-like activity
HPLC-EC	High performance liquid chromatography-electrochemical
dBcAMP	Dibutyryl cyclic adenosine monophophate
PG	Prostaglandin
Brdu	Bromodeoxyuridine
DMSO	Dimethylsulfoxide
Dx	Dexamethasone
HMBA	Hexamethylene bisacetamide
GCM	Glial conditioned medium
SDS	Sodium dodecyl sulfate

Abbreviation

PAGE	Polyacrylamide gel electrophoresis
TEMED	Tetramethyl-ethylenediamine
IEF	Isoelectric focusing
EDTA	Ethylene diamine tetraacetic acid
g	gram
mg	milligram
ug	microgram
ng	nanogram
pg	picogram
M	Molar
mM	millimolar
uM	micromolar
nM	nanomolar
pM	picomolar
l	liter
ml	milliliter
ul	microliter
hr	hour
min	minute
x g	Gravitational force
ND	Not detectable
SEM	Standard error mean
mA	milliamper
V	Volt

SUMMARY

We have used the neuroblastoma x glioma hybrid NG108-15 cell as a model to study the biochemical and morphological changes upon differentiation, induced by dBcAMP.

The specific binding of [^{3}H]-QNB to muscarinic acetylcholine receptors on intact NG108-15 cells increases gradually to 130% over that on control cells during the first 24 hrs of differentiation. The Kd and Bmax are 5.6 nM and 0.23 nM, respectively, for differentiated cells, and 4.4 nM and 0.10 nM, respectively, for undifferentiated cells. Analysis of data indicates a single class of binding sites on both differentiated and undifferentiated cells. Hill coefficient of 1.0 and 0.91 for differentiated and undifferentiated cells, respectively, suggest no receptor cooperativity. These results indicate that an increase of muscarinic acetylcholine receptors on intact cells correlates with the degree of cellular differentiation.

The presence of B-adrenergic receptor on intact NG108-15 cells has been identified by using [^{3}H]-DHA and unlabelled L-alprenolol. The binding is saturable, rapid, and reversible, having t 1/2 of 1.0 min for association and 3.5 min for dissociation. By Scatchard analysis, Kd and Bmax have been estimated to be 2.5 and 0.23 nM, respectively. Analysis of data reveals a single receptor type on intact NG108-15 cells, and a Hill coefficient of 1.0 indicating no cooperativity between binding sites. Other kinetic properties, including on-rate (K+1), off-rate (K-1)

and inhibition (Ki) constants have been determined to be 0.7×10^{-9} M min^{-1}, 0.19 min^{-1} and 8.9×10^{-6}M, respectively. Concentration of L-alprenolol to inhibit 50% of total binding (Ic50) has been estimated to be 10 uM. Rank order potency of catecholamine agonists reveals a subtype of beta 2-adrenergic receptor. A comparison of the Ic50 values of beta-adrenergic receptors on isolated plasma membranes and intact cells under differentiated and undifferentiated condition, revealed a greater potency of catecholamines (agonists)to bind to the receptors on differentiated cells. However,the maximum receptor binding on differentiated cells remained unchanged as compared to those on undiffeentiated cells.

Studies of proteins extracted from differentiated and undifferentiated NG108-15 cells by SDS-PAGE revealed several minor differences in protein patterns. A comparison of the protein profiles by 2-D PAGE (two-dimensional gel electrophoresis) labelled by either [^{3}H]-leucine or [^{35}S]-methionine, showed at least 12 protein spots varied in concentrations between differentiated and undifferentiated cells. It is speculated that some of these proteins may have a role(s) in the differentiation of NG108-15 cells, since the radiolabelled amino acid was incorporated into newly synthesized proteins during the early stages (24 hrs) of cellular differentiation.

Information on catecholamine content in NG108-15 cells, particularly during differentiation, has been lacking. In

the present study, the content of 5-HT, catecholamines and their major metabolites in differentiated and undifferentiated cells has been determined by HPLC-EC. Cellular contents of L-Dopa, NE, EPI, and Dopac are 149%, 40%, 129%, and 124%, respectively, higher in differentiated cells. Dopac, the major metabolite of DA, is detectable only in differentiated cells. A significant amount of Dopac has also been observed in the culture medium of differentiated cells. In contrast, 5-HT is detectable only in undifferen-tiated cells, and 5-HIAA, the major metabolite of 5-HT is markedly higher in undifferentiated cells. Our data indicate a shift in neurotransmitters from 5-HT to DA in NG108-15 cells during differentiation.

A serum factor, which induces rapid neurite retraction of morphologically differentiated NG108-15 cells, has been identified. This neurite retraction factor (NRF) has a MW of 70 Kda, and appears to be acid and heat stable. The neurite retraction activity is dose-dependent with t 1/2 of 1.8 min. NRF is present in serum of various species but not in tissue extracts of many organs of the rat except spleen and liver, which cause slight neurite retraction. It is noteworthy that NRF is not detectable in cerebral spinal fluid. Our data indicate that serum NRF differs from all of the well established growth factors, namely, NGF, EGF, PDGF, FGF, NSILA, ECGF, and TGF. The biological role(s) of this serum factor in the process of maturation and differentiation of developing neurons remains to be studied.

In summary, results of these studies reveal further information on correlation between morphological changes and biochemical modification, such as mAchR and neurotransmitters during neuronal differentiation. Our results on the identification and partial characterization of a serum NRF reveal the presence of a new factor restricting the process of maturation of the neuroblast cells.

INTRODUCTION

I. NEUROBLASTOMA

1-General Backgound

Neuroblastoma continues to be one of the most complex and challenging form of malignant disease in children (1). Neuroblastoma in infants was first described by Pepper (2) in 1901, when he reported a study of " Congenital Sarcoma of the Liver and Suprarenal " in an infant aged 4 weeks, who subsequently died in convulsions. This investigator further reviewed reports of other five infants with similar tumors and died. The disease was thought to be a congenital sarcoma of the liver and suprarenal.

In 1907, Hutchison (3) described a series of seven children who had tumors of the suprarenal gland that had metastasized to the orbit, a characteristic clinical picture in children with neuroblastomas. Pediatricians gradually became aware of neuroblastomas and its association with a better prognosis in younger children than older ones (1).

In 1971, D' Angio et al. (4) drew their attentions to a characteristic form of the disease found only in infants. However, later reports have revealed that neuroblastomas can occur rarely in adults (5). D'Angio et al. (4) segregated children with regionally limited primary disease as stage 1 and 2, who were found to have tumor involving the liver, skin, or subcutaneous tissue and / or bone marrow (IV-S category). Most of these children (18 out of 20) who were under 13 months of age at the time of diagnosis survived. After approximately Seventy

years (1974), neuroblastomas have been reported to be the third commonest neoplasm among children (6), exceeded only by leukaemia and cerebral tumors.

Neuroblastoma has been of great interest for many years because of its development, arising not only in the adrenal medulla but also from the sympathetic ganglia. In the embryo, neuroblast arises from the primitive neural crest and develops into the mature sympathetic ganglion cells. Neuroblastomas may arise from neuroblast, which is not undergoing differentiation and remains as tumor cells. Neuroblastoma is a tumor, composed mainly of immature, and/or poorly differentiated sympathoblasts (7). Although the tumor may consist of both malignant and differentiated cells, the metastatic lesions contain only malignant cells (8). The occurence of tumors in sympathetic sites and the presence of adrenergic cells containing tyrosine hydroxylase are taken as supporting evidence for their sympathetic origin (9). However, later reports (10) indicated that tumors may arise from any dividing neuronal cells. This has been demonstrated by the fact that mouse neuroblastomas always contain more than two types of nerve cells including adrenergic, cholinergic and serotonergic cells (11). The other important biologic behavior of neuroblastomas includes a high rate of spontaneous regression and maturation from neuroblastomas to benign ganglioneuroma (12). Evidence for the spontaneous regression of malignant tumor is now considerable, and by no means are such regressions due to immune responses,

endocrine influences, and interference with the nutrition of a tumor (13). In some instance, particularly well illustrated in neuroblastomas, regression may involve a maturation and differentiation of neuroblastoma cells into ganglion cells, which have lost their malignant properties (14).

In vitro investigation of the differentiation phenomenon of neuroblastomas began in 1947, when the first *in vitro* cultivation of neuroblastoma aspirates was demostrated. Murry and Scout (15) for the first time were able to explant the human neuroblastoma aspirates into plasma clot cultures and demonstrated the capacity of neurite outgrowth of these cells. The potentiality of neuroblastoma cells to undergo morphological changes initially in plasma clot cultures and later in tissue culture system has been confirmed by many investigators (16, 17, 18, 19). It is now believed that the malignancy of neuronal cells may be the result of an abnormal regulation of differentiation. This is demostrated by the fact that many differentiating functions, which are characteristics of mature neurons, are expressed in these cells in varying degrees (9). It has been reported (20) that when the differentiation of neuroblastoma cells was actively induced,their oncogenicity disappeared. Evidence for differentiation and maturation comes from histological examination of spontaneous and experimental tumors as well as studies with tumor cell lines *in vitro* (21). Therefore, the ability of malignant cells to initiate the normal program of differentiation is

of interest not only in model system to study development but also for insights into the reversible nature of neoplasia.

2. Incidence

Neuroblastoma, the commonest extracranial solid malignancy in children, rarely occurs in adults (5). This primitive neuronal malignancy generally arises during fetal development or in early childhood (22). Neuroblastoma has been estimated to constitute approximately 7 - 14 % of childhood malignancies and 15 - 50 % of neonatal malignancies (23). The peak incidence of this disease is the first three years of life. The majority (68 %) of these patients are under two years of age (5). The incidence of metastases in infants under 1 year of age is 60% as compared to an 87% incidence in patients over 1 year of age.. Bone metastases, an ominous prognostic sign, is also less frequent in the patients under 1 year of age; 36 % in contrast to 60 % in the older group (24). It has also been reported that neuroblastomas occured in one of every 10,000 live-born infants in USA (25) and one of every 20,000 infants screened in Japan (26). The early age distribution of the disease indicates that prenatal factors may play an important role in tumor induction particularly in prezygotic or prenatal exposure to carcinogens, such as petrochemicals, ionizing radiation and other suspected carcinogens (27). Studies on data obtained from 157 children, who died in Texas from neuroblastomas, revealed

that children of fathers employed in occupations with electromagnetic field exposure were at significantly increased risk of having neuroblastomas (27).

3. Familial Neuroblastomas

Knudson and Amronin (28) reviewed 504 unselected cases of neuroblastomas and estimated that as many as 22 % of all neuroblastomas could be hereditary. Regression analysis of the data by Knudson (29) indicated that this disease fits the two-mutation hypothesis for the origin of childhood cancer. According to this hypothesis, the non-hereditary form of neuroblastomas would result from two postzygotic mutations in a single cell, causing malignant transformation of the cell to develop into a single tumor. Hereditary tumors would arise in individuals who have either inherited or spontaneously acquired the first mutation as a prezygotic event (29). Later reports support the Knudson and Strong's observations (30).

When the nature of the genetic events in neuroblastomas is considered as non-random chromosomal abnormality, terminal deletion or rearrangement of the short arm of chromosome (1p) exists in a large percentage of neuroblastomas (31). Amplification and translocation of the N-myc oncogene have recently been detected as a frequent feature of human neuroblastomas (32). Abnormality in the proto-oncogene expression (possibly N-myc) causes inappropriately induction of cell replication and/or differentiation during embryogenesis (33). The association

of human neuroblastomas with the N-ras oncogene (34) has also been reported. However, despite of an estimated 22% incidence (28) of a predisposing germline mutation randomly in patients with neuroblastomas, a hereditary component to the disease is most likely to be present in a relatively selected group of patients (30). This leaves the large majority of neuroblastoma patients, in whom the likelihood of an inherited susceptibility is very low and whose siblings and offsprings are, therefore, at a correspondingly low risk for developing the disease (31).

4. Prognosis and Regression

Neuroblastoma is the classic tumor that illustrates the phenomenon of spontaneous regression (34). When dissemination of neuroblastomas occurs the malignancy is often fatal (35). However, a number of children have been reported to have spontaneous regression of this tumor despite of the presence of metastases. The most dramatic cases of regression occur in those infants who have disseminated disease involving liver, skin, or bone marrow (23). Bundey et al.(36) found that 47 patients of total of 630 cases of this disease recovered from either neuroblastomas or ganglioneuromas. However, in one case, recurrence of neuroblastomas was found in the liver with widespread of metastases, 20 years after treatment of a 3 month-old infant for stage IV-S disease (37).

Those patients, who eventually recover usually have certain characteristics in common. The most important

predictive features are the age of the child at the time of diagnosis and the pattern of organ involvement (35). Other investigators also confirmed that the age at which diagnosis is established is a major determinant in prognosis (38). Cure is more frequently encountered in children under one year of age. The sharp contrast in survival between the divergent younger and older age groups may lead to the suggestion of an intrinsic differences in the disease between the older and younger patients (23). Beckwith and Perrin (39) have estimated that the incidence of neuroblastomas in situ is about 40 times greater than the number of cases of clinically diagnosed disease. Based upon their findings, they proposed that the great majority of these tumors either degenerated or underwent differentiation to normal tissue. The site of origin has also been demonstrated to be a factor influencing survival of these children. A more favourable prognosis has been observed in children with mediastinal (neck and thorax) neuroblastomas (23,40). In contrast, tumors arising in the abdomen,particularly the adrenal gland, yield significantly poor results. The reason for this discrepancy is not known. Prognosis also depends on the degree of maturation. Thus the prognosis is better in a well differentiated ganglioneuroblastoma than in the malignant undifferentiated form of neuroblastomas (1). Further, the fact remains that infants with neuroblastomas with either a localized infiltered tumor or with a characteristic pattern of metastases have the prognosis much better than older

children. The survival rate for children under two years of age is about 75 %, as compared with only about 20 % in older children (1).

5. Stage of neuroblastoma

The stage system for neuroblastomas initially proposed by D'Angio et al.(4) is based principally on extent of the disease.

Stage I. Tumor confined to organ or structure of origin.

Stage II. Tumor extends in continuity beyond the organ or structure of origin but does not cross the midline. Homolateral regional lymph nodes may be involved.

Stage III. Tumor extends in continuity beyond the midline. Bilateral regional lymph nodes may be involved.

Stage IV. Remote disease involves skeleton, parenchymatous organs, soft tissues or distant lymph node groups.

Stage IV-S (S for special). Stage I or II with remote disease confined to one or more of the following sites: liver, skin, or bone marrow with radiologic evidence of bone metastasis.

The relationship between stage and prognosis has been analysed in depth. Many patients with stage IV-S demonstrated spontaneous tumor regression (34, 41). Those patients with remote metastases involving skeleton, soft tissue, or distant lymph nodes had the worst prognosis (34). Reasons for spontaneous regression of neuroblastomas are still controversial. Necrobiosis of tumors (41), degeneration and maturation of neuroblastoma

cells into ganglioneuroma cells and ganglioneuroblastoma cells (38) are some of the reasons considered for spontaneous disappearance of neuroblastomas in children.

6. Primary Sites

Neuroblastomas are derived from the neural crest, and develop into any site, at which one would normally find elements of the sympathetic nervous system. Thus the adrenal medulla and any segment of the sympathetic chain of the neck, thorax, abdomen and pelvis can be primary site of neuroblastomas. The occurance of tumor at these sites and the presence of adrenergic cells containing tyrosine hydroxylase have been taken as supporting evidence for their sympathetic origin (9). However later report (10) indicated that tumors may arise from any dividing neuroblast.

Studies of 144 patients by Stella et al.(24) on the distribution of primary neuroblastomas in children showed that the most common sites for neuroblastomas were abdomen (43 %), and adrenal (29.2 %). Other tumor sites were chest (13.6 %), neck (3.2 %), head (0.2 %) and other organs (11.3 %). These investigators also showed that bone and lymph node metastases were also frequent sites of occurance. The liver was less frequently involved, and lung metastases were uncommon.

7. Differentiation of Neuroblastoma

The term " differentiation " refers to the processes, involved in producing cells to become different in some way

from the parent cells. Many characteristic functions in differentiated and mature neurons have been identified and measured, including formation of neurites, increase in size of soma and nucleus associated with a rise in total RNA and protein content, an electrically excitable membrane, inhibition of cell division and high activities of neural enzymes (9). Prasad and Hsie (42) and Prasad (43) treated mouse neuroblastoma with dBcAMP and PGE1 to induce differentiation, using the formation of neurites (cytoplasmic extensions greater than 50 um long) as an expression of morphological differentiation, and the enlargement of the nucleus and cell size as an indication of morphological maturation. The most common parameter for measuring morphological differentiation has been neurite extension, which begins within a few hours after exposure of cells to neurite outgrowth inducing agents including dBcAMP (9).

Studies of Beckwith and Martin (44) on the relationship between maturation and prognosis have revealed the existence of various degree of differentiation in neuroblastoma tumors. A system of histologic grading has been derived on the basis of degrees of maturation as follows:

Grade I. Predominantly differentiated: over 50% differentiating elements.

Grade II. Predominantly undifferentiated: 5 - 50% differentiating elements.

Grade III. Slightly differentiated: under 5 % differentiating elements.

Grade IV. Undifferentiated: no recognizable neurogenesis. Using this system, these investigators have shown a clear relationship of maturation to survival (44) and thus explained the poor prognosis observed for adrenal neuroblastomas with undifferentiated cells.

Reynolds and Smith (45) have compared the behavior of neuroblastoma cells explanted into culture medium under standardized conditions and that of the same tumors remained in the patients. Based on the behavior of the tumor cells in culture, two biologic classes of neuroblastomas have been identified. Tumor cells that attach and extend neurites in culture, but do not proliferate in culture, are designated class I. Tumor cells that proliferate and could be subcultured are designated as class II. These investigators have shown that none of the patients with class II neuroblastomas survived more than 15 months with an average duration of 9 months; whereas, 67% of the class I patients survived over 38 months with an average duration of 18 months (45). This biologic classification correlates well with other clinical parameters affecting prognosis, including age, stage of disease, and site of primary tumors.

II. NEUROBLASTOMA CELLS IN CULTURE SYSTEM

1. Cell Culture

The ability to culture neural cells provides a unique tool for studies of certain aspects of developmental process, including differentiation of neuronal cells.

Neural cultures were first established by Harrison in 1907 (46), when fragments of frog neural tube were maintained *in vitro* for a week, utilizing frog lymph as a culture medium. Since that time, synthetic media supplemented with serum and sometimes other undefined components have been routinely employed for both mammalian tissue and cell cultures. At the present time, neural explant cultures can be maintained for several months and dissociated neural cells cultured for periods of a week to several months.

Tumorgenic neuronal and glial cell lines proliferate indefinitely when subcultured at confluence (47). Murray and Scout (15) showed the first explantation of neuroblastoma cells in plasma-clot cultures and observed a rapid outgrowth of neurites from clumps of neuroblastoma cells. In 1958, Goldstein and Pinkel (16) obtained neuroblastoma cells from bone-marrow aspirates of young children and cultivated *in vitro* into the medium containing 10 % fetal calf serum (FCS) and 10 % human ascitic fluid under cellophane for long periods. These investigators showed neurite extension, which formed dense, interlacing networks, grew out from the surviving fragments of neuroblastoma tissue. In addition to many immature neuro-blasts, in which mitoses were observed, large ganglion-like cells appeared. They concluded that the morphological changes in the cells during *in vitro* cultivation indicated the potentiality of neuroblastoma to differntiate into more mature-looking ganglion cells. In 1964, Goldstein and Burdman (17) cultured neuroblastoma cells

obtained from 13 young children in a medium containing 10 % FCS and human ascitic fluid for a period of 1 week to over a year. They demonstrated that *in vitro* culture of neuroblastoma cells resulted in differentiation and maturation of these cells within 5 months. The morphological evidence for maturation of neuroblastoma cells demonstrated to be the formation of neurofibrils and hypertrophy of axons. They also observed that when tissue from a more mature-appearing tumor was explanted, cells with the morphology of mature ganglion cells developed within 20 days (17). From this observation, they suggested that neuroblastoma cells might be the results of a biochemical defect which prevents maturation of nerve cells.

In 1969, two groups of investigators, Augusti-Tocco and Sato (18) and Klebe and Ruddle (19), described the first adaptation of a murine neuroblastoma C 1300 tumor line into *in vitro* culture. The C 1300 mouse neuroblastoma is a spontaneous tumor of the spinal cord, and has a similar morphology with neuroblastoma cells. Augusti-Tocco and Sato (18) demonstrated that when C 1300 neuroblastoma cells were placed into culture, the cells underwent a striking change in morphology, exhibiting a large number of elongated processes. Klebe and Ruddle (19) treated a clonal cell line of Neuro-2a, derived from C 1300 mouse neuroblastomas, with 10^{-4} M FudR and uridine for 4 days and observed a population of cells with neuronal morphology and a simultaneous elimination of all dividing

ameboid cells. It is concluded that the neuroblastoma stem cells could differentiate into a neuronal cell type without physical contact with other cells.

Since the initial reports of the establishment of neuronal cell lines that expressed differentiation properties, neurobiologists have made extensive use on these cells for studies involving the regulation of neuronal differentiation. Several hundred clones and subclones of mouse, human, and rat neuroblastomas which vary in their morphological, biochemical, and electrophysiological properties have subsequently been isolated and characterized (47). In spite of mouse neuroblastoma cell lines which have all been derived from a single C 1300 tumor, human neuroblastoma cell lines have been obtained from a variety of peripheral nervous system tumors (48). The mouse and human neuroblastoma cell lines constitute valuable models for investigations of neuronal differentiation of the peripheral nervous system (47).

2. Factors Inducing Differentiation

The availability of mouse and human neuroblastoma cells in cultures has provided a new biological tool for studies of the controlling mechanism(s) of differentiation in these cells. In 1971, Prasad and Hsie (42) showed that dBcAMP induced axon formation as early as 24 hrs after the addition of this agent in the culture medium. The maximum number of cells with axons, however, was seen 3 - 5 days after dBcAMP treatment. These investigators concluded that

metabolic changes were necessary for the expression of differentiation phenotype. Later reports (49) confirmed the hypothesis that cAMP may be an important molecule in the differentiation process of mouse neuroblastomas. Upon the initial report of the possible role of cAMP in morphological changes in neuroblastoma cells, the information of agents causing neurite formation in neuroblastomas dramatically increased. In 1972, Prasad and Sheppard (50) reported that inhibition of cyclic-nucleotide phosphodiesterase, an enzyme which degrades 3'5' cyclic AMP to 5'AMP, using papaverine induced _in vitro_ differentiation of mouse neuroblastoma cells. Prostaglandins (PGE 1 and 2) were also reported (43) to induce irreversible morphological changes of mouse neuroblastoma cells in culture as shown by axon formation, whereas prostaglandin F 2 alpha did not. Seeds et al. (51) demonstrated that removal of serum from culture medium resulted in axon outgrowth of neuroblastoma cells. Neurite extension is not inhibited by cyclohexamide but was sensitive to colchicine or vinblastine, suggesting that neurite formation is dependent upon the assembly of microtubules or neurofilaments from preformed protein subunits. These investigators observed that 70 - 85 % of neuroblastoma cells extended processes within 60 min after removal of serum, suggesting that cells are capable of extending processes during most of the cell cycle. It has also been reported (52) that X- irradiation induced morphological differentiation of mouse neuroblastoma cells

even in medium containing fetal calf serum. Morphological changes were evident as early as 24 hrs after irradiation and by the third day most differentiated cells had matured morphologically as shown by formation of axons, and enlargement of the nucleus and cell size. These investigators believed that X-irradiation inhibits the growth of neuroblastoma cells and this effect is dose dependent. Radiation is known to inhibit cell division and thus apparently inhibition of cell division is mandatory for morphological differentiation. The fact that serum-free medium blocked cell multiplication and induced axon formation in neuroblastoma cells (51) tends to support this contention.

Schubert and Jacob (53) demonstrated that 5-bromodeoxy uridine (Brdu) induced differentiation of mouse neuroblastoma C 1300 cells to morphologically resemble mature neurons. Induction of differentiation of neuroblastoma cells, even in the absence of DNA synthesis, indicates that the halogenated pyrimidine is not necessary to be incorporated into DNA to alter the phenotype of neuroblastoma cells. These investigators speculated that Brdu causes metabolic changes, which indirectly induce differentiation of the neuroblasts. In addition, it has been demonstrated that the expression of the differentiated state is dependent on protein and microtubule synthesis. The possible involvement of macromelecular synthesis on cAMP levels, but not RNA or DNA synthesis has been reported (49). From the inhibitors of macromolecular synthesis

tested, only cyclohexamide has any noticeable effect on the basal level of cAMP. Agents such as actinomycin D, thymidine, cytochalasin B,has been shown to inhibit growth of neuroblastoma cells but have no effect on cellular cAMP levels, nor do they induce morphological differentiation (49).

Sheppard and Prasad (49) studied the possible mechanism(s) of some of the inducing agents or conditions on morphological differentiation, and demonstrated that inhibitors of phosphodiesterase, PGE1 and serum deprivation increased cellular levels of cAMP. However, the rate of increase in the level of cAMP differed depending on the agent or condition used. These investigators concluded that all agents and conditions which stimulate axon extension also increase the cell's cAMP level and that all agents which increase cAMP levels of neuroblastoma cells continue to do so although axons may or may not be extended. The reason why cAMP levels change in serum-free medium is not yet known (49). But it becomes clear that inhibition of phosphodiesterase, which degrades 3'5' cAMP to 5'AMP would naturally lead to an increase of endogeneous cAMP level. Prostaglandin E1 is thought to stimulate adenylate cyclase activity (42) and could thus account for its stimulation of the cAMP level. Subatance P has also been reported (54) to increase the level of cAMP. However, the mechanism(s) of dimethylsulfoxide (DMSO)(55), hexamethylene bisacetamide (HMBA)(56),and haemin (57) to induce differentiation in neuroblastoma cells remains undetermined, though

haemin has been reported to cause membrane stabilization or inhibit protein kinase of neuroblastoma cells.

Nerve growth factor which has been established to have biological effects on sympathetic and sensory ganglion cells (58), causes nerve processes and synthesis of microtubule proteins in human neuroblastoma cells (59). However, neurite outgrowth has not been observed in mouse neuroblastoma cells upon in vitro treatment with nerve growth factor (58).

The role of cell membrane in morphological diferentiation of neuroblastoma cells has also been studied. In 1978, Koike (60) demonstrated that K+ ionophore, valinomycin treatment caused mouse neuroblastoma cells to undergo morphological differentiation. This effect was blocked by increasing concentration of KCl in the medium, suggesting that changes in resting membrane potential and ion fluxes may be involved in the mechanism of the formation of neurite outgrowth in neuroblastoma cells.

3. Reversibility and Irreversibility of Morphological Differentiation

It has been reported that dBcAMP (42), PGE1 (43), X-irradiation (52) and cAMP phosphodiesterase inhibitors (50) induce irreversible morphological differentiation of neuroblastoma cells, and this irreversibility is time dependent. Prasad and Sheppard (50) demonstrated that when papaverine, a cAMP inhibitor, was removed and fresh growth medium was replenished at 1 or 2 days after treatment, the

papaverine effect, as measured 2 days later, was reversible. But when the drug was removed 3 days after treatment, the number of differentiated cells as measured 2 days later remained unchanged. This observation indicates that the differentiation phenotype is irreversible after 3 days of exposure to papaverine. Similar property has also been reported for PGE1 (43). Upon removal of PGE1 at day 3 after treatment, the number of differentiated cells, 2 days later, was approximately 50 % of the control, in which PGE1 had not been removed. The reduction in the number of differentiated cells after the removal of PGE1 is explained to be due partially to death of the differentiated cells and partially to retraction of some of the axons. If PGE1 was removed 1 day after treatment, the number of morphologically differentiated cells was similar to the untreated control cells, indicating that differentiated cells are capable of retracting axons during the initial period of differentiation. Irreversibility of morphologically differentiated neuroblastoma cells induced by dBcAMP has also been explored (42). Removal of this agent at day 3 did not cause renewal of cell division, indicating that morphological differentiation of neuroblastoma cells induced by dBcAMP becomes irreversible.

In most studies, reversibility of morphologically differentiated neuroblastoma cells is demonstrated to be due to neurite retraction after replenishment of fresh growth medium. However, the cause of neurite retraction has not been extensively explored. It has been well

established that serum plays a major role in cell proliferation. Studies of Seeds et al.(51) revealed that most neuroblastoma cells cultured in the presence of 10 % FCS exhibit round morphology and adhere to one another, forming clusters; whereas, cells incubated in serum-free medium show rapid extension of axon-like processes. These investigators demonstrated further that the rate of appearance of neurites was inversely related to serum concentration. Addition of 10 % FCS to neuroblastoma cells grown in medium containing low concentration of 1 % FCS for 4 days resulted in disappearance of neurites followed by cell multiplication, suggesting that neuroblastoma cells retract their processes prior to mitosis. However, the cause of neurite retraction upon addition of serum to morphologically differentiated cells has not been extensively studied.

III- BIOCHEMICAL CHANGES IN DIFFERENTIATION

1 - Membrane Changes

Differentiation of mouse neuroblastoma cells _in vitro_ is correlated with alterations in some cell surface components. Cells that extend neurites show differences in cell surface glycopeptides. This also occurs during the transition from logarithmic to stationary growth phases (61). In 1979, Littauer et al. (62) demonstrated the modulation of a membrane glycoprotein with MW of approximately 200 Kda in concert with active ionic flux in a human neuroblastoma cell line upon induced differentia-

tion with 2% DMSO treatment, suggesting a membrane maturation similar to neuronal cells. Clone PC12 cells (pheochromocytoma), derived from a rat adrenal medullary tumor, form functional cholinergic synapses with L6 myotube cells upon differentiation induced by either NGF or dBcAMP (63). Electrical excitability of mouse neuroblastoma NIE-115 is markedly increased, concomitant with morphological maturation (64). Upon treatment with B-NGF, human neuroblastoma SY.5Y cells undergo morphological changes similar to those of dissociated chick embryonic dorsal root ganglion and dissociated sympathetic ganglion cells (65). In the presence of NGF, SY.5Y cells extend neurites, cease multiplying, and aggregate into clumps, similar to those observed in primary cultures of sympathetic ganglion cells treated with NGF. Further analysis of these differentiated cells has shown that they have developed electerically excitable membranes. Treatment of SY-5Y cells with dBcAMP also induces neurite outgrowth and enhances electrical excitability.

Changes of external surface membrane proteins have also been observed in differentiated mouse neuroblastoma cells induced by 2% DMSO (61). Most of these changes are clonal-specific, while others are common to several clones. A group of proteins with molecular weights between 150 Kda and 200 Kda has been found to be related to the transition of cells from logarithmic to confluent growth phases. An additional protein, with an apparent MW of 95 Kda, is observed to be common to the differentiated cells of the

two inducеable clones of N-18 and NIE-115 studied (61). However, in these studies (61), the expression of protein(s) at an early phase of differentiation, as a possible signal for initiation of differentiation, has not been reported. Changes in membrane phospholipids in neuroblastoma clone NB41A have also been reported (66) upon cellular differentiation induced by liposome treatment, suggesting a possible role of phospholipids in cellular differentiation.

2- Receptors

A variety of receptors for putative neurotransmitters and neuropeptides has been identified on C 1300 neuroblastoma clones (47). Matsuzawa and Nirenberg (67) have identified adenosine, muscarinic acetylcholine and prostaglandin E1 receptors on adrenergic NIE-115 neuroblastoma cells, and shown further that levels of both cAMP and cGMP increased upon activation of these receptors. The adrenergic NB-A2 clone exhibits a dopamine receptor-mediated increase in adenylate cyclase activity, but no effect is observed upon treatment with micromolar concentrations of acetylcholine or norepinephrine (NE) (9). Although various numbers of receptors, including PGE, A, B, F, I, nicotinic, muscarinic acetylcholine, histamine, opiate, and gamma-aminobutyric acid (GABA) receptors, have been identified on different neuroblastoma cell lines, namely, N4TG3, NB-A2, NIE-115 and N4TG1, but the characteristics of these receptors during morphological

differentiation of these cells, induced by neurite enhancing agents, have not been extensively studied. In general, human and rat neuroblastoma cell lines have not been studied as extensively as those of mouse origin (9), particularly in regard to charaterization of receptors during cellular differentiation.

3 - Neurotransmitters and Related Enzymes

Schubert et al.(63) have demonstrated that the activity of choline acetyltransferase (CAT) increased significantly, parallel to accumulation of acetycholine (Ach) in PC12 cells upon morphological differentiation induced by NGF. Other neurite enhancing agents, such as cAMP derivatives and phosphodiesterase inhibitors (thiophyline), have similar effects on the activity of CAT. Morphological differentiation induced by neurite enhancing agents, enable these cells to form cholinergic synapses with the clonal L6 myoblasts (63). Prasad (9) has demonstrated an increase of activity of tyrosine hydroxylase (TH) in NB-A2 and NB-P2 cells upon treatment with cAMP analogues or papaverine, a phosphodiesterase inhibitor. However, X-irradiation and serum deprivation do not affect the activity of this enzyme. Acetylcholinesterase (Achase) in neuroblastoma cells is enhanced upon differentiation induced by neurite outgrowth inducing agents (9). The activity of this enzyme is also increased in cells, which have stopped cell division after treatment with 5' AMP and sodium butyrate (47). These observations

suggest that morphological differentiation of neuroblastoma might not be the necessary event for the increase of Achase. Although it has been confirmed (47) that several inducers of morphological differentiation increased enzyme activities of CAT, TH, dopamine beta-hydroxylase (DBH) and glutamic acid decarboxylase (GAD) in some neuroblastoma cell lines, studies of Kimhi et al.(55) on NIE-115 cells have not revealed a correlation between activities of TH and Achase with morphological differentiation induced by dimethylsulfoxide (DMSO). However, differentiated cells, induced by either PGE1 or RO20-1724 (cAMP phosphodiesterase inhibitor), have relatively high activities of adeneylate cyclase and cAMP phosphodiesterase, parallel to the intracellular level, of cAMP (68, 69). It is interesting to note that cerebral adenylate cyclase and phosphodiesterase activities in adult hamster become doubled during maturation of brain cells (70), similar to those observed in *in vitro* differentiated neuroblastoma cells (9).

4 - Cytoplasmic Changes

Relatively few studies have been reported on cytoplasmic changes of neuroblastoma cells in relation to differentiation (9). Croizat et al.(71) have analysed the poly(A)- containing mRNA from neuroblastoma cells at two different developmental stages: either as round immature neuroblasts grown in suspension condition, or as differentiated cells with morphological similarities to mature

neurons, attaching to the culture dish. Results of these studies have revealed an expression of 16S RNA transcribed by cells grown in suspension condition, but not by cells grown in monolayer (71). A significant increase in tubulin and actin, subunit proteins of microtubules and microfilaments, upon morphological differentiation of N18 and N103 mouse neuroblastoma cells induced by serum deprivation was reported (72). However, a later report (73) has not confirmed such an increase in content of these proteins, and instead, revealed by immunofluoresecent microscopy that structure reorganization of these proteins might have occured during morphological differentiation (73).

IV - DIFFERENTIATION DURING NEURONAL DEVELOPMENT

Considerable effort has been expended in endeavours to elucidate the mechanisms underlying the transformation of undifferentiated and apparently identical neural crest cells into various cell types constituting the peripheral nervous system (74). Manipulation of the cellular and hormonal environment of cultures of dissociated primary neurons have been used to explore the developmental potential of neurons and to investigate factors required for neuronal development (75). It is believed that neuronal maturation involves both morphological and biochemical changes. Smith et al.(76) have cultured the trunk neural crest of 3-day old chick embryos in the presence and absence of hydrocortizone and observed that

migrating neural crest cells differentiated into neuron-like cells with ability to synthesize and store considerable amounts of catecholamine. It is concluded that neural crest derivates would be potentially receptive, directly or indirectly, to glucocorticoids at the moment of undergoing gangliogenesis, with the induction of a number of developmental processes. Zurn et al.(77) have observed that the content and synthesis of catecholamine in dissociated superior cervical ganglion (SCG) neurons from 8-day and 12-day chick embryos increased with time upon 1, 2, and 3 weeks in culture. This increase is not due to neuronal growth but probably a specific increase in adrenergic differentiation. Seeds (78) has demonstrated a marked increase in specific activities of neuronal enzymes, such as Achase, CAT and TH, in primary culture of cells prepared from whole mouse fetal brain at 17 days of gestation. *In vitro* studies of Seeds (78) have also indicated that the highest increase in biochemical activities related to cholinergic and gabaergic development in brain occur between the first and third postnatal weeks, a time of active dentritic growth and synaptogenesis. Baetge et al.(79) have purified polysomal ploy(A)$^{+}$-mRNA for TH from PC12 neuroblastoma cells and shown that the expression of TH mRNA paralleled the enhanced TH activity upon differentiation of these cells, induced by dexamethasone (DX).

Louis (80) has described the ultrastructural development, together with the significant changes in number of

cellular organelles and neurotransmitter enzymes of neurons from 8-day old chick embryos. Tyrosine hydroxylase activity shows a 3-fold increase between 2 - 8 days of culture. It is surprised to detect TH activity in 8-day old chick embryonic brain cells *in vitro* after only 2 days in culture, because *in vivo* it can not be detected in chick embryos younger than 14 days. During the subsequent 6 days of culture, the TH activity increases rapidly, parallel to the development of neurites and appearance of synaptogenesis. It has also been documented that the phenotype expressed by the precursors of autonomic neurons depends on environmental cues arising from non-neuronal embryonic tissues through which they have had migrated. Fauquet et al. (81) have investigated the ability of isolated neural crest in culture to differentiate into autonomic neurons as a function of other embryonic tissues. When neural crest is explanted alone into tissue cultures, cells become attached to the bottom of the culture dish, but no morphological changes occur during 48 hrs of inoculation. The levels of neurotransmitters, catecholamines and acetylcholine, at this state are at the lowest level as compared to those treated with extracts of other embryonic tissues. In contrast to the relatively modest degree of neuronal differentiation occurring in neural crest cultures and co-cultures with heart, somite and hindgut tissues, large numbers of morphologically and biochemically identifiable nerve cells become developed in cultures of isolated sclerotome, which consists of trunk neural crest and

tissues into which they would have normally migrated. It is also demonstrated that when the crest cells are still migrating, no visible signs of neuronal differentiation appears and no catecholamine storage and synthesis can be detected histochemically or biochemically. However, once in culture, differentiation is extremely rapid, catecholamine related enzymes become functional within 24 hrs.

Maderspach et al.(82) have investigated the effect of Beta-adrenergic agonist of norepinephrine (NE) or glial condition medium (GCM) on Beta-adrenergic receptors and influencing the concentration of B-adrenergic receptors of neuronal cells. In contrast, morphological studies have revealed that neurons treated with either B-adrenergic agonist or GCM have longer and thicker processes than control. Prolonged treatment of neurons to 48 hrs has resulted in a 4-fold increase in receptor concentration, and a simultaneous increase of morphological differentiation. It is concluded that cellular differentiation and the development of B-adrenergic receptors are related.

V - MODELS FOR STUDIES ON NEURONAL DIFFERENTIATION

1 - Neuroblastoma cell lines

The anatomical, physiological and biochemical complexities of the mammalian nervous system and the difficulties in separating the various individual neural components have proven to be the major obstacles in elucidating the cellular and molecular mechanisms involved in the development of the nervous system. Not only are

there an abundance of complex cellular phenotypes, but also the interactions of neural cells are exceedingly complex (83, 84). One approach to this problem is to use culture methods in an attempt to simplify and control experimental variables (83). There are two basic types of cell cultures:1) primary culture in which tissue are removed from an organ, placed in a culture vessel in an appropriate fluid medium, and have a finite life time (up to many months); and 2) continuous cell lines which proliferate and can thus be subcultured and stored. Dissociation of brain cells and subsequent fractionation into cell populations enriched in neurons and glia, yield still impure materials of essentially nonviable cell bodies (85). Therefore, such preparations may be of limited use in biochemical and pharmacological studies. Continuous cell lines derived from neuronal or tumorgenic tissues have generated large numbers of cells in a short period of time for biochemical analysis (84).

Neuronal cell lines, particularly clonal lines derived from single cells, represent the simplest model available for neuronal differentiation. The ability of neuronal cell lines to proliferate indefinitely distinguishes themselves from primary cultures, in which neurons do not continuously divide (9). Goldstein and Pinkel (16) have established neuroblastoma cells in culture, which generated morphological differentiation and could be maintained _in vitro_ for long time. In intact tumor, neuroblastoma cells lack processes, but when established (18,84) in culture and

subcultured, these cells form rich networks of long processes. Further, these cells, characteristically as mature neurons, contain many microtubules and dense core vesicles,and when grown in suspension cultures, they retain their round process-free configuration, characteristic of solid tumor (86). Characterization of established mouse neuroblastoma has shown that these cells have excitable membranes and other neuronal properties (87), indicating the expression of genes for neuronal properties. Clonally inherited differences in phenotype have reported (88) that some neuroblastoma cell lines synthesize acetylcholine (Ach) (10), whereas, others catecholamines. Cells from neuroblastoma cell lines that synthesize Ach have failed to synapse with striated muscle cells when co-cultured with myotubes, which possess abundant nicotinic acetylcholine receptors, or with cardiac muscle cells, which possess muscarinic acetylcholine receptors (88). From these observations, Amano et al.(10) have concluded that neuroblastoma cells fail to form synapses with myotubes because of two reasons : 1) the extent of neural maturation and the ability of cells to form synapses are regulated and highly sensitive to environmental conditions, making it necessary to find conditions to yield populations of differentiated cells; 2) most, but not all, of the cholinergic neuroblastoma cell lines, indeed lack the reactions which are required for synapse formation.

Some populations of neuroblastoma cells can be shifted from a poorly differentiated, synapse incompetent state, to

a well differentiated, synapse competent state by fusion of two different cell types (10). These attempts have led to search for hybrid cells with strong expression of neuronal properties (83). Cell hybridization has been involved both clones derived from mouse neuroblastoma C 1300 and cell line C6, which has been cloned from a chemically induced rat glioma (89). These attempts have led to establishment of several hybrid cell lines, resulting from mouse neuroblastoma x mouse fibroblast, mouse neuroblastoma x human fibroblast and mouse neuroblastoma x rat glioma. Each of these cell line has its own characteristics. Hybrid cell lines from neuroblastoma x glioma cells have been the most studied models to answer questions pertinent to the nervous system (85). The usefulness of neuroblastoma x glioma cells relies on the simultaneous presence of many neuronal properties in a single cell. Their superiority is based on the fact that such properties are more strongly expressed in the hybrids than in the neuroblastoma clones (85).

2 - Neuroblastoma x Glioma NG108-15 Cell Line

Hybrids between mouse neuroblastomas and rat glioma cells display remarkable neuronal properties (90) and have been used as tools to obtain information on the mechamism underlying cellular differentiation (91). In contrast to their parental lines, they contain CAT activity (90), and are rather large cells capable of extending long processes with ability to fire action

potential in response to depolarization by either electrical current or acetylcholine (85). One hybrid cell line, which has been widely used for neuronal differentiation studies, is the NG108-15 cell line. This cell line is obtained (85) by somatic cell hybridization technique using Sandai virus-induced fusion of a rat neuroblastoma clone N18TG-2, a clone resistant to 6-thioguanine, and a mouse glial clone C6 BU-1, a 5-bromodeoxy-uridine resistant clone (92, 93). NG108-15 cells form synapses with cultured myotubes (93, 94), synthesize acetylcholine (95), have functional voltage-sensitive Ca^{+2} channels (96), small clear vesicles approximately 60 nm in diameter, and large dense core vesicles approximately 180 nm in diameter (97). Further, NG108-15 cells possess a variety of receptors, including PGE1 (98), adenosine (99), alpha-2-adrenergic (100), met-enkephaline (67),depolarizing muscarinic (101) and serotonin receptors (102). Some of these receptors, such as those for PGE1, mediate activation of adenylate cyclase; other receptors, such as met-enkephaline and muscarinic acetylcholine receptors, mediate inhibition of adenylate cyclase (88).

Treatment of NG108-15 cells with dBcAMP for 1-3 weeks produce extensive changes in their light and electromicroscopic appearance (97). Observations of Furuya et al.(103) have confirmed that NG108-15 cells treated with dBcAMP exhibit typical neuronal characteristics of having ultra-structure maturation within one week of dBcAMP treatment. Nirenberg et al. (88) have demonstrated that the level of

cAMP in NG108-15 cells increase for 5 -7 days, upon treatment with either PGE1 or thiophylline, followed by increases in innervation of myotubes by neurites and spontaneous secretion of Ach from these cells at synapses. Therefore,the NG108-15 cell line has been established to be a good model for neuronal studies including differentiation.

From the above litrature review of neuroblastoma, it is clear that disorder of differentiation of neuroblasts is one of the major problem in malignancy of neuroblastoma. To this important problem, two approaches seem to be logical: 1) to search for a way to stimulate neuroblastoma cells to regain their potentialities to undergo differentiation and 2) to search for a possible factor(s) which may restricts normal neuroblast to undergo differentiation as a possible cause for disorder of neuroblast differentiation. To achieve the first aim, several investigators (42,43,50,51,52,53) have attempted to induce neuroblastoma to undrego morphological differentiation similar to normal neuronal cells using various neurite inducing agents. Results of these studies showed that these agents including dBcAMP were able to potentiate neurite outgrowth with concomitant with a decrease in growth rate of neuroblastoma cells. However, morphological differentiation dose not mean biochemical differentiation. To our judgement, studies of correlation between morphological and biochemical differentiations are required to confirm _in vitro_ differentiation of neuroblastoma cells. The present study, therefore, is

proposed to examine the correlation betweem these two important phenmena using NG108-15 cells, which has been approved to be a very useful model for neuronal differentiation. To acieve this goal, it is proposed to treat NG108-15 cells with one of the neurite inducing agent, such as dBcAMP, to induce morphological changes, followed by analyses of some neuronal molecules, including acetylcholine and beta-adrenergic receptor, indolamines and their major metabolites, and newly synthesized proteins. The profiles of any changes in these molecules would be comparable to those of neuroblasts during developmental processes.

Our second prosposal is to search for a factor that possibly restricts differentiation of neuroblastom cells. Since removal of serum from culture medium of neuroblastoma cells leads to neurite extention of these cells, it is logical to speculate that such a factor(s) may exsist in serum. The second part of this study, therefore, is proposed to subculture NG108-15 cells in serum-free defined medium (104) in the presence of dBcAMP, followed by addition of aliquots of serum to concentrations of 1 - 10% into the culture medium. If serum causes dedifferentiation of morphologically differentiated neuroblastoma cells, the presence of neurite retraction factor (NRF) is assumed. Our preliminary experiments favour the presence of such a factor in FCS. This study is undertaken to identify and characterize this serum factor.

B - MATERIALS AND METHODS

MATERIALS

The neuroblastoma x glioma hybrid NG108-15 cell line was a gift from Dr. B.Schrier, Laboratory of Developmental Neurobiology, National Institute of Health, Bethesda, MD, USA. [^{3}H]-quinuclidinyl benzilate (34.7 Ci/mmol), [^{3}H]-dihydroalprenolol (103.5 Ci / mmol), [^{3}H]-isoleucine (56.5 Ci / mmol), [^{3}H]-tyrosine (53.8 Ci /mmol), [^{3}H]-norepinephrine and [^{35}S]-methionine (1000 Ci / mmol) were purchased from New England Nuclear, Boston, USA. Tissue culture supplies, including Dulbeco's modified Eagle's medium-high glucose (DMEM), fetal calf serum (FCS), penicillin-streptomycin, and L-glutamate were obtained from GIBCO, Canada. Insulin, transferrin, oleic acid, norepinephrine hydrochloride (NE), L-epinephrine (EP), 3,4-dihydroxyphenethylamine hydrochloride (dopamine), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydoxytryptamine creatine sulfate (5-HT), homovanillic acid (HVA) and dibutyryl cAMP, mercaptoethanol, hypoxanthine, aminoptrine, thymidine, DL-dithiothreitol (DTT), trypsin, atropine sulfate, L-alprenolol, plasminogen activator, plasmin, N-alpha-P-tosyl-L-lysine chloromethyl kelton (LALCK) were obtained from Sigma Co. St. Louis, USA. Molecular weight protein standards for SDS-PAGE, bis-acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS) and tertramethyl-ethylenediamine (TEMED) were products of Bio Rad Canada Laboratories (Mississauga, Ontario, Canada). Ultrogel AcA-44, ampholines (PH = 3.5 - 10 and 3 - 5) were purchased from LKB (Bromma, Sweden). Silver nitrate,

methanol, acetic acid, bromophenol blue, glycerol, formaldehyde (37 %) and glutaraldehyde were products of Fisher Scientific Co.(Winnipeg,Canada). Urea was obtained from Bethesda Reaserch Laboratories Inc. Gathersburg, USA. Molecular weight markers were obtained from Pharmacia (Picaraway, New Jersey).

All other standard laboratory reagents were purchased either from Fisher Scientific Co. (Winnipeg, Canada) or Sigma Chemical Co.(St.Louis, MO, USA).

Human and cat cerebral spinal fluids were gifts of Dr. L. Sekla, Cadham Provincial Laboratory, Winnipeg, Man. and Dr.B.Nago, Department of Physiology, University of Manitoba, Winnipeg, Man.

METHODS

1 - Cell Culture

i) - Standard Monolayer Culture

Neuroblastoma x glioma NG108-15 cells were maintained in medium D at 37° C in a humidified atmosphere of 10% CO2 and 90 % air according to Hamprecht (84) with slight modifications. Medium D, consists of 90 % DMEM, 10 % FCS, 0.1 mM hypoxanthine, 10 uM aminoptrine, 16 uM thymidine, 100 units / ml penicillin, 100 ug / ml streptomycin, 2 mM L-glutamate and 3.7 gram / l glucose at pH 7.4 with osmolality of 335 mosm at room temperature.

To subculture into either plastic flasks or 24-well multiwell plates, the cultured cells were routinely dislodged, collected by centrifugation (500 x g for 3 min)

and resuspended in appropriate volumes of prewarmed (37°C) medium D at a density of 4 - 5 x 10^4 cells per ml unless otherwise indicated. Cell number and viability were determined by hemocytometer and trypan blue staining, respectively.

ii) - Induction of Differentiation

Neuroblastoma x glioma hybrid NG108-15 cells were routinely induced to differentiate by subculturing the cells in medium E, which is identical to medium D with the exception of having 5% FCS and 1mM dBcAMP (42). Other culture conditions were identical to those described in " Standard Monolayer Culture".

In some experiments, NG108-15 cells were also induced to differentiate by PGE1 (43) or serum deprivation (51). For PGE1 treatment, cells were subcultured into a medium identical to medium D, except having 5% FCS and 1uM PGE1. Serum deprivation treatment of NG108-15 cells were achieved by subculturing the cells into a medium, identical to medium D except FCS.

iii) - Induction of De-differentiation

To revert differentiation, morphologically differentiated NG108-15 cells were de-differentiated by replacing medium E (with dBcAMP) to medium D (without dBcAMP) for 24 hrs.

iv) - Suspension Culture

Neuroblastoma x glioma NG108-15 cells were cultured in medium E, containing 5 % FCS and 1mM dBcAMP, and incubated

at 37° under constant gentle shaking. Cells under this suspension condition remained undifferentiated in spite of the presence of 1mM dBcAMP.

v) - Cultures in Serum-Free Defined Medium.

For studies on neurite retraction of morphologically differentiated cells, NG108-15 cells were cultured in serum-free defined medium, similar to that described by Wolfe and Sato (104). The serum-free medium consists of a mixture of three parts of DMEM and one part of Ham's F12 medium (DMEM / F12 [3:1]), supplemented with insulin (25 ug / ml), transferrin (50 ug / ml) and oleic acid (10 ug / mg albumin /ml). NG108-15 cells were subcultured into 24-well multiwell plates at a density of 2-3 x 10^{4} cells per ml and maintained under similar conditions as described under " Standard Monolayer Culture ". Differentiation of NG108-15 cells was induced by culturing for 48-72 hrs after adding 1mM dBcAMP into the serum-free culture medium.

2 - Receptor

i) Receptor Binding on Intact Cells

Binding of [^{3}H]-QNB and [^{3}H]-DHA to muscarinic and beta-adrenergic receptors, respectively, were assayed according to the procedure of Repke and Maderspach (105) with slight modifications. The cultured cells were mechanically dislodged and collected by centrifugation (500 x g for 3 min). The pelleted cells were resuspended

in appropriate volumes of fresh serum-free medium at a density of 1x 10^5 cells per ml, transferred into 1.5 ml plastic microcentrifuge tubes, and preincubated at 37° C for 15 -30 min. Binding reaction was initiated by adding 0.2-0.5 nM radioligand, unless otherwise indicated. After an additional incubation of 30 min at 37° C, binding was terminated by removal of free radiolabelled ligands by centrifugation, followed by two superfacial washings of the pellet with 1 ml each of ice-cold PBS, according to the procedure of Strange (106). The amount of non-specific binding was determined by assaying in the presence of excess amounts of an unlabelled ligand. Specific binding was calculated by subtracting the non-specific binding from the total binding (107). The non-specific bindings of [^{3}H]-QNB and [^{3}H]-DHA were measured in samples containing 0.5 mM atropine sulfate and 10 uM L-alprenolol,respectively. The pelleted cells were extracted with 0.1 ml of 0.1N NaOH and radioactivity was measured by liquid scintillation counting. Specific bindings were expressed as pmole per mg protein.

ii) - Characteristics of Muscarinic and Beta-adrenergic Receptors

To estimate the total binding (Bmax), affinity constant at equilibrium (Kd), dissociation rate constant (K-1), association rate constant (K+1), and Ic50 value (the concentration of non-radiolabelled ligand that displaces 50% of total binding), the following kinetic

properties were measured.

$[^3H]$-QNB and $[^3H]$-DHA bindings to muscarinic and beta-adrenergic receptors, respectively, on both dBcAMP treated (differentiated) and untreated (control) cells were determined. For saturation bindings of radiolabelled ligand, as a function of protein content, cell extracts at various protein concentrations were incubated with a constant amount of radioligand (0.3 nM) in the presence or absence of a constant concentration of non-radioligand (0.5 mM atropine sulfate for muscarinic and 10 uM L-alprenolol for beta-adrenergic receptors). Binding was carried out under conditions described in " Receptor Binding Assay ". Experiments on saturation of bindings as a function of radioligand concentration were carried out similarly as described above, except various concentrations of the radioligand were used.

1 - Scatchard (Rosental) Analysis

The total binding (Bmax) and affinity constant at equilibrium (Kd) were determined by plotting bound radioligand versus (bound / free radioligand) according to Scatchard (108) and Rosenthal (109). The affinity constant at equilibrium (Kd) and total binding (Bmax) were determined as the negative reciprocal of the Scatchard plot and the intercept on the abscissa (when [bound / free] = 0), respectively.

2 - Hill Plot Analysis

To determine the co-operativity of binding between

radioligands and receptor molecules, the Hill plot analysis was used (110). Data were plotted as log [bound / (Bmax - bound)] versus log (free) according to Hill (110). The slope of the Hill plot was calculated as the Hill coefficient (nH). A coefficient of (nH) less than, equal to, or greater than one was interpreted as negative, neutral, or positive co-operativity, respectively, between receptor molecules.

3 - Dissociation Rate Constant (K - 1)

Binding of $[^3H]$-DHA to adrenergic receptors on intact NG108-15 cells was allowed to proceed for 30 min to equilibrium as the total receptor binding of zero time (equilibrium time). At this time, all samples recieved excessive amounts of non-radioligand L-alprenolol (10 uM), and specific bindings were then determined upon additional incubation for various time intervals. Specific bindings at various time intervals were plotted against incubation time. The t 1/2 for dissociation, the time required for the displacement of 50 % of total binding, was calculated from regression of the curve. The dissociation rate constant (K -1) was then estimated from equation : (K -1) = ln 2 / (t 1/2).

4- Association Rate Constant (K + 1)

The association rate constant was determined by estimating the amount of $[^3H]$-DHA bound to intact NG108-15

cells at various time intervals after incubation. The t1/2 for association was determined as the time required for radioligand to occupy 50% of the total receptor sites. The association rate constant (K+1) was estimated from equation : (K+1) = [Kob - (K-1)/([^{3}H]-DHA)]. (K-1) is the dissociation rate constant, and Kob is the slope of the plot of Time versus ln [Beq / (Beq - Bt)] where Beq is the [^{3}H]-DHA binding at equilibrium and Bt is the [^{3}H]-DHA binding at various time intervals.

5- Inhibition constant (Ki)

In these experiments, constant number of NG108-15 cells ($5x10^4$ per tube) were incubated with a constant concentration (0.3 nM) of [^{3}H]-QNB or [^{3}H]-DHA for muscarinic and beta-adrenergic receptors, respectively, in the presence of various concentrations of non-radioligand (atropine sulfate and L-alprenolol for muscarinic and beta-adrenergic receptors, respectively) under standard experimental conditions. Specific binding was plotted against the concentration of non-radioligand. The concentration of unlabelled ligand that displaced 50 % of the total binding at equilibrium was determined as Ic50 (111). The inhibition constant (Ki) was derived from equation : Ki = Ic50 / [(1 + free radioligand) / Kd].

iii) - Type of Beta-adrenergic Receptor

To determine the type of beta-adrenergic receptors on plasma membrane preparations and intact NG108-15 cells, a

measurement of relative affinities of beta-adrenergic receptors for various agonists was determined (112).

Plasma membranes were prepared by homogenization of NG108-15 cells in 50 mM Tris-HCl buffer, PH 8, by a polytron homogenizer. Homogenates were washed three times by centrifugation at 50,000 g for 15 min, followed by resuspension in the same buffer according to the procedure of Kohn et al.(113). The pelleted membranes were resuspended in appropriate volumes of serum-free DMEM. For assays, aliquats of 150 ul each of membrane suspension (which equals to approximately 50,000 cells) were used.

To determine the relative affinity of β-adrenergic receptor, plasma membrane preparations or intact cells were incubated with [^{3}H]-norepinephrine in the presence of various concentrations of different agonists, namely, (-)-isoproterenol (-ISO), (+)-isoproterenol (+ISO), epinephrine (EPI) and norepinephrine (NE), under standard binding assay conditions. The potency of each agonist for beta-adrenergic receptor was determined by estimation of the Ic50 values for each non-radioligand for 50% displacement of the total binding of [^{3}H]-NE. A comparison of the order of potency of various agonists to bind beta-adrenergic receptor was ranked in order to determine the type of beta-adrenergic receptor.

iv - Protein Determination

The protein content of NG108-15 cells was determined by the method of Lowry et al. (114) after solublizing the cell

pellet with 0.1 ml of 0.1N NaOH. Bovine serum albumin was used as protein standard in the assay.

3 - Analysis of Proteins by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

i) - One dimensional gel electrophoresis

Harvested cells were washed twice in 0.9 % saline by centrifugation at 500 x g for 3 min. Pelleted cells were extracted in appropriate volume (0.5 ug protein / ul) of sample buffer, containing 0.06 M Tris-HCl (PH 6.8), 2 % SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromo-phenol blue, boiled for 5 min and stored at -20°C until use. Molecular weight markers of phosphorylase B (94 Kda), bovine serum albumin (67 Kda), ovalbumin (40 Kda), carbonic anhydrase (30 Kda),soybean trypsin inhibitor (20.1 Kda) and lactalbumin (14.4 Kda) were pre-treated identically for use.

polyacrylamide SDS-gel electrophoresis of cell extracts was carried out according to Laemmli (115). The separating gel contained 10 % acrylamide, 0.8% N,N'-Bis-methylen acrylamide, 0.375 M Tris-HCl (PH 8.8) and 0.1 % SDS. The gel was polymerized chemically by the addition of 0.025 % of tetramethyl-ethylenediamine (TEMED) and 0.05 % freshly prepared ammonium persulfate. The stacking gel consisted of 4% acrylamide, 0.125 M Tris-HCl(PH 6.8) and 0.1% SDS. Electrophoresis was carried out with a current of 20 mA / slab gel.

After electrophoresis, proteins were stained with silver staining according to the procedure of Morrissey (116).

ii - Two Dimensional Gel Electrophoresis

Cells were cultured for 24 hrs in 24 well multiwell plate at density of $5x10^4$ cells / well in 0.5 ml of either L-leucine- or methionine-free medium, supplemented with $[^3H]$-leucine (1 uCi/well) or $[^{35}S]$-methionine (10 uCi/ well), respectively. At the end of incubation, cells were washed with ice-cold DMEM, and dissolved with 50 ul / well of the lysis buffer (117), containing 2 mM N-alpha-P-tosyl-L-lysine chloromethyl keltone (TLCK Sigma), a protease inhibitor. After 3 cycles of quick freeze and thaw, the lysates were stored at - 70°C and used within a week.

Two dimensional SDS-PAGE was carried out according to the procedure of O'Farrell (117). For the first dimension of gel isoelectric focusing, an equal volume of lysis buffer (9.5 M urea, 2 % NP40, 2% ampholines of 1.6 % pH ranged 3 - 10 and 0.4 % pH ranged 5 -7, and 5% beta-mercaptoethanol) was added to the cell extract and applied to the gel column. The isoelectric focusing (IEF) of proteins was carried out on gels (16 x 0.3 cm) for 15 min at 200 V, followed by 18 hrs at 400 V. After focusing, the gel was equilibrated for 1 hr in sample buffer (10% glycerol, 5 % beta-mercaptoethanol, 2.3 % SDS and 0.062 M Tris-HCl with PH 6.8) for the second dimension of electrophoresis or stored at - 20° C for use within a week.

The first dimensional gel column was applied to the SDS slab gel with 1% agarose in SDS sample buffer for the second dimensional SDS-gel electrophoresis. After electrophoresis, the gel was fixed in a solution of 50 % methanol

and 7.5 % acetic acid and then dried by a Bio-Rad slab dryer. The dried gel was autoradiographed by using X-MAR Kodak film for 10 days or 24 hrs for [^{3}H]-leucine and [^{35}S]-methionine labelled proteins, respectively.

4 - Analysis of Indolamines by High Performance Liquid Chromatogaphy

The cultured neurobalstoma x glioma hybrid NG108-15 cells were mechanically dislodged, and collected by centrifugation at 500 x g for 5 min. The pelleted cells were washed twice in PBS and disrupted by sonication (4 x 20 Sec). Indolamines were either extracted directly into 0.1N perchloric acid (containing 0.1 mM sodium metabisulfite and 0.1 mM EDTA), followed by centrifugation at 35000 x g for 30 min according to Kotake C. et al. (118), or absorbed onto activated alumina (119), which was then washed twice with distilled water, containing 5mM sodium metabisufite, and extracted by perchloric acid. Alumina was used at ratios of 100 mg alumina / 10 ml of medium. For analysis of indolamines, 20 ul of the 0.1N perchloric acid extracts (direct extraction or alumina extraction) were injected into the HPLC system.

Indolamines were separated by HPLC-EC using Altex C18-IP reverse phase column (25 cm x 4.6 mm ID; 5um particle size; Beckman, Berkely, California). The mobile phase consisted of 75 mM sodium phosphate monobasic, 1.0 mM sodium octylsulphate, 50 uM EDTA and 11.5 % acetonitrile. The buffer was adjusted to PH 3.25 with H3PO4, filtered and

degassed prior to use. A flow rate of 1.0 ml / min. was provided by a Bekman Model 114 solvent delivery module. The indolamines were identified by coulometeric detection using ESA model 5100 A detectors with detector 1 set at reduction potential of 0.05 V and detector 2 at an oxidation potential of 0.40 V. A pre-injector guard cell was set at 0.45 V. Indolamine peaks were identified by relative retention time compared to standards.

The concentration of neurotransmitters and their major metabolites was determined by comparimg peak areas using a Shimadzu integrator, interfaced with detector 2 of the detector unit. An spike control of samples containing indolamine at 1 ng / 20 ul of injection volume was used to determine the rate of recovery. The cellular content of L-dopa, NE, EPI, DA, Dopac, 5-HT, HIAA, HVA, and 3-MT was expressed as ng per 10^6 cells. The content of NE, EPI and DA in culture medium was expressed as ng per volume of medium obtained from 10^6 cells. Blank medium was similarly extracted and used as control.

5 - Assay for Tyrosine Hydroxylase

Tyrosine hydroxylase (TH) activity was determined by measuring the formation of [^{3}H]-H2O from L-[^{3}H]-tyrosine as described by Nagatsu et al. (120). The TH assay was performed at 37°C in a total volume of 0.2 ml PBS solution, pH 7.3, containing 110 mM NaCl, 5.3 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 2.0 mM Na2HPO4, 25 mM glucose and 70 mM sucrose, with osmolality of 335 mosm. Prior to assay,

cells were washed once with 0.15 ml of PBS at 37° C. and reaction was initiated by the addition of [^{3}H]-tyrosine (1x10^{5}cpm / sample) to a final volume of 0.2 ml of PBS and incubated at 37° C for 20 min. The reaction was terminated by adding 75 ul of 1 M acetic acid, followed by 500 ul of ice-cold 10 mM L-phenylalanine in distilled water. The entire reaction mixture, including lysed cells was transferred to a tube, followed by an additional washing of 500 ul of ice-cold 10 mM L-phenyalanine. The content of each tube was then placed onto a Dowex 50 - Hydrogen form column and the eluate was collected into a scintillation vial. Radioactivity was counted by a beta-counter. The TH activity was expressed as pmole / min / mg protein.

6 - Neurite Retraction Factor

i) - Bioassay for Neurite Retraction

Samples of serum or tissue extract were added to the culture of differentiated NG108-15 cells in serum-free medium containing 1mM dBcAMP. Neurite retraction was monitored by measuring neurite length in photographs taken at various time intervals within 2 min after addition of test sample unless otherwise indicated. A unit of activity was expressed as the amount of protein that causes neurites to shorten 50% in 2 min.

ii) - Acid and Heat Stability

For acidification, the pH of FCS was adjusted to 2.5

with 1N HCl, followed by centrifugation and reneutralized with 1N NaOH to 7.4. For heat stability, the serum sample was heated to 56°, 75° and 100°C for 15 min. Both acidified and heated serum samples were centrifuged at 150,000 x g for 60 min to obtain clear supernatants for assays.

iii) - Fractionation of Serum by Ultrafilteration

FCS was filtered through centricon 10X by centrifugation or millipore immersible CX30 under vacuum to separate molecules of MW < 10 Kda or < 30 Kda, respectively. Fractions of serum containing molecules of < 10 Kda, between 10 - 30 Kda and > 30 Kda were assayed for neurite retraction and growth prolifration rate of NG108-15 cells

iv) - Gel Filteration

For gel filtration, the FCS was boiled (100°C) for 15 min and centrifuged at 150,000 x g for 60 min. The supernatant was applied onto a column (1.6x 110 cm) of LKB Ultrogel AcA - 44 in PBS (pH 7.4) and eluted at 16 ml/hr. Fractions of 1 ml / tube were collected, and aliquots of 50-100 ul of each fraction were used directly for neurite retraction bioassay.

v) - Trypsinization

After boiling and centrifugation, the clear supernatant of serum samples was desalted through centricon 10X, and trypsin was then added to a final concentration of 2%. Digestion was carried out by incubating the reaction

mixture for 16 hrs at 37° C and terminated by boiling the mixture for 15 min.

vi) - Extraction of tissues

Rat lung, brain, kidney, spleen, heart, liver, and skeletal muscle were washed and homogenized in 3 volumes of PBS buffer. The crude homogenate was centrifuged at 9000 x g for 1 hr. Appropriate volumes of the supernatant, containing 1 mg of protein, was added to the morphologically differentiated NG108-15 cells in culture for neurite retraction activity.

vii) - Effect of Growth Factors and Other Chemical Agents on Neurite Retraction

Various known growth factors and chemical agents were tested for their effects on neurite retraction on morphologically differentiated NG108-15 cells in culture. These agents included nerve growth factor (10 ug / ml), platelet derived growth factor (1,10, and 50 units / ml), plasminogen (100 ug /ml) and plasmin (100 ug /ml), and hormones ($1x10^{-5}$ M), including PGE1, E2, A, F2a and dexamethasone.

Statistical Analysis

Differences of statistical significance between receptor bindings on differentiated and undifferentiated cells were automatically determined during computer analysis of data using the EBDA program.

C - RESULTS

I - Cell Culture

Fig. 1 shows that NG108-15 cells grown in medium D containing 10 % FCS (undifferentiated cells) are flat to slightly rounded with only few cells bearing short processes (Fig. 1A), which did not increase in length with time up to 5 days in culture. When cells were grown in medium E containing 5 % FCS and 1mM dBcAMP, extensive neurite formation occurred, mainly from singlets (Fig.1B).

Although various neurite inducing agents and treatment of serum deprivation promote neurite outgrowth in NG108-15 cells, yet the number, diameter and ramification of their neurites vary, depending on the type of agents used for induction. Fig.2A shows that serum deprivation caused NG108-15 cells to produce short neurite with low density, whereas dBcAMP induced long neurite with high density (Fig.1B). PGE1 and theophyline (Fig.2B and 2C) caused cells to produce long neurite with low density. Fig. 2D indicates that inspite of the presence of dBcAMP in the medium, NG108-15 cells grown in suspension condition remained undifferentiated in clusters of round cells.

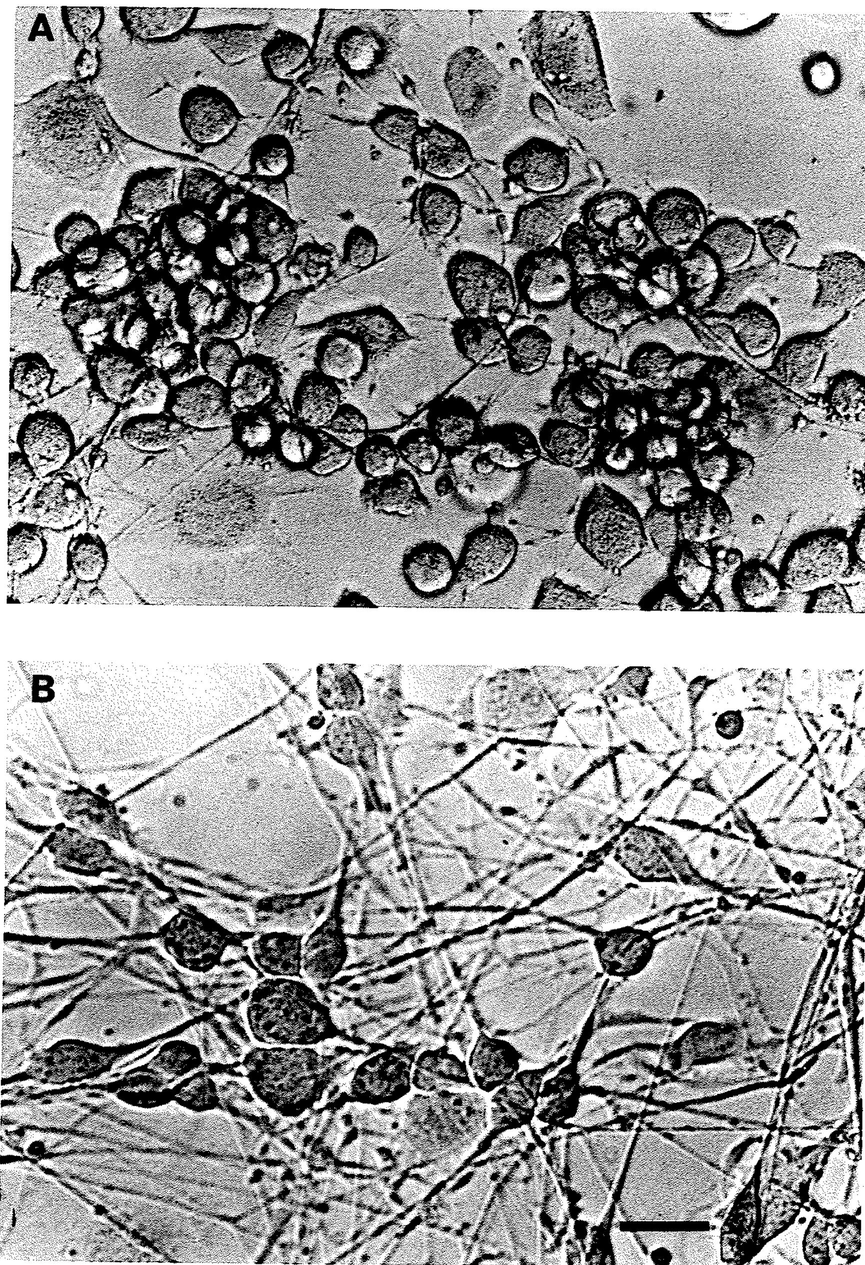

Fig. 1. Comparison of phase contrast micrographs of undifferentiated (control) and differentiated (dBcAMP-treated) NG108-15 cells. Cells were cultured for 5 days in medium D containing 10% FCS (A) or medium E containing 5 % FCS plus 1mM dBcAMP (B). Bar = 50 um.

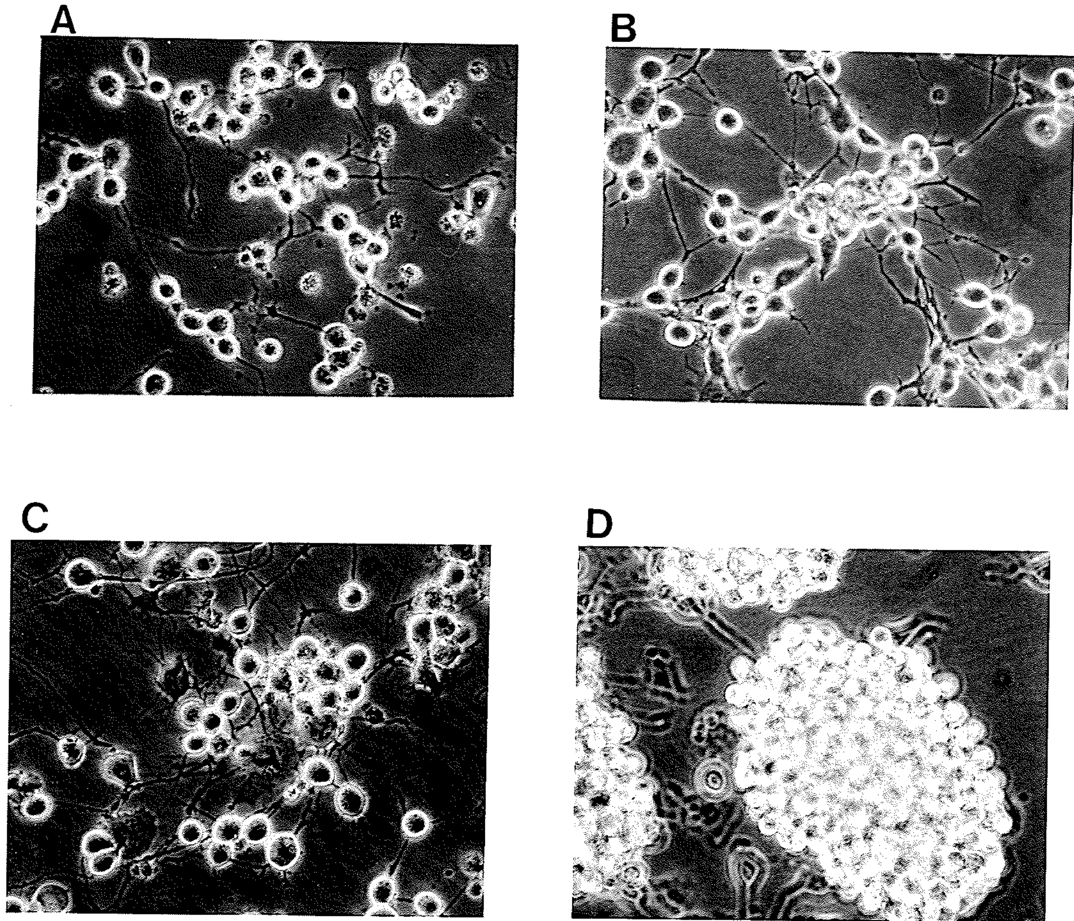

Fig.2. Comparison of phase contrast micrographs of NG108-15 cells cultured for 72 hrs in serum-free medium D (A), medium E containing 5 % FCS plus 10 uM PGE1 (B), medium E containing 5% FCS plus 1mM thiophyline (C) and suspension culture in medium E containing 5% FCS plus 1 mM dBcAMP (D).

II - Muscarinic Acetylcholine Receptors on Differentiated and Undifferentiated NG108-15 Cells

1 - Muscarinic Acetylcholine Receptor (AchR) Binding

Fig.3 shows that undifferentiated NG108-15 cells contained a substantial amount of specific AchR binding and that differentiated NG108-15 cells, induced by treatment with 1.0 mM dBcAMP for 5 days, possessed approximately 120% more specific receptor sites.

2 - Receptor Binding as a Function of Protein Content

Fig.4 indicates that specific [^{3}H]-QNB binding to muscarinic AchR increased proportionately to the contents of protein in sample and thus the number of cells present. Receptor binding reached saturation at a protein content of approximately 200 ug. Accordingly, for routine muscarinic AchR assays, protein concentration of 100 - 150 ug per sample was used.

3 - Muscarinic Acetylcholine Receptor Binding on NG108-15 Cells During Differentiation

Fig.5 reveals that the number of AchR increased gradually after 4 hrs of treatment with 1.0 mM dBcAMP, and reached a maximum increase of 140 % in specific binding after 24 hrs as compared to that of untreated control cells. Only a further slight increase of 20% in receptor binding was observed after this initial 24 hr period, even on prolonged treatment up to 7 days (data not shown). Fig.6 dipicts the morphological changes in neurite

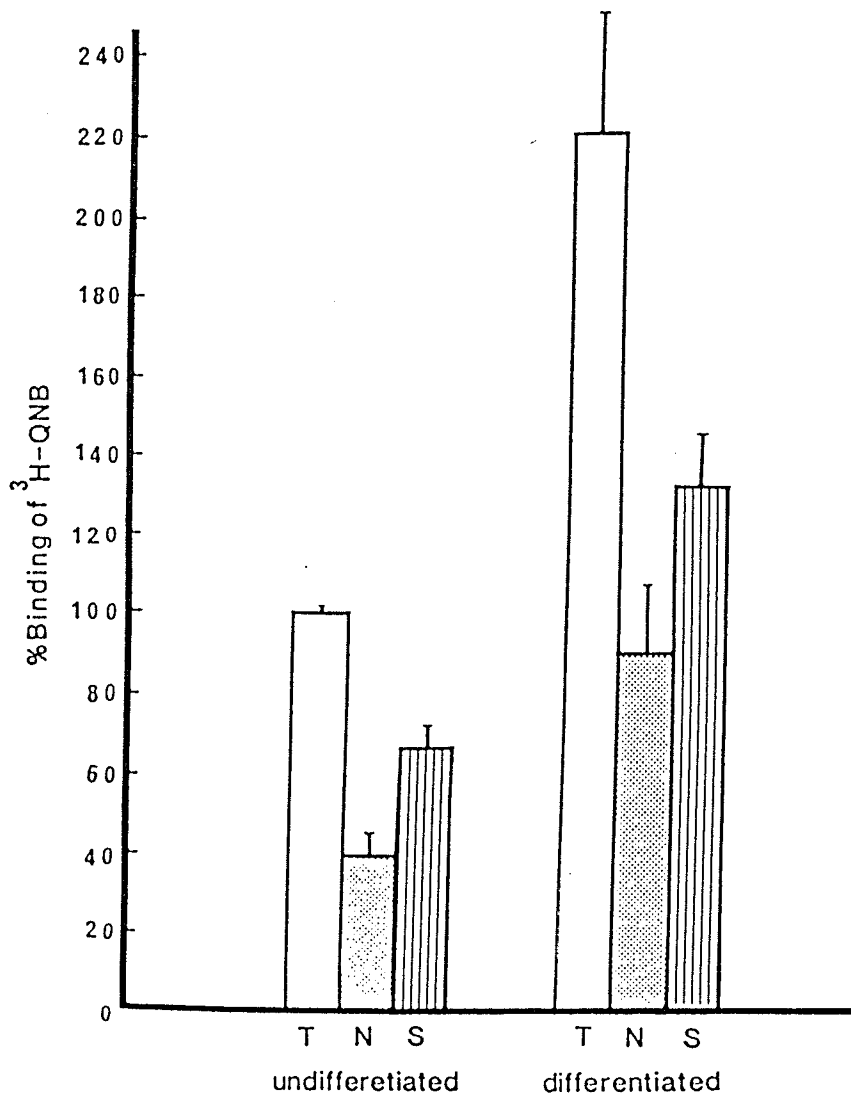

Fig.3. Muscarinic acetylcholine receptor binding on NG108-15 cells. Cells were cultured in medium D or medium E for 5 - 7 days. Undifferentiated and differentiated cells were incubated with 0.3nM [^{3}H]-QNB in the presence or absence of 0.5 mM atropine sulfate for 30 min at 37° C. Binding in pmole/mg protein is expressed as a percent of the total binding. Data represent means + SEM of 2 experiments in triplicates. (T), total binding ; (N), non-specific binding ; (s), specific binding.

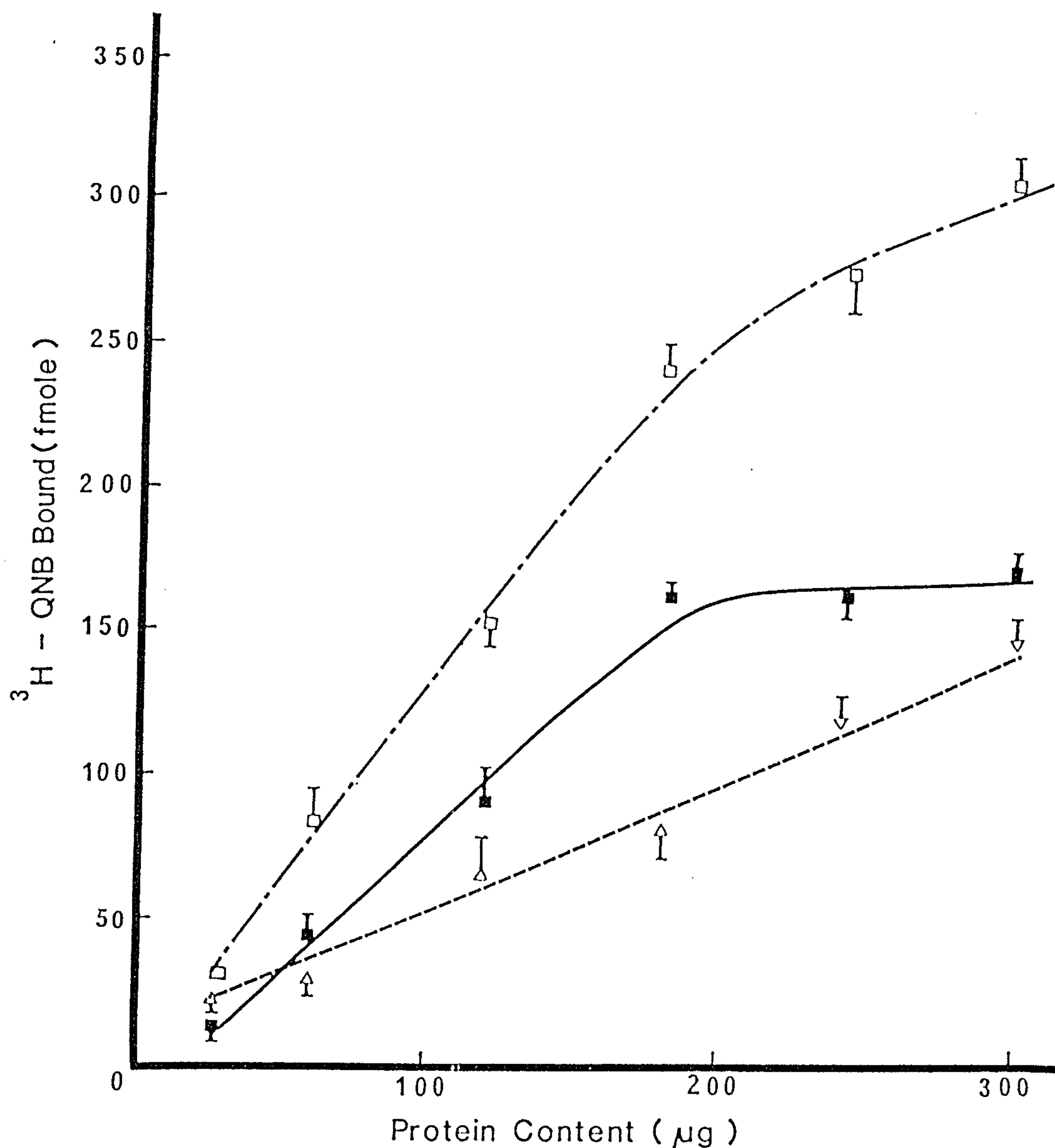

Fig.4. Muscarinic acetylcholine receptor binding on NG108-15 cells as a function of protein content. Samples of various concentrations of protein were incubated with 0.3 nM [^{3}H] QNB in the presence or absence of 0.5 mM atropine sulfate for 30 min at 37° C. Data represent means + SEM of a typical experiment in triplicates. (□) total binding, (▽) non-specific binding, (■) specific binding.

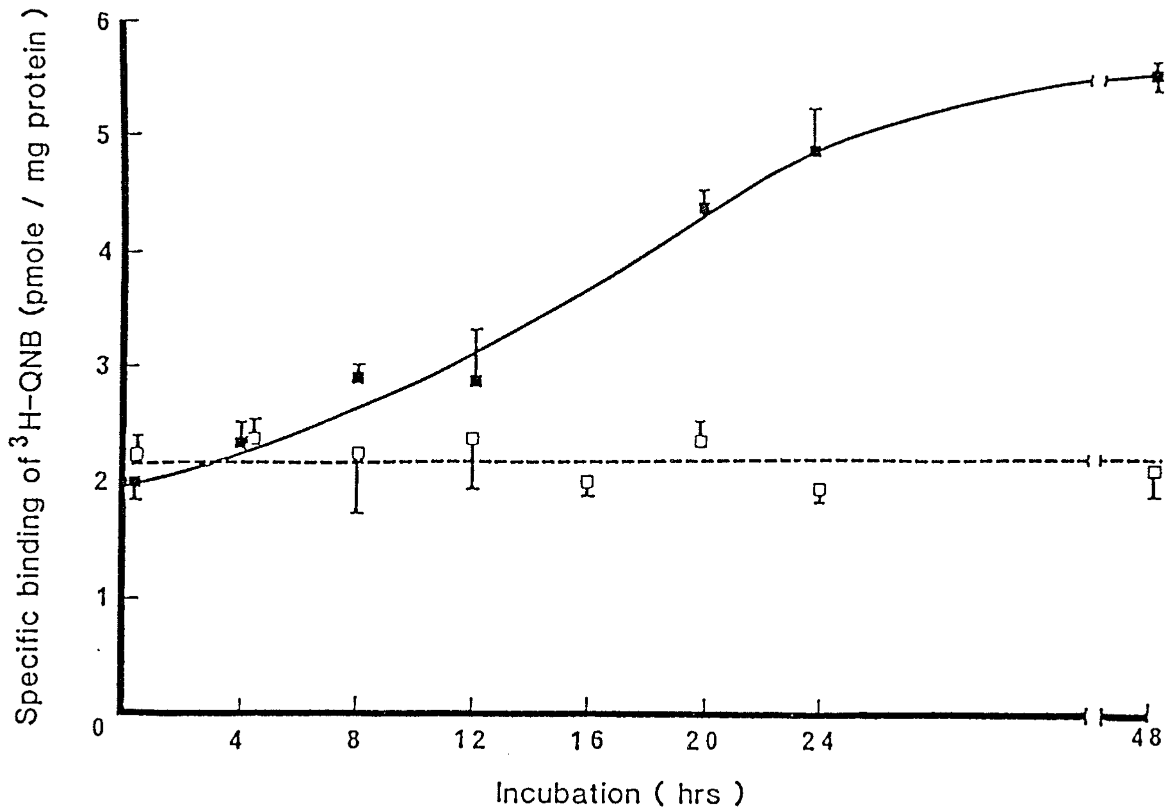

Fig.5. Time course for acetylcholine receptor binding on NG108-15 cells upon induced differentiation. Cells were treated with 1.0 mM dBcAMP at 0 hr of incubation. Specific binding at various incubation time intervals was determined and expressed as pmole per mg protein. Data represent means + SEM of 4 determinations. (■), dBcAMP-treated cells; (□), untreated control cells.

extension of NG108-15 cells at different time intervals of the initial 24 hr period of dBcAMP treatment. It was observed that the extent of neurite extension (Fig.6) coincided with the increase of AchR binding (Fig.5) during this critical period of cellular differentiation upon dBcAMP treatment.

4 - Binding Characteristics of the Muscarinic AchR on NG108-15 Cells

Specific binding of [^{3}H]-QNB to intact NG108-15 cells, either differentiated (dBcAMP-treated) or undifferentiated (control), was saturable as shown in Fig.7. Data obtained from the saturation experiments were analysed by computer, using the EBDA Program (121). The maximum binding sites at saturation, as measured by the Scatchard plot analysis (Fig.8), for differentiated and undifferentiated NG108-15 cells were estimated to be 0.23 and 0.10 nM, respectively. The differentiated cells possessed 118 % more muscarinic AchR sites at equilibrium. Analysis of data on Fig.7 revealed that the equilibrium dissociation constant (Kd), the concentration of [^{3}H]-QNB at which half maximal binding occurred, was 5.6 nM for differentiated and 4.4 nM for undifferentiated NG108-15 cells. Further, the Kd values of 7.5 and 5.5 nM obtained from Scatchard plot analysis as calculated by computer (Fig.8) for differentiated and undifferentiated cells,respectively, were in good agreement with those (5.6 and 4.4 nM) obtained from saturation analysis (Fig.7).

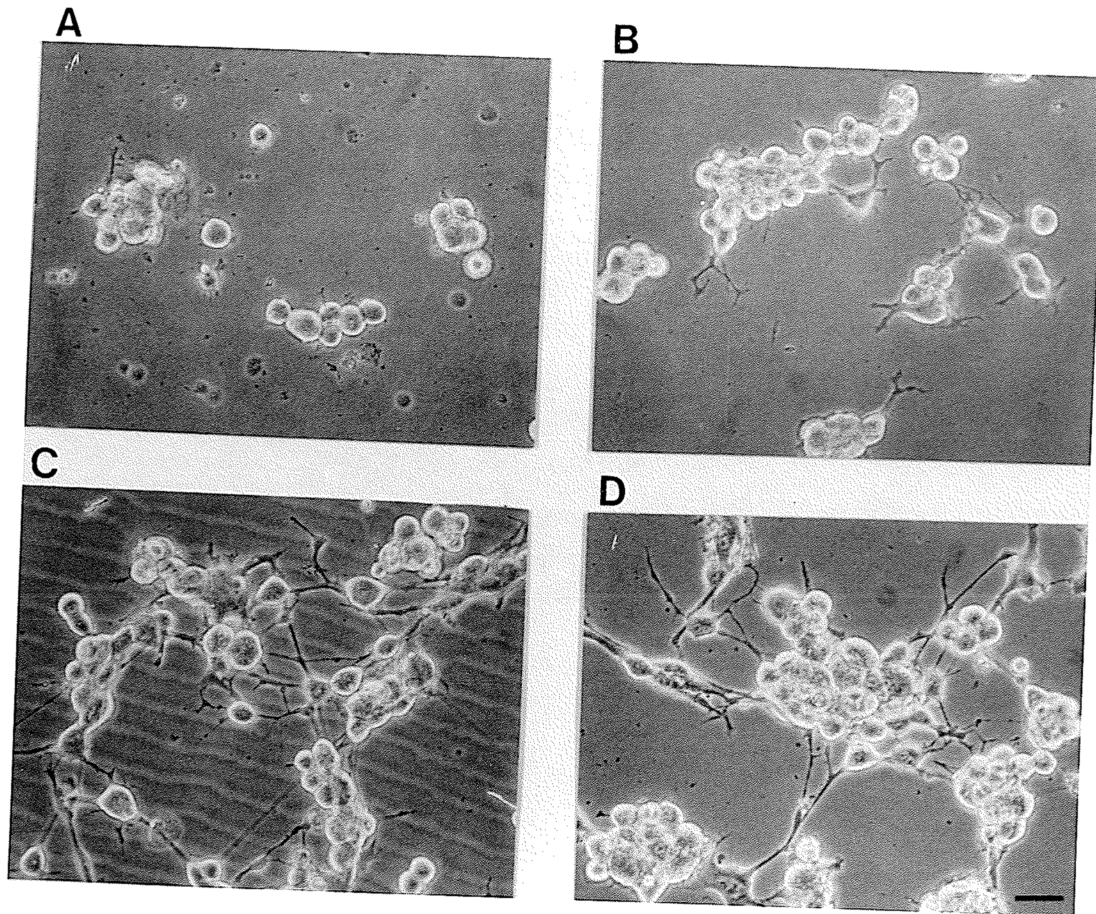

Fig.6. Light microscopy of NG108-15 cells at various time intervals upon induced differentiation. NG108-15 cells were treated with 1.0 mM dBcAMP at o hr up to 24 hrs of incubation. Morphologically differentiated cells were photographed at various time intervals for neurite extension. A = 0 time, B = 4 hrs, C = 16 hrs, and D = 24 hrs. Bar = 50 um.

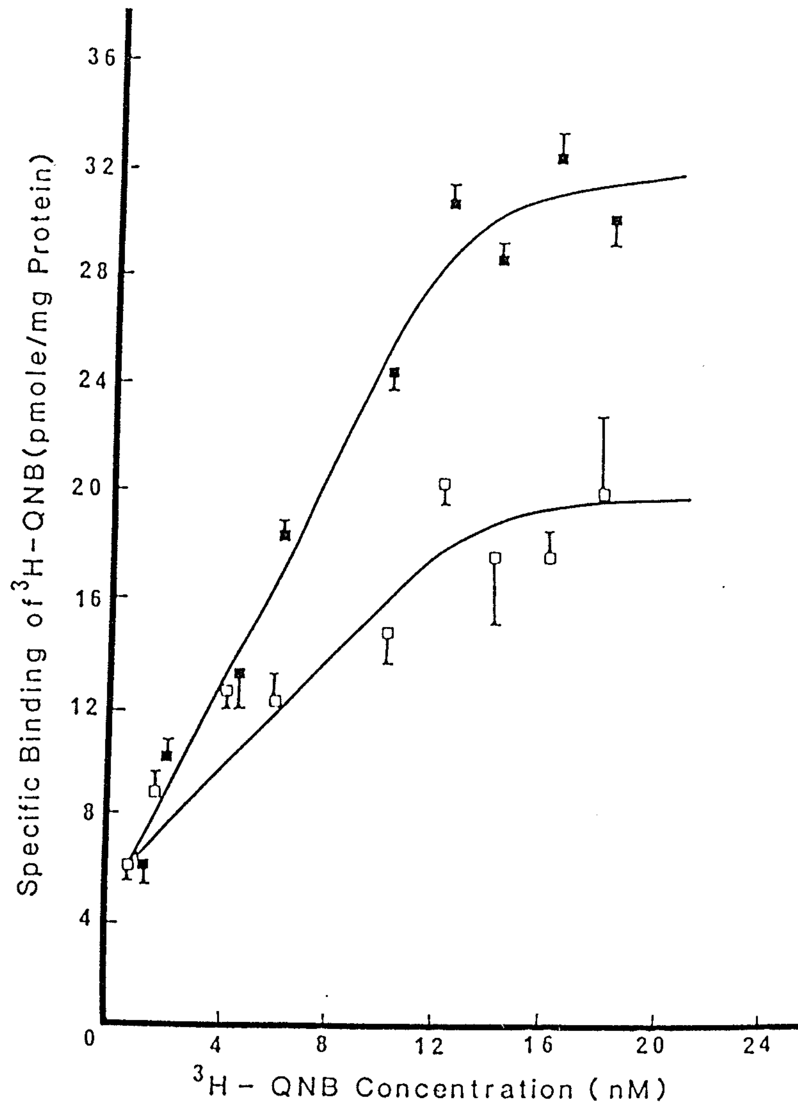

Fig.7. Equilibrium [^{3}H]-QNB binding to intact NG108-15 cells. Differentiated and undifferentiated cells were incubated with various concentrations of [^{3}H]-QNB in the presence or absence of 0.5 mM atropine sulfate for 30 min at 37° C. Specific binding is expressed as pmole per mg protein. Data represent means +SEM of 2 experiments in triplicates. (■), differentiated cells; (□), undifferentiated cells.

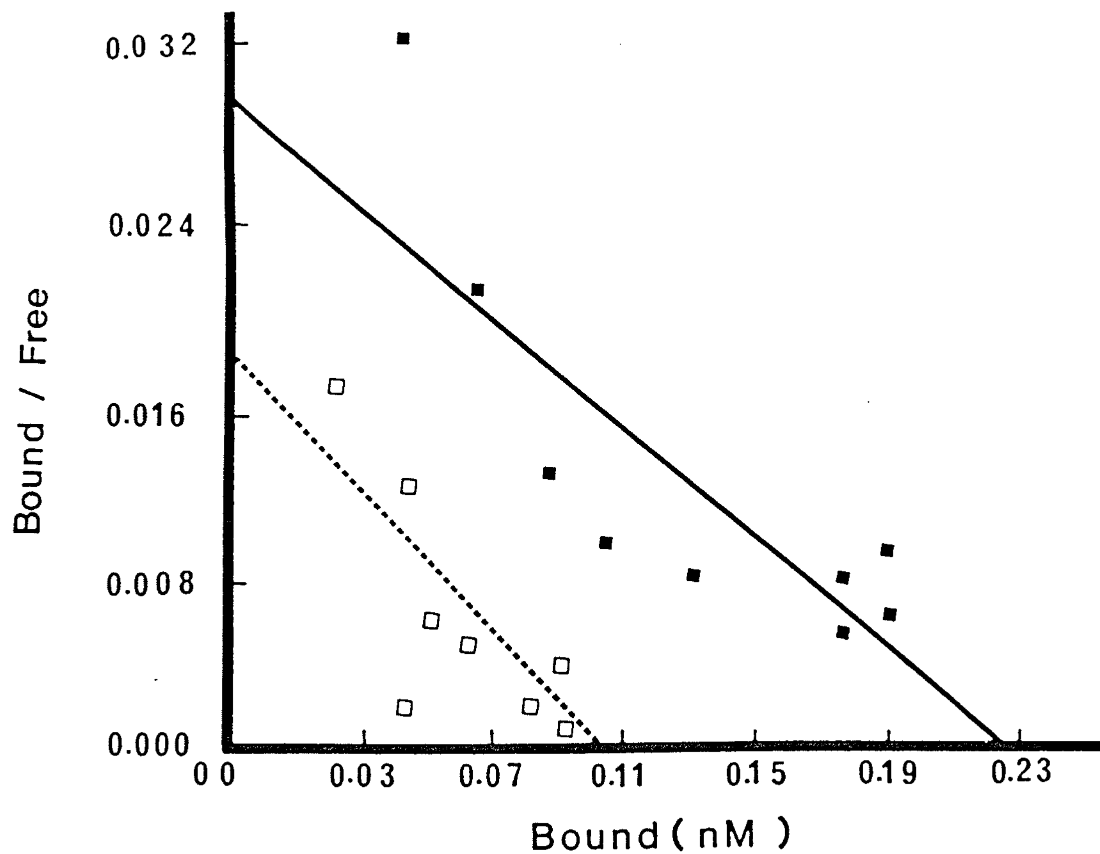

Fig.8. Scatchard plot analysis of [^{3}H]-QNB binding on differentiated and undifferentiated NG108-15 cells. Data from saturation experiments (Fig. 7) were analysed and plotted by the computer EBDA program as a function of [^{3}H] - QNB bound (pM) versus ratio [bound / free]. (■), differentiated cells; (□), undifferentiated cells.

Computer analyses of the specific [^{3}H]-QNB binding to muscarinic AchR on NG108-15 cells by Hill plot (Fig.9) provided Hill coeficient (nH) values of 1.00 and 0.91 for differentiated and undifferentiated cells,respectively. These binding data of having Hill coeficient (nH) values approximately 1.0 indicate no negative co-operativity for AchR bindings on both dBcAMP-treated and untreated cells. Computer analyses of data obtained from saturation experiments revealed a single receptor type on both differentiated and undifferentiated NG108-15 cells.

5 - Inhibition of [^{3}H]-QNB Binding by Atropine Sulfate

Fig. 10 reveals that inhibition of [^{3}H]-QNB binding to AchR on NG108-15 cells was proportional to the concentrations of added atropine sulfate. The concentration of atropine sulfate to displace 50 % of [^{3}H]-QNB binding at saturation (IC50) was calculated by the computer EBDA program to be 55 uM and 259 uM for differentiated and undifferentiated cells, respectively.

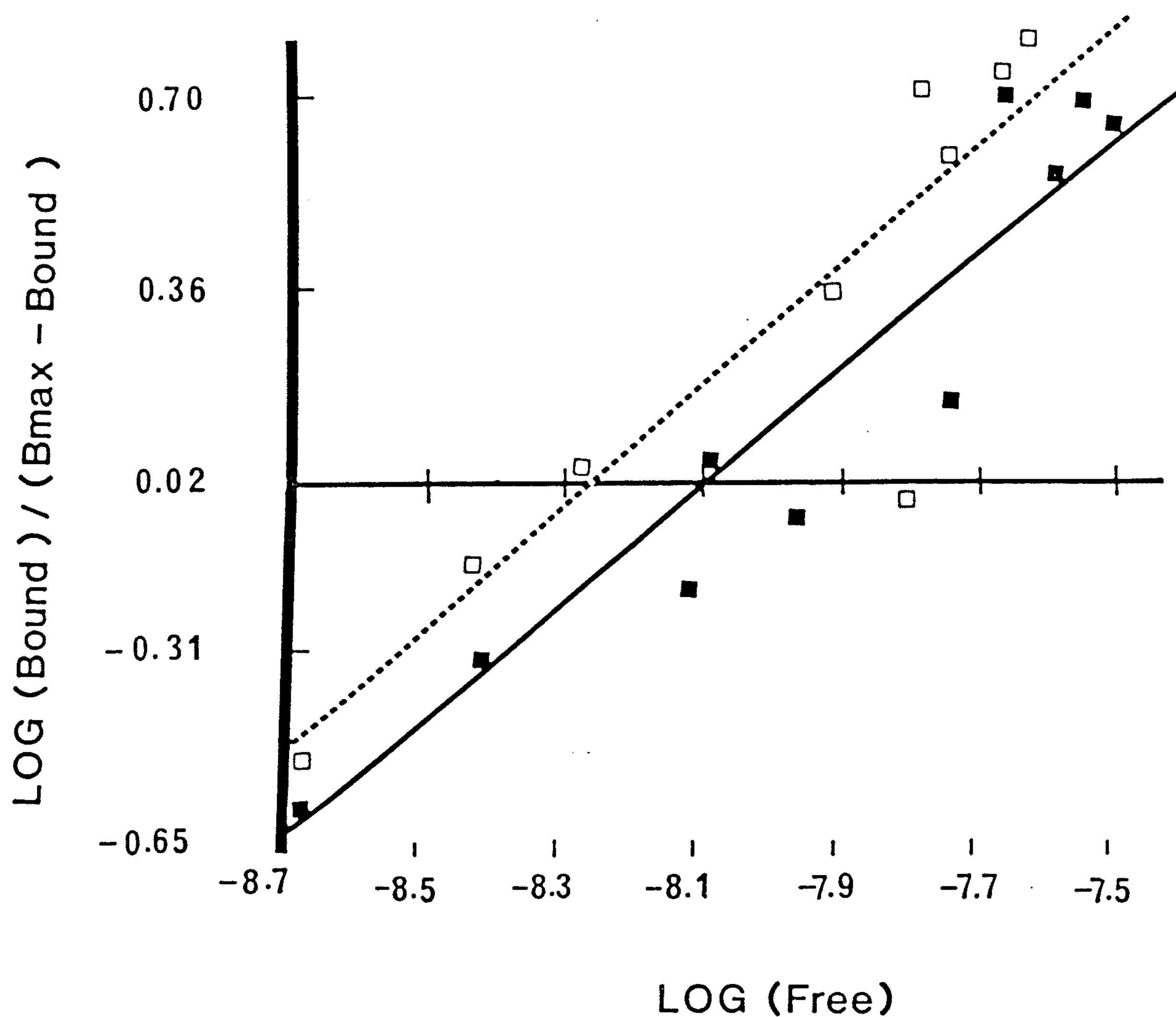

Fig.9. Hill plot of [^{3}H]-QNB binding on intact NG108-15 cells. Data obtained from saturation experiments on differentiated and undifferentiated cells were analysed and plotted by the computer EBDA program as a function of Log (Free) versus log [bound / Bmax - bound]. (■), differentiated cells; (□), undifferentiated cells.

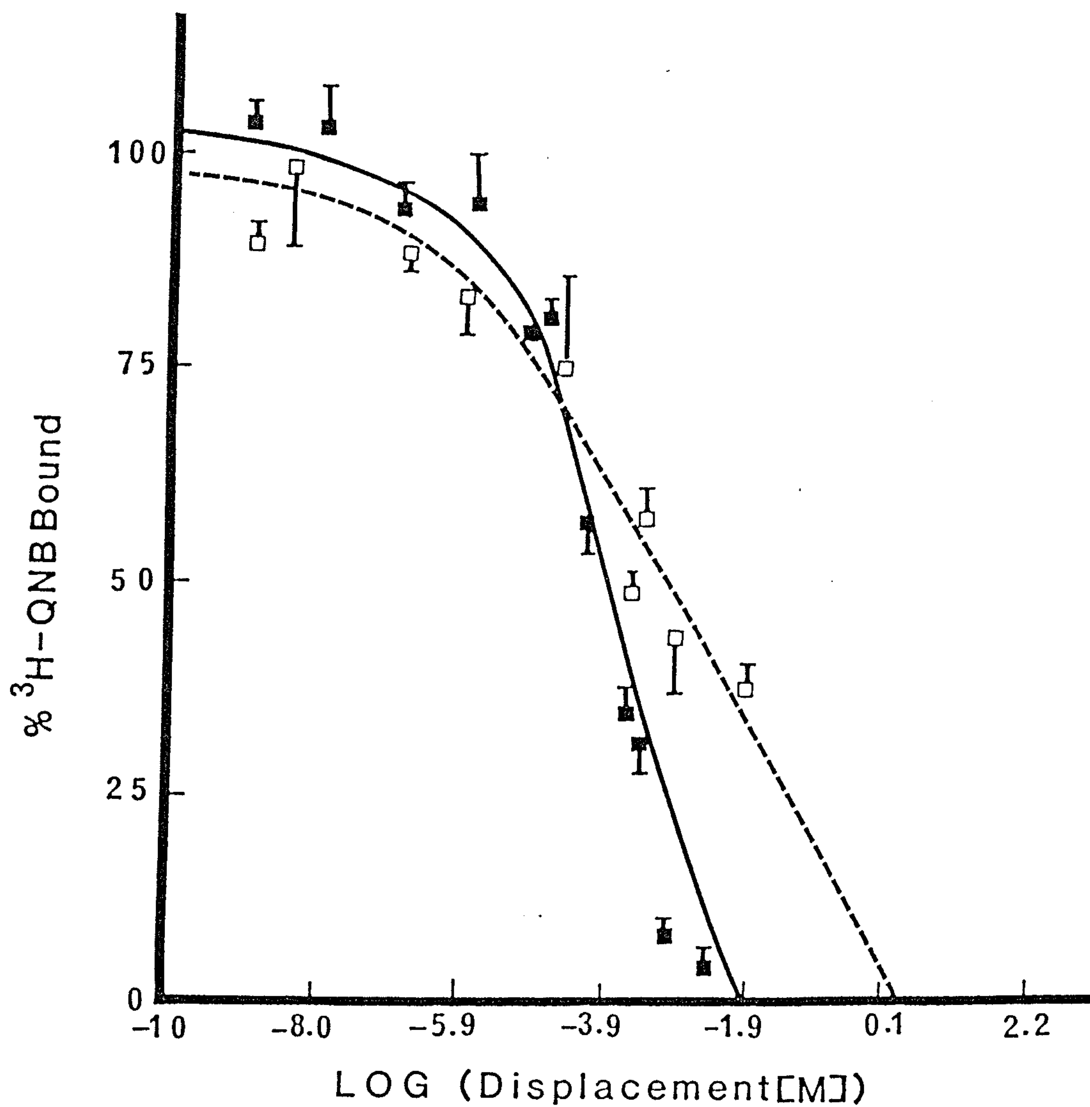

Fig.10. Inhibition of [^{3}H]-QNB binding by atropine sulfate on NG108-15 cells. Inhibition of [^{3}H]-QNB binding was determined by incubating 0.3 nM [^{3}H]-QNB in the presence of various concentrations of atropine sulfate under standard assay conditions. Data were analysed and plotted by computer using the Ligand Program. Data represent means + SEM of 4 determinations. (■), differentiated cells; (□), undifferentiated cells.

III - Beta-Adrenergic Receptors on NG108-15 Cells

1 - [^{3}H]-DHA Binding to B-adrenergic Receptors on NG108-15 Cells

Studies of β-adrenergic receptors using [^{3}H]-DHA revealed that NG108-15 cells possessed a substantial amount of β-adrenergic receptors as shown in Fig. 11. The specific binding for [^{3}H]-DHA, the difference between total and non-specific bindings, constitute 83 % of the total binding.

2 - [^{3}H]-DHA Binding as a Function of Protein Content

Fig.12 indicates that specific [^{3}H]-DHA binding to β-adrenergic receptor sites on intact NG108-15 cells increased proportionately to the protein content and thus cell number in the sample, and reached saturation at a concentration of approximately 120 ug protein. Accordingly, for routine β-adrenergic receptor assays, protein concentrations of 40-60 ug per sample were used.

3 - [^{3}H]-DHA Binding on Differentiated NG108-15 Cells

A comparison of total, non-specific and specific [^{3}H]-DHA bindings to differentiated and undifferentiated cells is shown in Fig. 13. This shows that differentiated cells, induced by 1mM dBcAMP, did not possess a significantly higher number of beta-adrenergic receptor as compared to undifferentiated cells. An increase of 13 % in the total

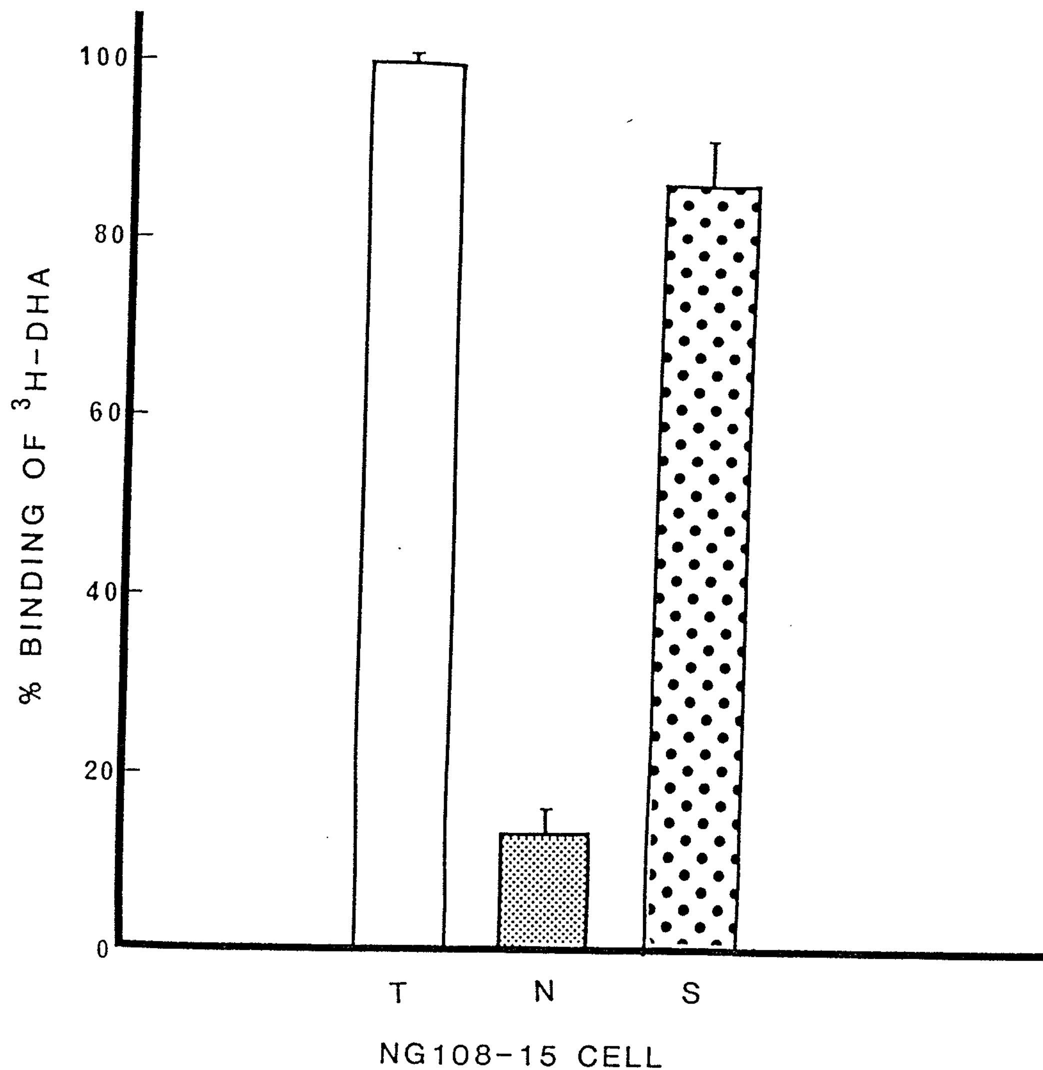

Fig.11. Beta-adrenergic receptor binding of [^{3}H]-DHA to intact NG108-15 cells. Cells were cultured in medium D for 5 days. For binding assays, cells were harvested, washed and incubated with 0.3 nM [^{3}H]-DHA in the presence or absence of 0.5 mM L-alprenolol for 20 min at 37° C. Specific and non-specific [^{3}H]-DHA bindings are expressed as a percent of the total binding. Data represent a typical experiment in triplicates (means ± SEM). (T), total binding; (N), non-specific binding; (S), specific binding.

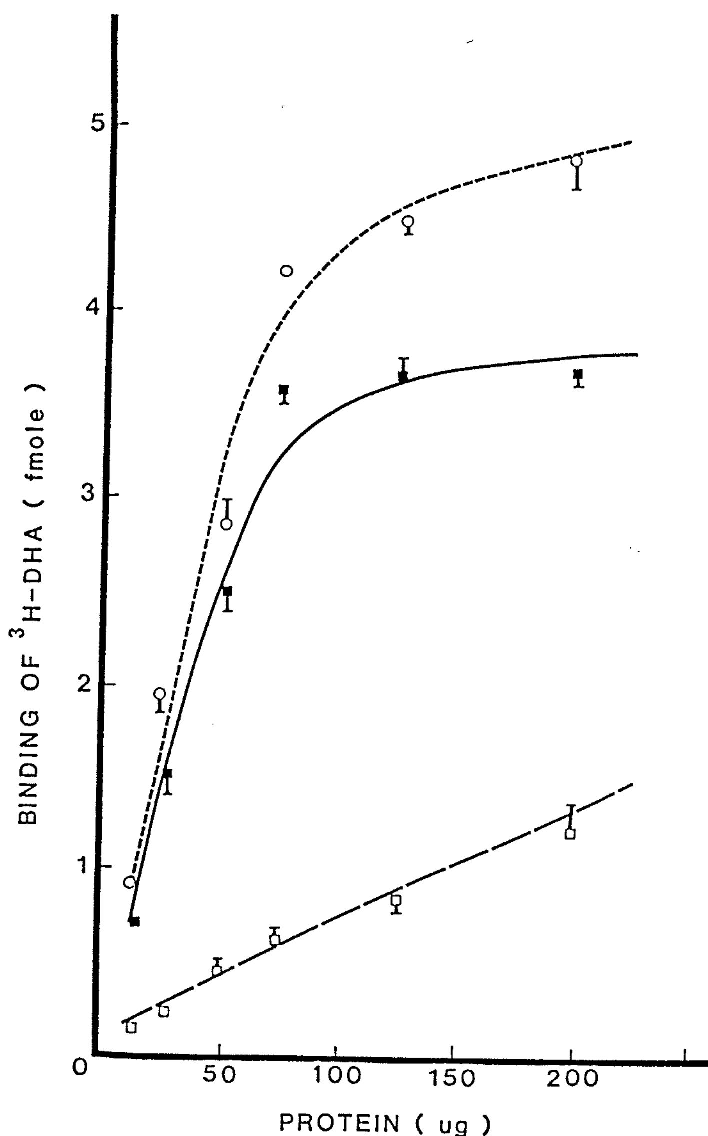

Fig.12. Beta-adrenergic receptor binding on intact NG108-15 as a function of protein content. Samples of various concentrations of protein were incubated with 0.3 nM [^{3}H]-DHA in the presence or absence of 0.5 mM L-alprenolol for 20 min at 37°C. Data represent a typical experiment in triplicates (means ± SEM). (○), total binding; (□), non-specific binding; (■), specific binding.

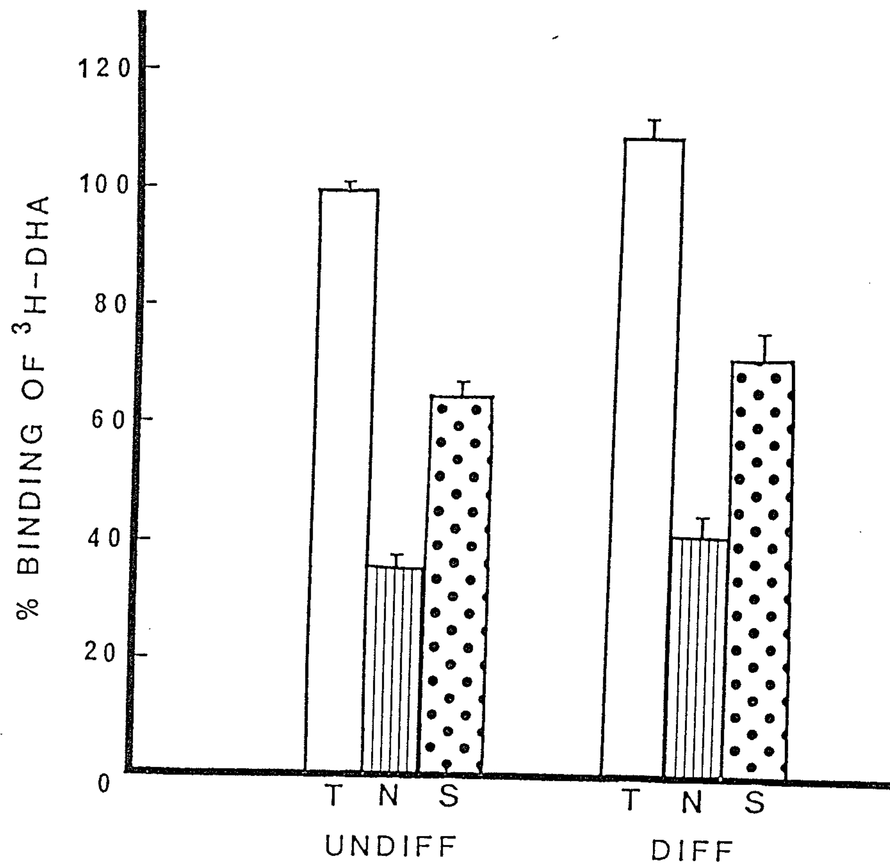

Fig. 13. Comparison of beta-adrenergic receptor bindings on intact differentiated and undifferentiated NG108-15 cells. Cells were cultured in either medium E (differentiated) or medium D (undifferentiated) under standard conditions. For binding assays, cells were harvested, washed and incubated with 0.3 nM [^{3}H]-DHA in the presence or absence of 0.5 mM L-alprenolol for 20 min at 37°C. Specific and non-specific [^{3}H]-DHA bindings were expressed as percent of the total binding. (T), total binding; (N), non-specific binding; (S), specific binding.

[^{3}H]-DHA binding on differentiated cells was observed. This contributed only 7 % and 6 % increase in non-specific and specific receptor bindings, respectively, on differentiated NG108-15 cells (Fig. 13). Beta-adrenergic receptor was further characterized becuase the presence of this receptor on NG108-15 cells has not been reported.

4 - Saturation Experiment

Fig. 14 shows that the specific binding of [^{3}H]-DHA to intact NG108-15 cells was a saturable process. The maximum number of binding sites at saturation, as assessed by the Scatchard plot analysis (Fig. 15), for intact cells was estimated to be 0.23 nM. There was no indication of interaction between receptor sites as evidenced by the linearity of the Scatchard plot (Fig.15). Computer analysis using the EBDA program (121) of data obtained from saturation experiments revealed a single receptor type for [^{3}H]-DHA on intact NG108-15 cells. Analysis of data on Fig.14 also revealed that the equili-brium association constant (Kd), the concentration of [^{3}H]-DHA to obtain half maximal binding occurred, was 2.5 nM, which is in excellent agreement with that of 2.9 nM calculated by Scatchard analysis (Fig.15). Analysis of the specific binding data by the Hill plot (Fig.16) provided a Hill coefficient (nH) of 1.0, indicating no co-operativity between [^{3}H]-DHA binding sites on NG108-15 cells. This Hill coefficient is another indication for the existence of a single type of B-adrenergic receptors on NG108-15 cells.

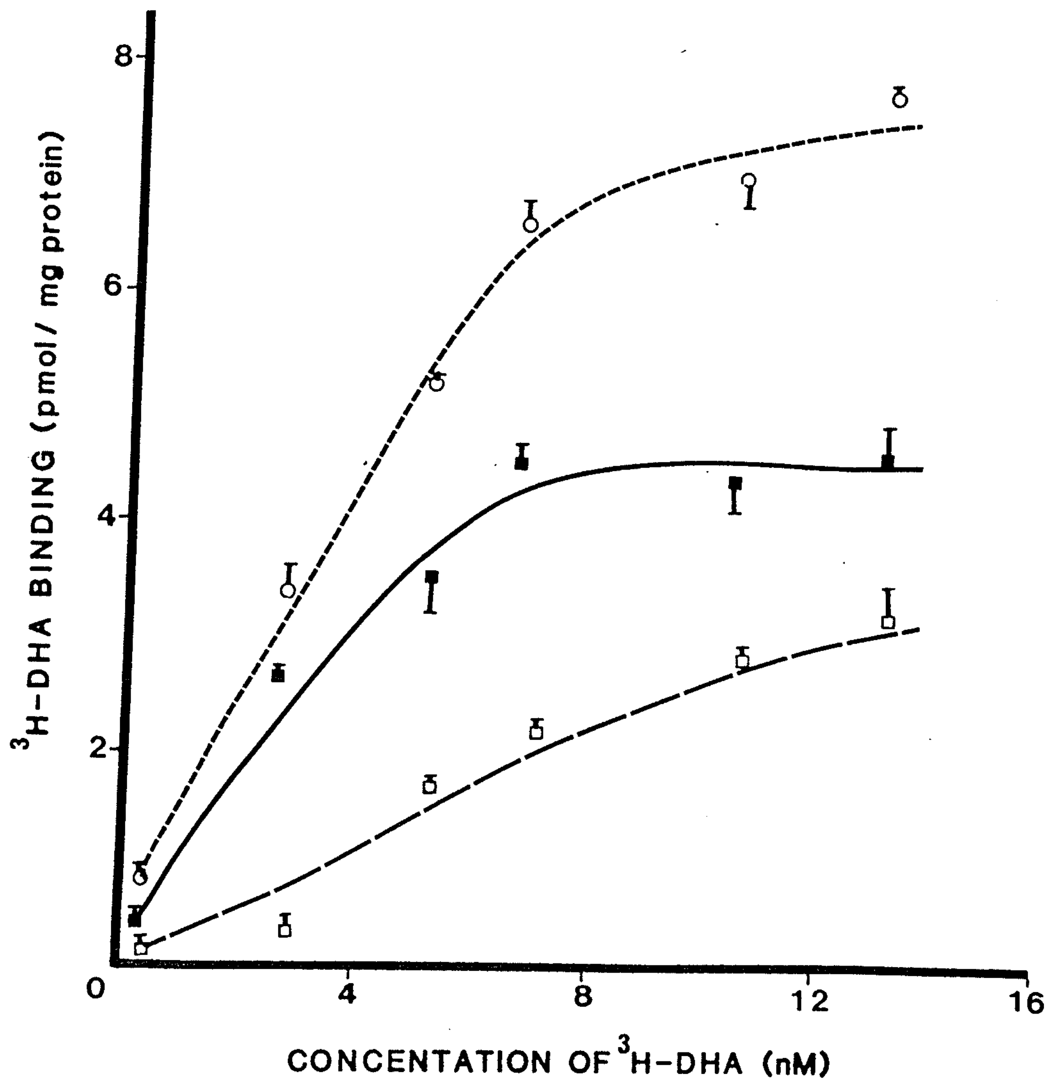

Fig.14.Equilibrium binding of [^{3}H]-DHA to intact NG108-15 cells. Cells were grown in medium D under standard culture conditions for 5 days. For binding assays, cells were harvested, washed and incubated with various concentrations of [^{3}H]-DHA for 20 min at 37° C. [^{3}H]-DHA binding was expressed as pmole/mg protein. Data represent a typical experiment in triplicates (means ± SEM). (○), total binding; (■), specific binding; (□), non-specific binding.

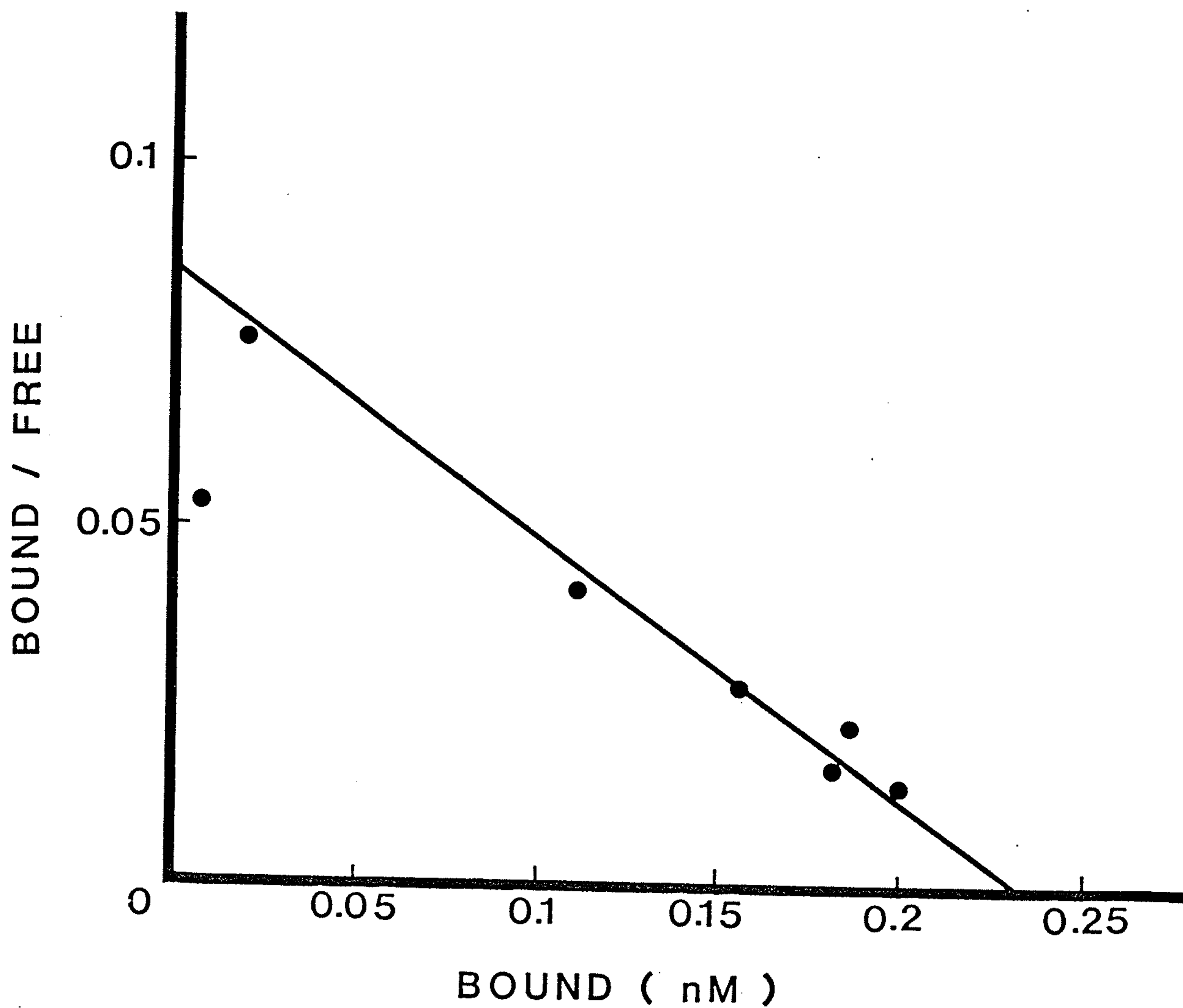

Fig. 15. Scatchard plot of [^{3}H]-DHA binding on intact NG108-15 cells. Data from saturation experiments at equilibrium (Fig. 14) were analysed and plotted as a function of [^{3}H]-DHA bound versus ratio (bound / free).

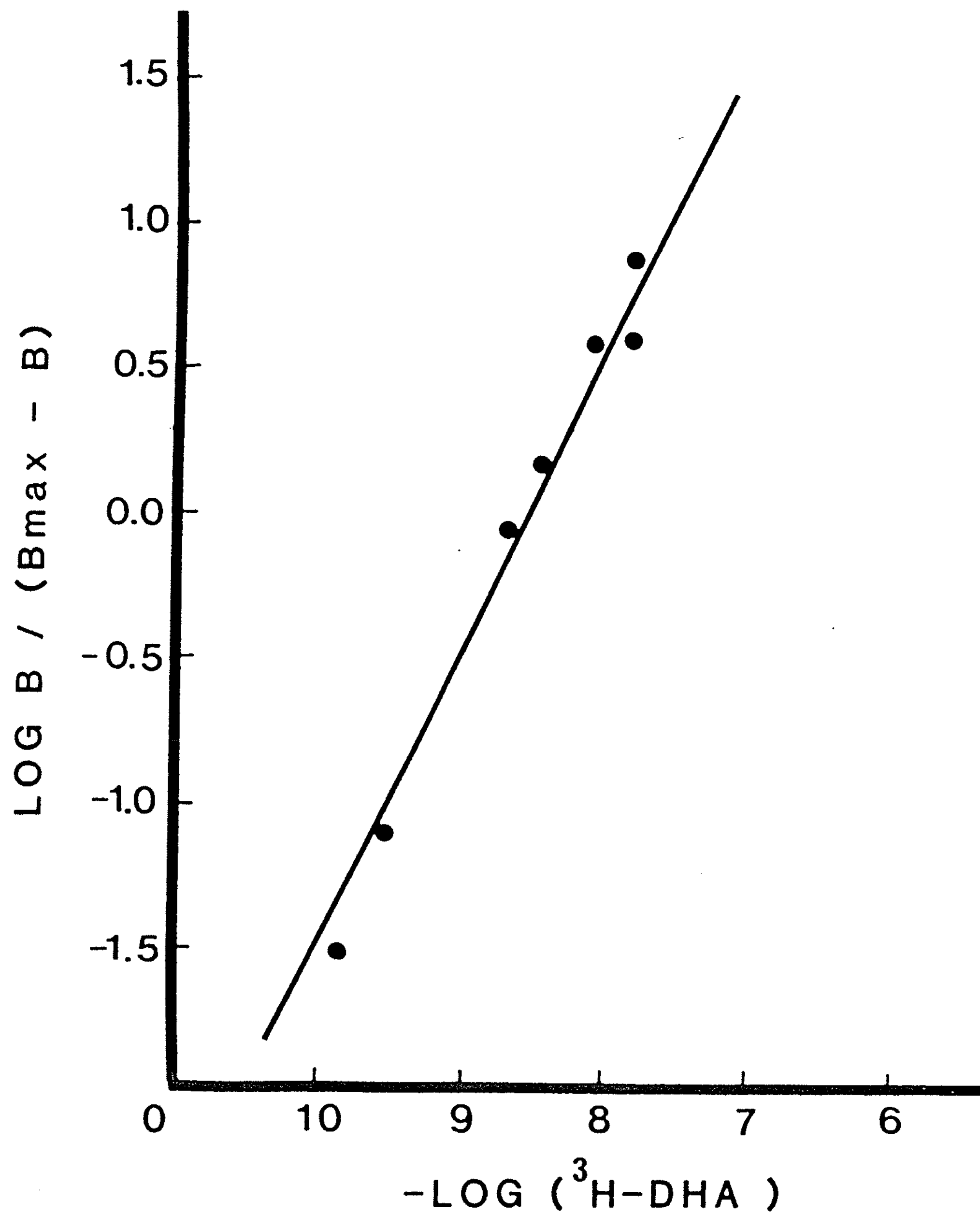

Fig.16. Hill plot of [^{3}H]-DHA binding to intact NG108-15 cells. Data obtained from saturation experiments at equilibrium (Fig.14) were analysed and plotted as a function of Log (Free) versus log (bound / Bmax - bound).

5 - Dissociation and Association of $[^3H]$-DHA Binding

The off rate of specifically bound $[^3H]$-DHA on intact NG108-15 cells after the addition of L-alprenolol is shown in Fig. 17. From these data, the t 1/2 for dissociation was estimated to be 3.5 min. The Dissociation Rate Constant (k-1) was calculated to be 0.19, using equation (K-1) = ln 2 / t 1/2.

The on rate constant (K+1) was determined by measuring the binding of $[^3H]$-DHA on intact cells at various incubation time intervals. Fig. 18 depicts that binding of $[^3H]$-DHA to β-adrenergic receptors reached equilibrium in 10 min with t 1/2 of approximately 1.0 min. The Association Rate Constant (K+1) was calculated to be $0.7 \times 10^{-9}\ M^{-1}\ min^{-1}$, using equation K+1 = (Kob - [K-1]) /($[^3H]$-DHA), where (K-1) is the Dissociation Rate Constant (Fig.17) and Kob is the slope of the plot of time versus ln [Beq / (Beq - Bt).

6 - Inhibition of $[^3H]$-DHA Binding by L-Alprenolol

Fig. 19 reveals that $[^3H]$-DHA binding to β-adrenergic receptors was inversly proportional to the concentrations of L-alprenolol added to the sample. The concentration of L-alprenolol that displaced 50 % of $[^3H]$-DHA binding at equilibrium (Ic50) was estimated to be 10 uM; and the inhibition constant (Ki) was calculated to be 8.9 uM, using equation Ki = Ic50 / (1 + $[^3H]$-DHA / Kd).

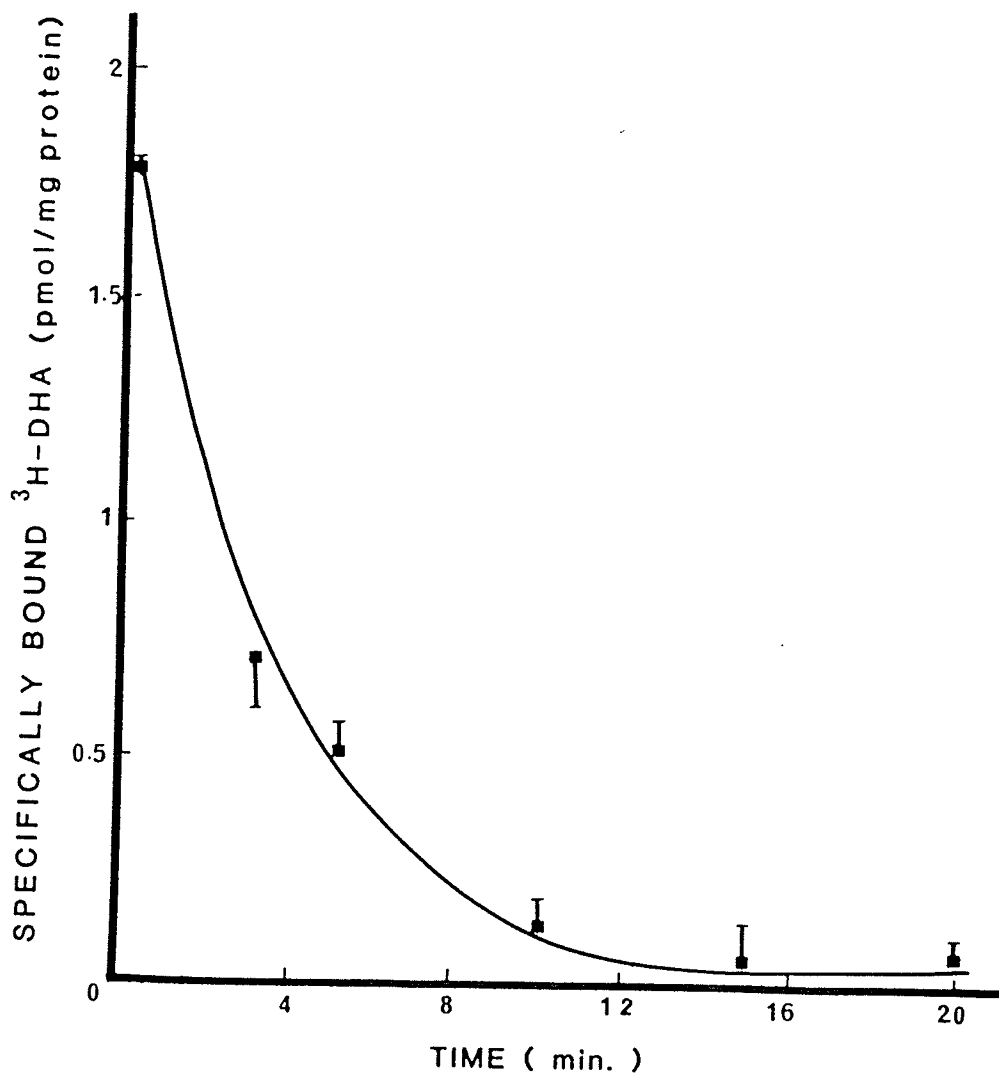

Fig.17. Time course for dissociation of receptor-bound $[^3H]$-DHA from intact NG108-15 cells. Cells were incubated with $[^3H]$-DHA for 20 min at 37°C to allow binding reaction to reach equilibrium. At 0 min time L-alprenolol was added, and at various time intervals the extent of specific binding was determined. t 1/2 was estimated as the incubation time for 50 % of bound $[^3H]$-DHA to be dissociated from binding. The Dissociation Rate Constant (K-1) was determined from equation: (K-1) = ln 2 / (t 1/2).

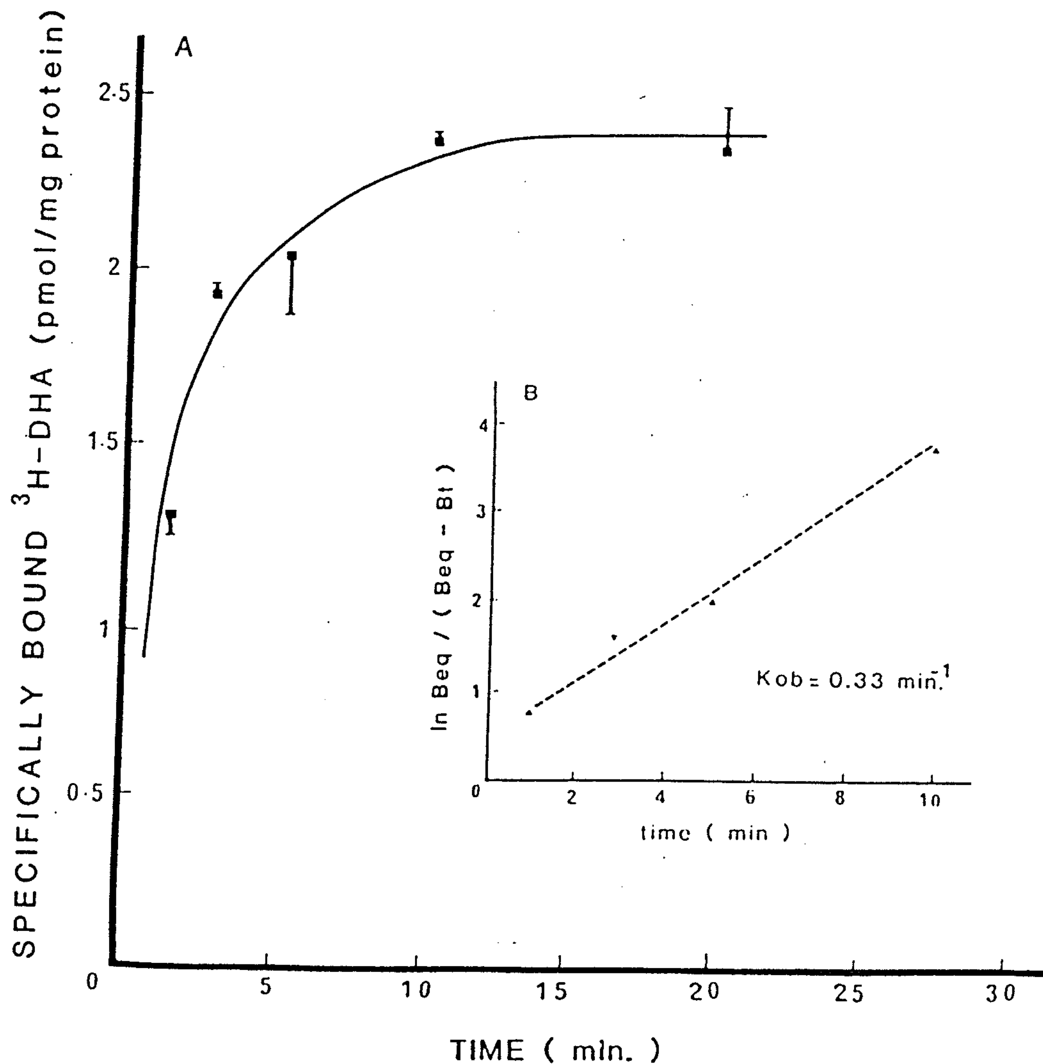

Fig.18. Time course for association of $[^3H]$-DHA to NG108-15 cells. Cells were incubated with $[^3H]$-DHA in the presence or absence of L-alprenolol at indicated time intervals at 37°C. t 1/2 was estimated as the incubation time required for $[^3H]$-DHA to occupy 50 % of the total binding. The Association Rate Constant (K+1) was determined from equation: (K+1) = [Kob - (K-1)] / $[^3H]$-DHA, where Kob is the slope of the curve B and (K-1) is the Dissociation Rate Constant estimated from Fig.17.

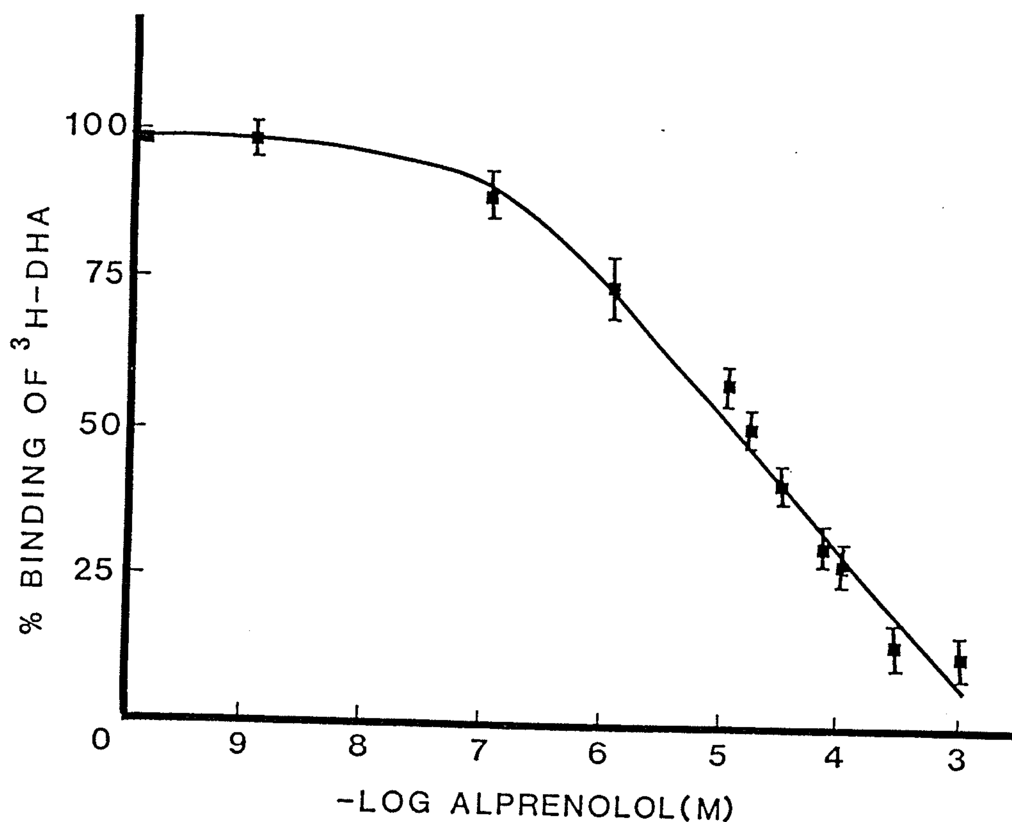

Fig.19. Inhibition of [^{3}H]-DHA binding by L-alprenolol on NG108-15 cells. Inhibition of [^{3}H]-DHA binding was determined by incubating 0.3 nM [^{3}H]-DHA with various concentrations of L-alprenolol under standard assay conditions. Data were analysed and plotted as percent inhibition of [^{3}H]-DHA binding. Ic50 value was calculated as the concentration of L-alprenolol to displace 50% of [^{3}H]-DHA binding at equilibrium. Data represent a typical experiment in triplicates (means ± SEM).

7 - Type of Beta-Adrenergic Receptor

Type of beta-adrenergic receptors on plasma membrane preparations and intact NG108-15 cells was determined according to the procedure of Land et al. (128), with slight modifications. The rank order potency of agonist: (-)(ISO), (+) (ISO), EPI and NE, to bind B-adrenergic receptors on membrane preparations and intact cells in differentiated and undifferentiated conditions was estimated with competition experiments of each individual agonist. Figs. 20 and 21 show a rank order potency of (-) ISO > (+) ISO > EPI > NE for B-adrenergic receptors on either plasma membranes (Fig. 20) or intact NG108-15 cells (Fig. 21). This rank order did not change when NG108-15 cells were induced to undergo morphological differentiation by dBcAMP treatment for 5 days (Figs.22 and 23). From the rank order potencies of agonists: (-) ISO > (+) ISO > EPI > NE,to bind B-adrenergic receptors, as shown in Figs.20, 21, 22, and 23, a beta-2 adrenergic receptor on both differentiated and undifferentiated cells is evident.

Stereoselectivity of beta-2 adrenergic receptor is shown by the greater potency (5X) in binding of L-isoproterenol to these receptors on both plasma membranes and intact cells under differentiated and undifferentiated conditions as compared to the D-isomer (Figs. 20, 21, 22, and 23). The Ic50 value for each individual agonist, (-)ISO, (+) ISO, EPI and NE, to bind B-adrenergic receptors on plasma membranes from undifferentiated cells (Fig. 20),

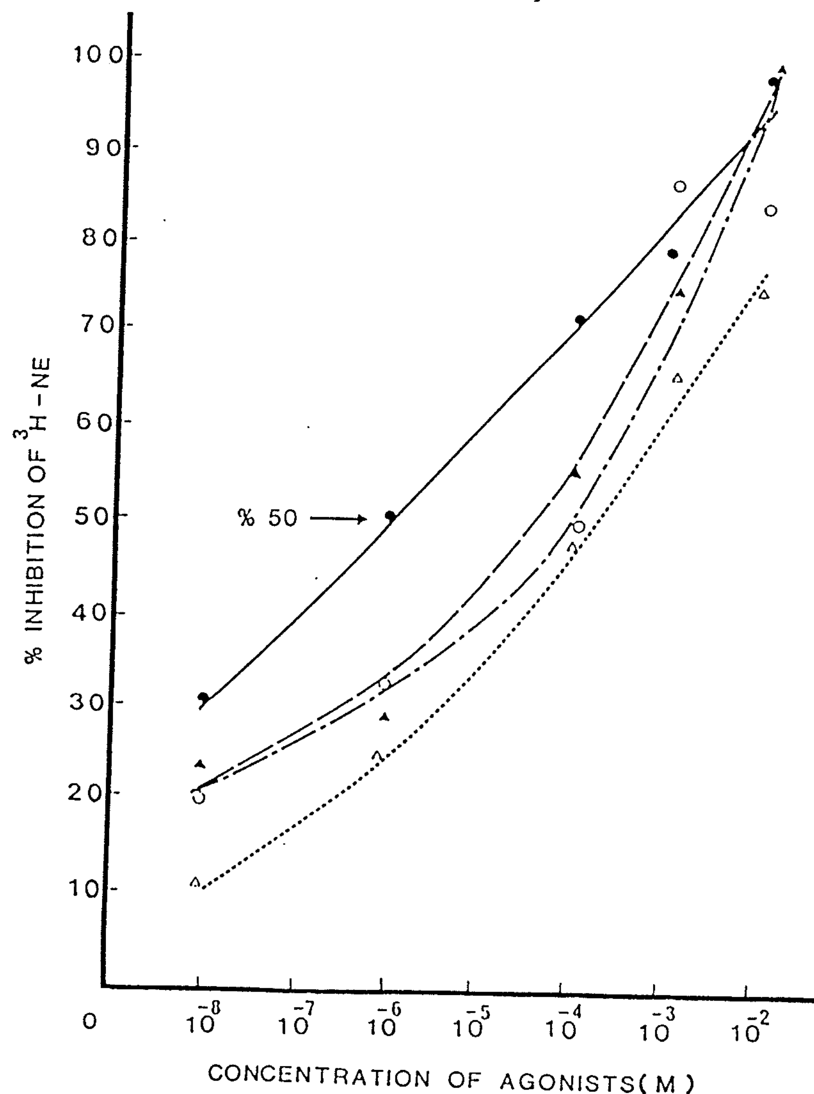

Fig. 20. Inhibition of [^{3}H]-norepinephrine binding by catecholamine agonists to plasma membranes from undifferentiated NG108-15 cells. Cells were cultured in DMEM containing 10% FCS for 5 days. Plasma membranes were prepared as described under " Materials and Methods ". For binding assays, plasma membranes were resuspended in DMEM and incubated with 6.8 nM [^{3}H]-NE in the presence of various concentrations of catecholamine agonists for 20 min, at 37°C. The Ic50 values from each agonist was determined as the concentration to displace 50 % of the specific binding of [^{3}H]-NE. (●), -ISO; (▲),+ISO; (O), EPI; and (▽), NE.

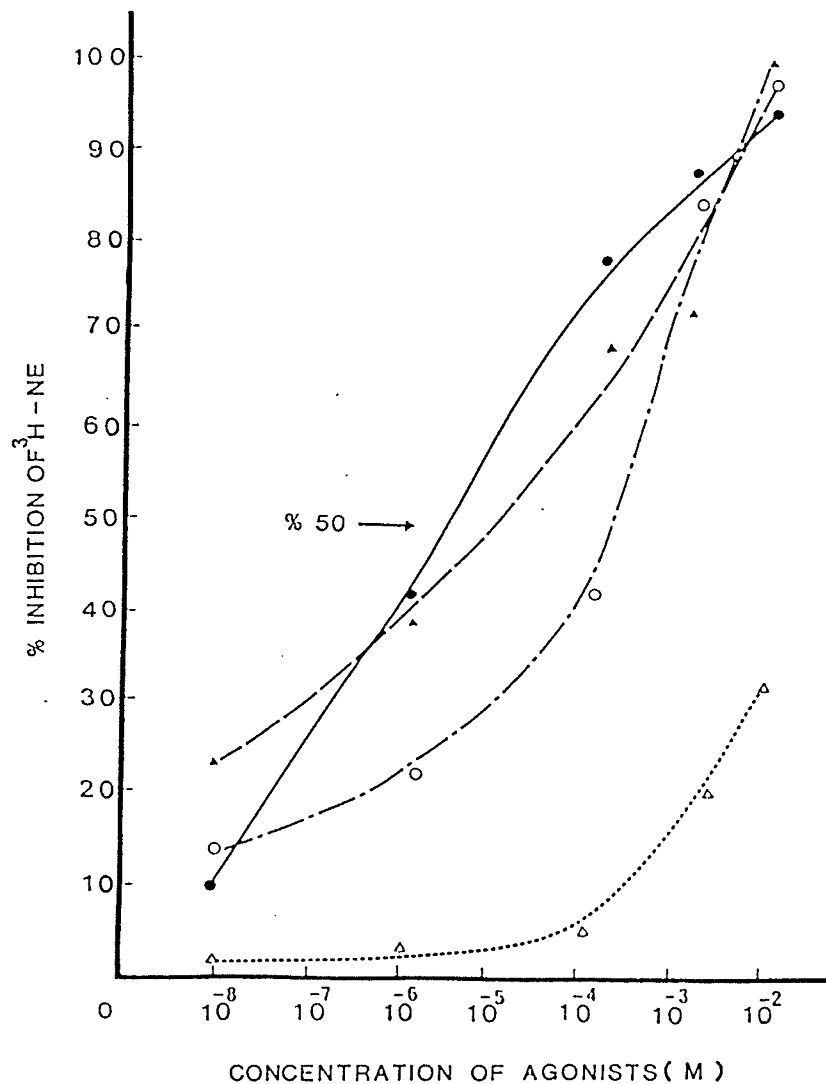

Fig. 21. Inhibition of [^{3}H]-norepinephrine binding by catecholamine agonists to intact undifferentiated NG108-15 cells. Cells were cultured in medium D containing 10% FCS for 5 days. Cells were washed and incubated with 6.8 nM [^{3}H]-NE in the presence of various concentrations of catecholamine agonists for 20 min at 37°C. The Ic50 value for each agonist was determined as the concentration to displace 50% of the specific binding of [^{3}H]-NE. (●), -ISO; (▲), +ISO; (○), EPI; (△) NE.

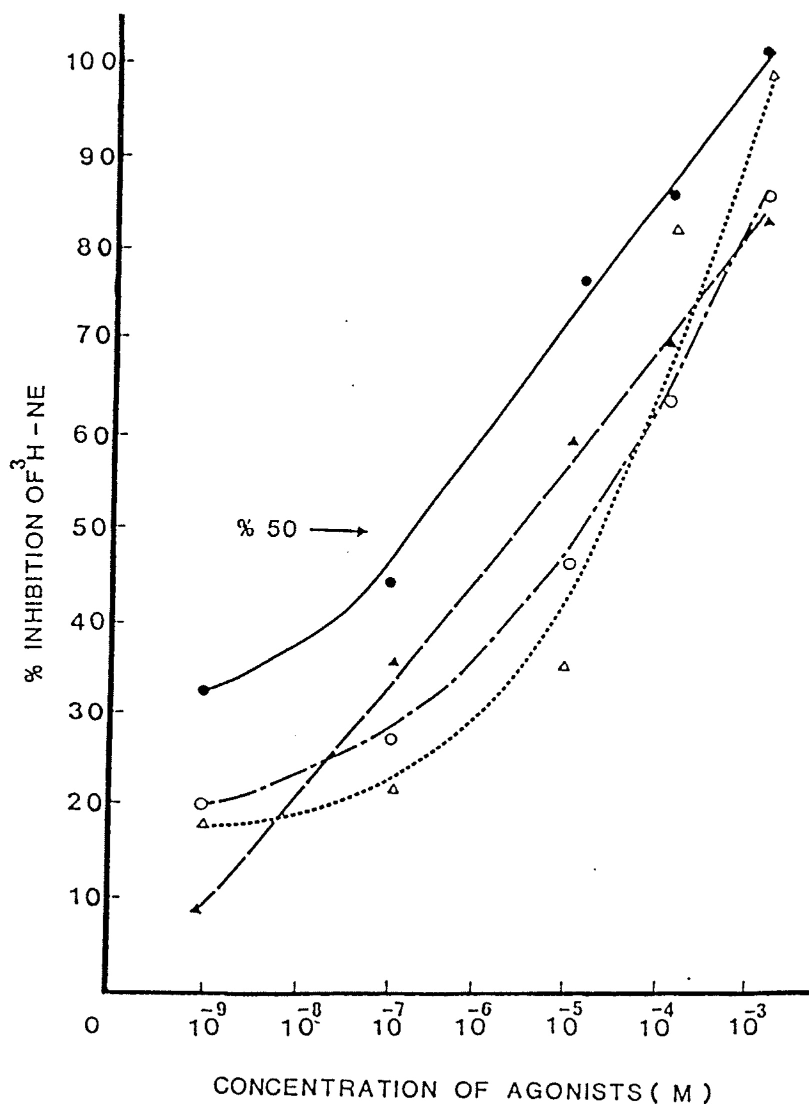

Fig.22. Inhibition of $[^3H]$-norepinephrine binding by catecholamine agonists to plasma membranes from differentiated NG108-15 cells. Cells were cultured in medium E containing 5% FCS plus 1mM dBcAMP for 5 days. Plasma membranes were prepared as described under " Materials and Methods ". Membranes were resuspended in DMEM and incubated with 6.8 nM $[^3H]$-NE in the presence of various concentrations of catecholamine agonists for 20 min at 37°C. The Ic50 value for each agonist was determined as the concentration to displace 50% of the specific binding of $[^3H]$-NE. (●), -ISO; (▲), +ISO; (○), EPI; (△), NE.

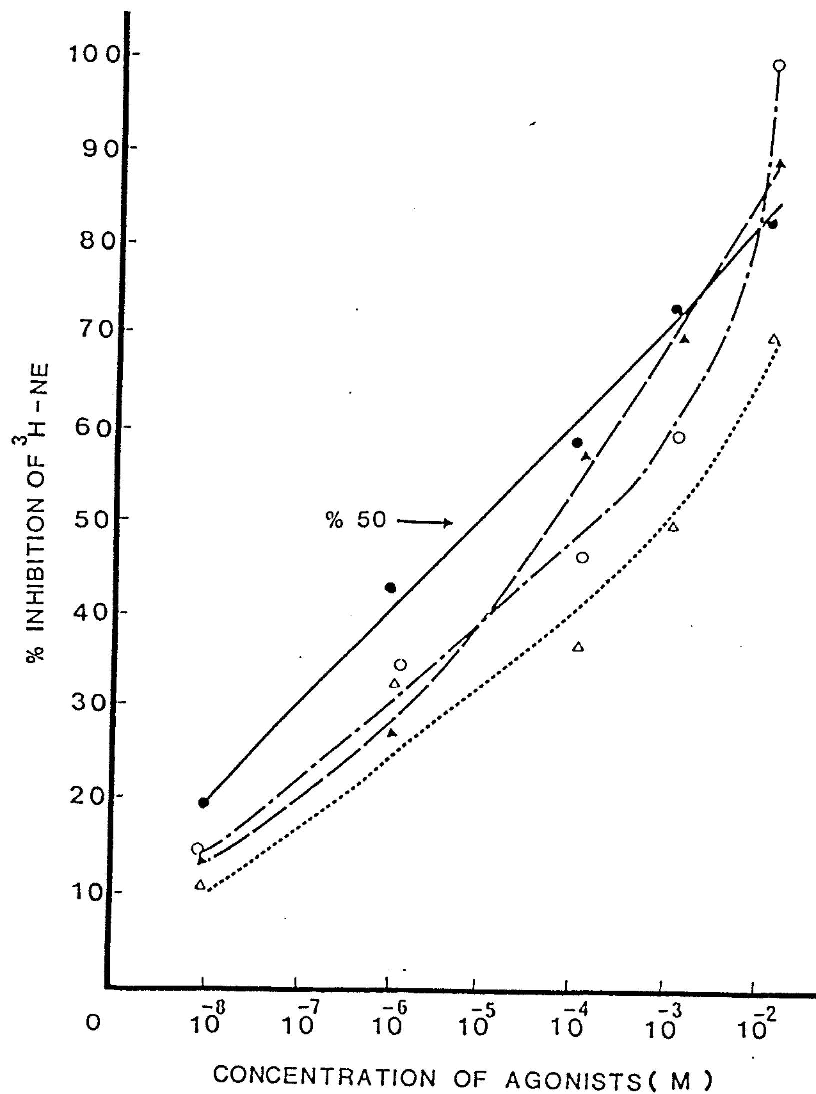

Fig. 23. Inhibition of [^{3}H]-norepinephrine binding by catecholamine agonists to the intact differentiated NG108-15 cells. Cells were cultured in medium E containing 5% FCS plus 1mM dBcAMP for 5 days. Cells were harvested, washed and incubated with 6.8 nM [^{3}H]-NE in the presence of various concentrations of catecholamine agonists for 20 min at 37°C. The Ic50 value for each agonist was determined as the concentration to displace 50% of the specific binding of [^{3}H]-NE. (●), -ISO; (▲), +ISO; (○), EPI; and (△), NE.

intact undifferentiated cells (Fig. 21), plasma membranes from differentiated cells (Fig.22) and intact differentiated cells (Fig.23) were summarized in Table 1. Comparison of these values indicates that almost all these agonists possessed greater potencies (3 - 100 times) to bind receptors on isolated plasma membranes from either undifferentiated or differentiated cells than those on intact cells. Table 1 and Fig. 21 show that NE possessed the lowest potency to bind β-adrenergic receptors on intact undifferentiated cells with Ic50 greater than 1mM. This indicates that NE with concentration of 1mM is not able to inhibit 50 % of the total radioligand binding to β-adrenergic receptors on intact undifferentiated cells. However, the potency of NE to bind β-adrenergic receptors enhanced more than 100-fold when plasma membranes from the same undifferentiated cells were used (Fig.20). Meanwhile Ic50 of NE decreased from > 1mM (intact cells) to 200 uM (isolated membranes). Although most agonists bound with greater potency to plasma membranes than on intact cells (Table 1), the overall comparison of the Ic50 values of β-adrenergic agonists as shown in Table 1 indicates that the potency of almost all agonists for β-adrenergic receptors on both membranes and cells was enhanced upon differentiation, induced by dBcAMP, as compared to that of undifferentiated cells.

CELL TYPE	AGONISTS	INTACT CELLS	PLASMA MEMBRANE
UNDIFF. CELL	(-) ISO	$5X10^{-5}$ M	$8X10^{-7}$ M
	(+) ISO	$5X10^{-5}$ M	$6X10^{-5}$ M
	EPI	$1X10^{-4}$ M	$1X10^{-4}$ M
	NE	-	$2X10^{-4}$ M
DIFF. CELL	(-) ISO	$8X10^{-6}$ M	$2.5X10^{-7}$ M
	(+) ISO	$8X10^{-5}$ M	$1X10^{-5}$ M
	EPI	$1X10^{-4}$ M	$5X10^{-6}$ M
	NE	$1X10^{-3}$ M	$1X10^{-5}$ M

Table 1. Comparison of the Ic50 values for beta-adrenergic receptors on isolated plasma membranes and intact NG108-15 cells under undifferentiated and differentiated conditions. Cells were cultured in medium D (undifferentiated) and medium E (differentiated) for 5 days. Specific bindings were carried out as described in "Materials and Methods" (see Figs. 20, 21, 22, and 23). The Ic50 value for each agonist was determined as the concentration to displace 50% of specific binding of $[^3H]$-NE to the beta-adrenergic receptor.

1V - Protein Contents of Differentiated and Undifferentiated Cells

1 - One Dimensional Gel Electrophoresis

A comparison of SDS-gel electrophoresis of proteins extracted from undifferentiated control cells and differentiated NG108-15 cells as induced by dBcAMP, DMSO or DX is shown in Fig. 24. More than 45 different proteins were detected upon gel electrophoresis after silver staining. The levels of several proteins appeared to vary between undifferentiated and differentiated cells, and among the differentiated cells treated with dBcAMP, DMSO, and DX. In lane 3 of the dBcAMP treated cells, which had the highest number of neurites, a protein with MW of approximately 32 Kda was strongly expressed, compared to those on lane 2, 4, and 5 of the undifferentiated , DMSO-, and DX-treated cells, respectively. A protein with MW of approximately 85 Kda was detected only on lane 4 and 5 of the DMSO- and DX-treated cells, respectively. A protein with MW of approximately 40 Kda was strongly expressed and another with MW of 35 Kda was weakly expressed in undifferentiated cells (lane 2), as compared to those proteins of identical electrophoretic mobilation in dBcAMP-,DMSO- and DX-treated NG108-15 cells (lanes 3, 4 and 5).

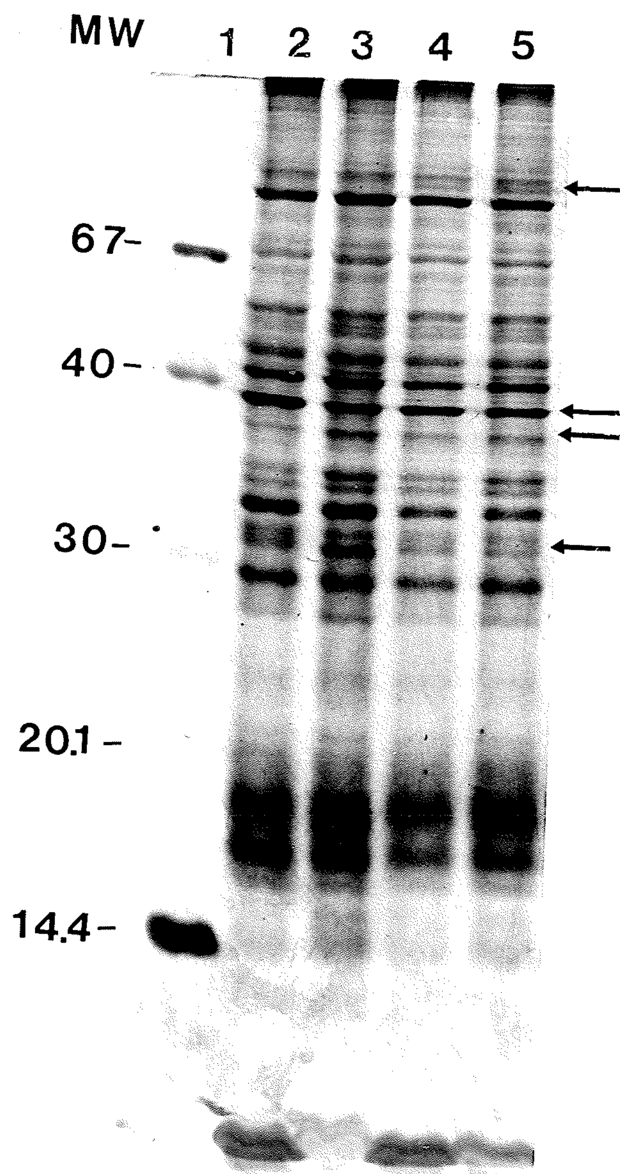

Fig. 24. SDS-Gel electrophoresis of proteins, from undifferentiated (control) and differentiated cells treated by various inducing agents. NG108-15 cells were cultured in medium D containing either 10% FCS (undifferentiated) or medium E containing 5% FCS with an appropriate amount of a neurite inducing agents (1mM dBcAMP, 1 % DMSO and 25 uM DX) for 5 days. Cells were extracted as described in " Materials and Methods ". Proteins were subjected to SDS-PAGE using 12 % polyacrylamide gel, followed by silver staining proteins. Lane 1, protein markers: (94 Kda),phosphorylase;(67 Kda),bovine serum albumin;(40 kda), ovalbumin; (30 Kda), carbonic anhydrase; (20.1 Kda),trypsin inhibitor; (14.4 Kda), lactalbumin ;lane 2, undifferentiated cells; lane 3,4,and 5 differentiated cells induced by dBcAMP, DMSO and DX respectively.

2 - Two Dimensional Gel Electrophoresis

Fig.25 shows the protein profiles of differentiated (upper panel) and undifferentiated (lower panel) NG108-15 cells cultured for 24 hrs in leucine-free medium, containing 1 uCi [^{3}H]-leucine. Levels of proteins labelled A, C and D were higher, whereas protein B was lower in differentiated (upper panel) as compared to those corresponding proteins in undifferentiated cells (lower panel). In these experiments with [^{3}H]-leucine, only minor differences in proteins were observed between differentiated and undifferentiated NG108-15 cells.

Fig. 26 presents the protein profiles of differentiated (upper panel) and undifferentiated (lower panel) NG108-15 cells cultured for 24 hrs in medium E or D, respectively, containing 10 uCi [^{35}S]-methionine. Many proteins in both cell types migrated identically on the basis of their isoelectric points (PI) and molecular weights. Comparison of the protein profiles of differentiated and undifferentiated cells reveals that at least 12 protein spots varied in concentrations between differentiated and undifferentiated cells. Concentrations of proteins labelled B,C,D,E, and K apparently decreased upon 24 hrs of dBcAMP treatment, whereas, concentration of proteins labelled A,F,G,H,I,J, and L increased upon induced differentiation (Fig.26). Since [^{35}S]-met. was incorporated into newly synthesized proteins at early stages of cellular differentiation (24 hrs), it is possible that some of these 12 proteins play an important role in the differentiation of the NG108-15 cell.

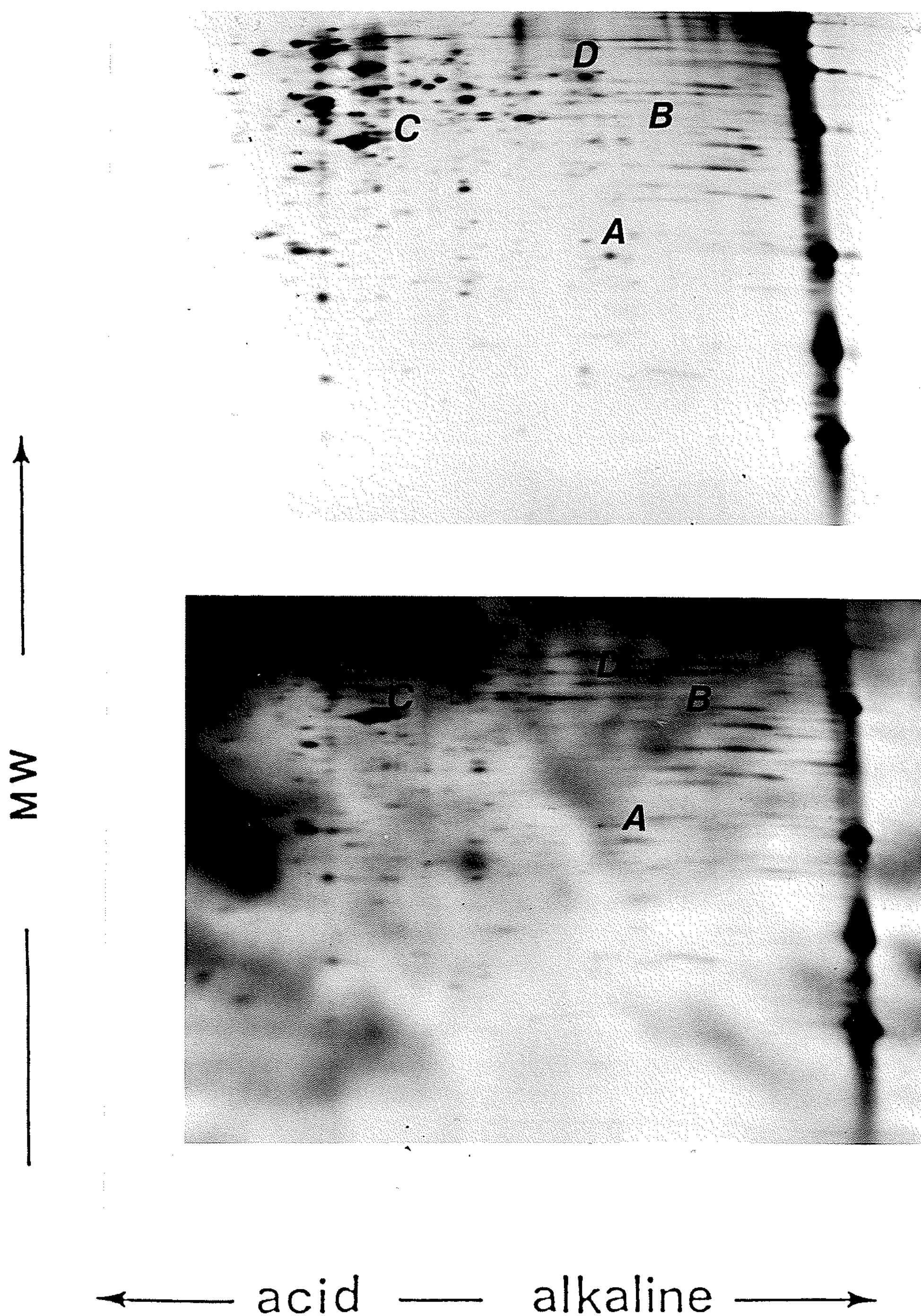

Fig. 25. Autoradiographs of $[^3H]$-labelled proteins in differentiated and undifferentiated NG108-15 cells. Differentiated (upper panel) and undifferentiated (lower panel) cells were cultured in leucine-free medium E or D, respectively, supplemented with 1 uCi $[^3H]$-leucine. After 24 hrs, cells were extracted and subjected to two-dimensional gel electrophoresis as described in " Materials and Methods ". Proteins of varying concentrations between differentiated (upper panel) and undifferentiated cells (lower panel) are labelled from A to D for identification.

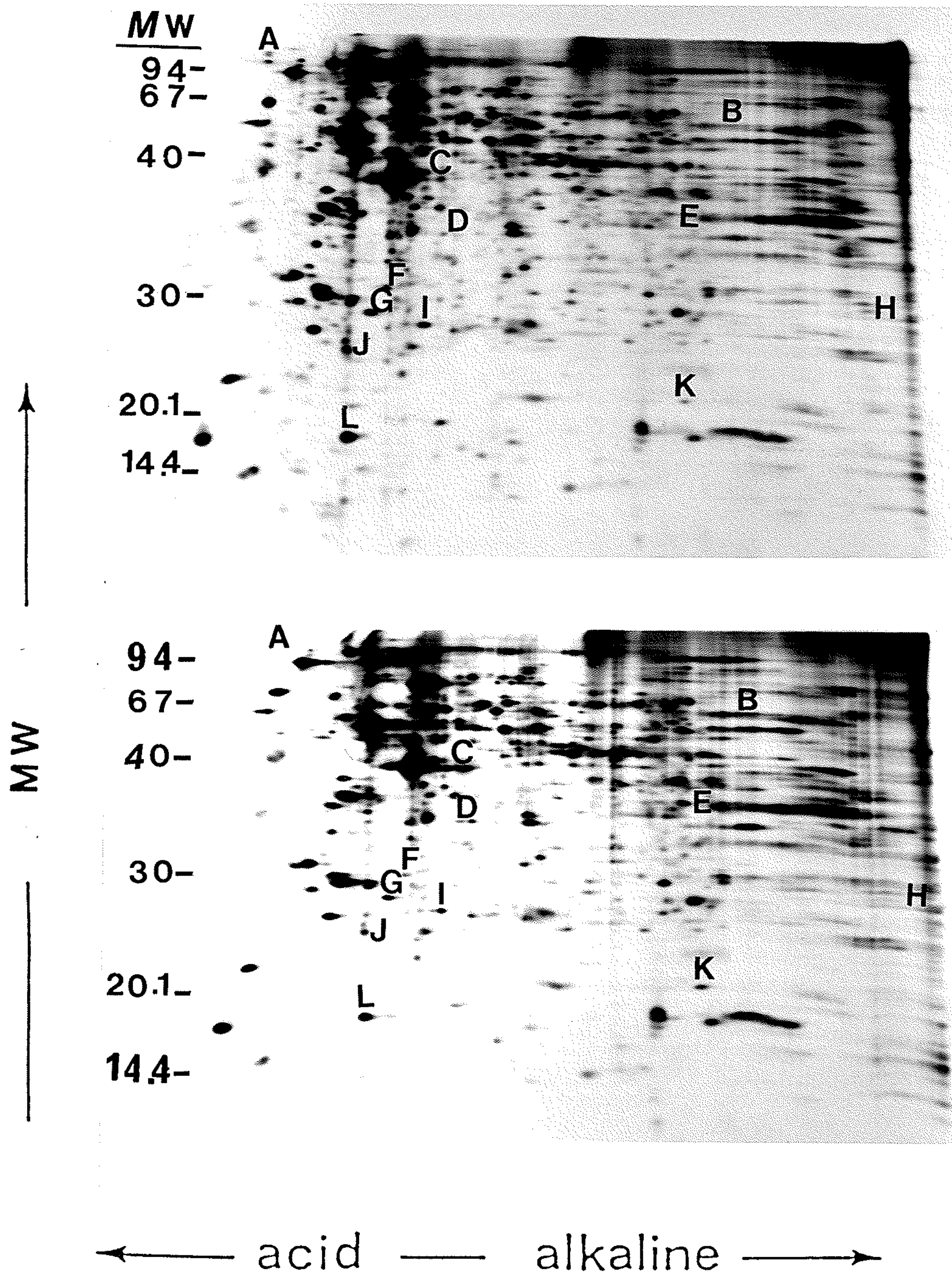

Fig.26. Autoradiographs of [^{35}S]-met.labelled proteins of differentiated and undifferentiated NG108-15 cells. Differentiated (upper panel) and undifferentiated (lower panel) cells were cultured in methionine-free medium E or D, respectively, supplemented with 10 uCi [^{35}S]-met. After 24 hrs, cells were extracted and subjected to two-dimensional gel electrophoresis as described under " Materials and Methods ". Proteins of varying concentrations between differentiated and undifferentiated cells are labelled from A to K for identification.

V- Contents of Indolamines and Thier Major Metabolites in Undifferentiated, Differentiated and De-differentiated Cells

Figure 27 shows a representative chromatogram of alumina extracts of differentiated and undiffererntiated NG108-15 cells. The content of NE, EPI, DA and Dopac in differentiated cells (Fig. 27 A) was 0.15 ng, 0.1 ng, 0.55 ng and 0.15 ng / 10^6 cells, respectively. Similarly in undifferentiated cells, the content of NE and DA was calculated to be 0.11 ng and 0.13 ng/10^6cells, respectively. EPI and Dopac were not detectable in the alumina extracts of undifferentiated cells under those experimental conditions. However, trace amounts of Dopac would be expected with increased cell number since DA was detected. NE and DA contents of differentiated NG108-15 cells extracted with alumina, increased 36.4% and 328%, respectively,as compared to those of undifferentiated cells.

Similar results in indolamine content were obtained with experiments using perchloric acid extraction of cells. Table 2. shows that NE and DA content in differentiated cells increased 40 % and 129%, respectively. Perchloric acid extraction was also used to study the cellular contents of 5-HT and several other metabolites, not recoverable by alumina extraction. Fig.28 shows representative chromatograms of perchloric acid extracts of cells. Fig.28 A shows the chromatogram of the standards of transmitters and their major metabolites. Fig.28 B reveals the absence of 5-HT in differentiated NG108-15 cells,

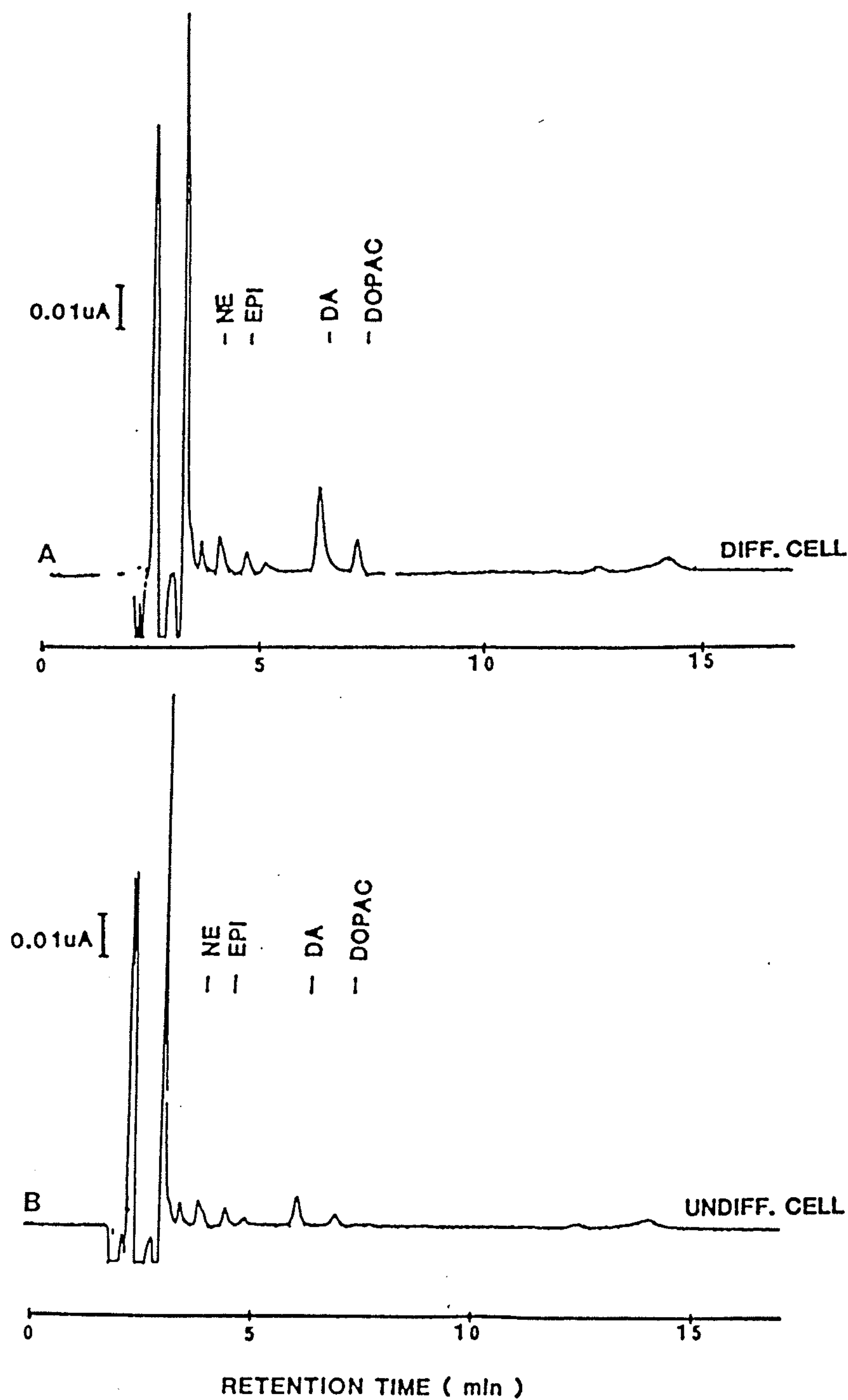

Fig. 27. HPLC chromatograms of alumina extraction of differentiated and undifferentiated NG108-15 cells. Cells were cultured in medium E containing 5 % FCS plus 1mM dBcAMP (differentiated) or medium D with 10 % FCS undifferentiated) for 5 days. Equal numbers of cells were extracted and analysed using acid washed alumina as described in " Materials and Methods". (A), differentiated cells and (B), undifferentiated cells.

transmitters or metabolites	ng / 10^6 cells		% increase in diff.cells
	Undiff	Diff	
L-DOPA	1.91 ± 0.01	4.75 ± 0.15	149
NE	0.54 ± 0.18	0.76 ± 0.23	40
EPI	0.61 ± 0.11	1.42 ± 0.21	129
DA	0.73 ± 0.12	1.64 ± 0.14	124
DOPAC	ND	0.64 ± 0.09	-
5 - HT	1.01 ± 0.09	ND	-

Table 2. Comparison of neurotransmitters and their major metabolites extracted from differentiated and undifferentiated NG108-15 cells. The cells were grown in either medium D (DMEM plus 10 % FCS) or E (DMEM plus 5 % FCS and 1mM dBcAMP). After 5 days of culture, cells were dislodged mechanically and collected by centrifugation. Cells were disrupted by sonication and extracted into per-chloric acid as described in " Materials and Methods ". An aliquot of 20 ul of cell extracts was injected into the HPLC system, and the concentration of each indolamine was calculated and expressed as ng per 1x 10^6 cells. ND = not detectable, L-Dopa = L-dihydroxyphenylalanine, NE = norepinephrine, EPI = epinephrine, DA = dopamine, DOPAC = 3, 4, dihydroxyphenet-hylacetic acid, and 5-HT = serotonin.

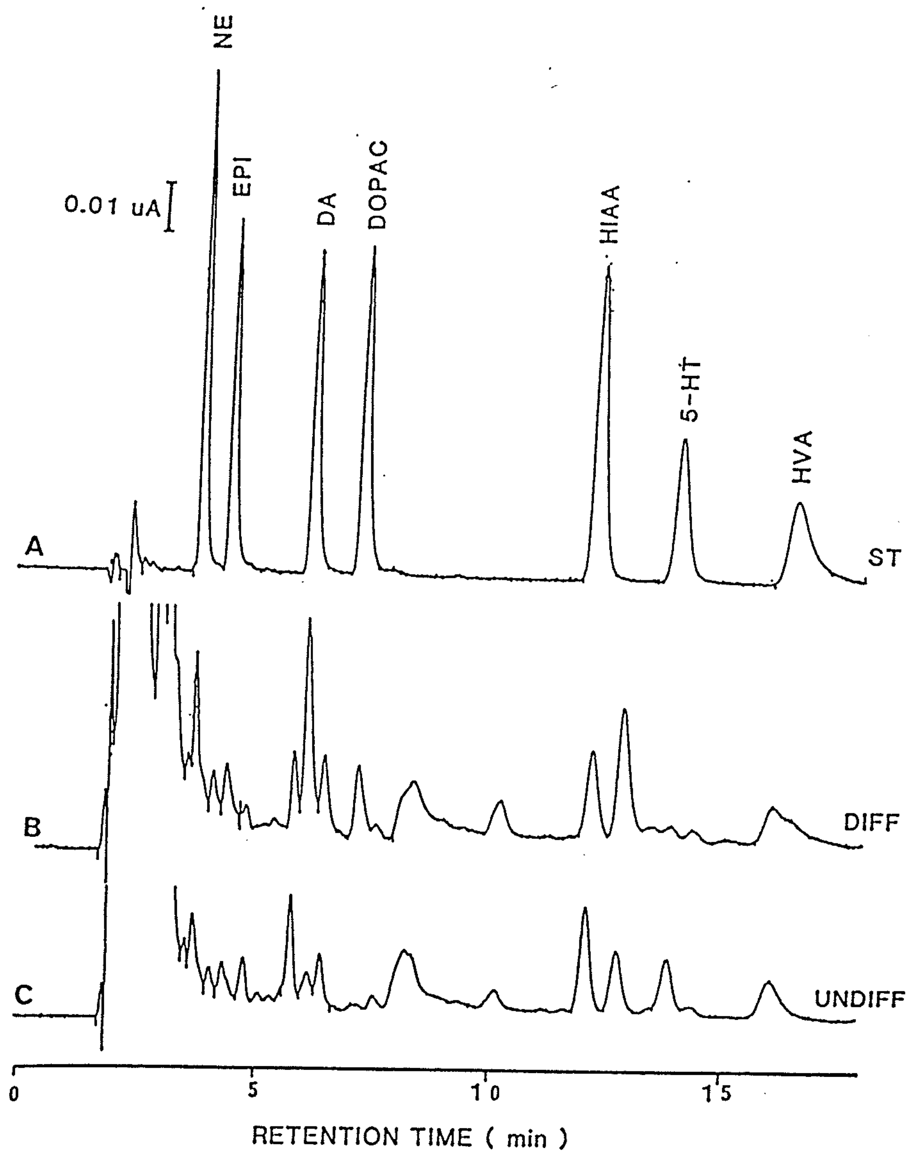

Fig. 28. HPLC chromatograms of indolamine in extracts of differentiated and undifferentiated NG108-15 cells. Cells were cultured in medium E containing 5 % FCS and 1mM dBcAMP (differentiated) or medium D with 10 % FCS (undifferentiated). At day 5, cells were extracted with perchloric acid as described under "Materials and Methods" for indolamine analysis. Indolamine standards were 2 ng of each. (A), standards; (B), differentiated cells; and (C), undifferentiated cells.

which 5-HT was detectable in the extracts of undifferentiated cells (Fig.28 C). In addition, 5-HIAA, which is a major metabolite of 5-HT,increased 12.7% in undifferentiated cells. An unidentified peak with a retention time of 12.8 min between HIAA and 5-HT was observed, being substantially higher in differentiated (Fig.28 B) than in undifferentiated cells (Fig. 28 C). This substance appears to migrate with a retention time close to that of 3-methoxytyramine (3-MT), a DA metabolite. In addition,the concentration of homovanillic acid, a major metabolite of DA, was revealed to be higher in differentiated cells (Fig. 28 B), as compared to that of undifferentiated cells (Fig.28 C). Similarly, as shown in Fig 29, L-dopa, a DA precursor, increased approximately 2-fold in differentiated cells.

To confirm the changes of catecholamine content (L-Dopa, DA, NE and EPI) in differentiated NG108-15 cells, the activity of TH, the rate limited enzyme in biosynthesis of catecholamines, was measured. Fig.30 indicates that the TH activity increased 104% in NG108-15 cells upon induced differentiation by dBcAMP treatment for 5 days.

To examine the possibility of reversible changes in the content of indolamine upon differentiation, morphologically differentiated NG108-15 cells were reverted to undifferentiated state by replacing the culture medium containing 1mM dBcAMP (medium E) to dBcAMP-free medium for 24 hrs. Changes in indolamine content were measured

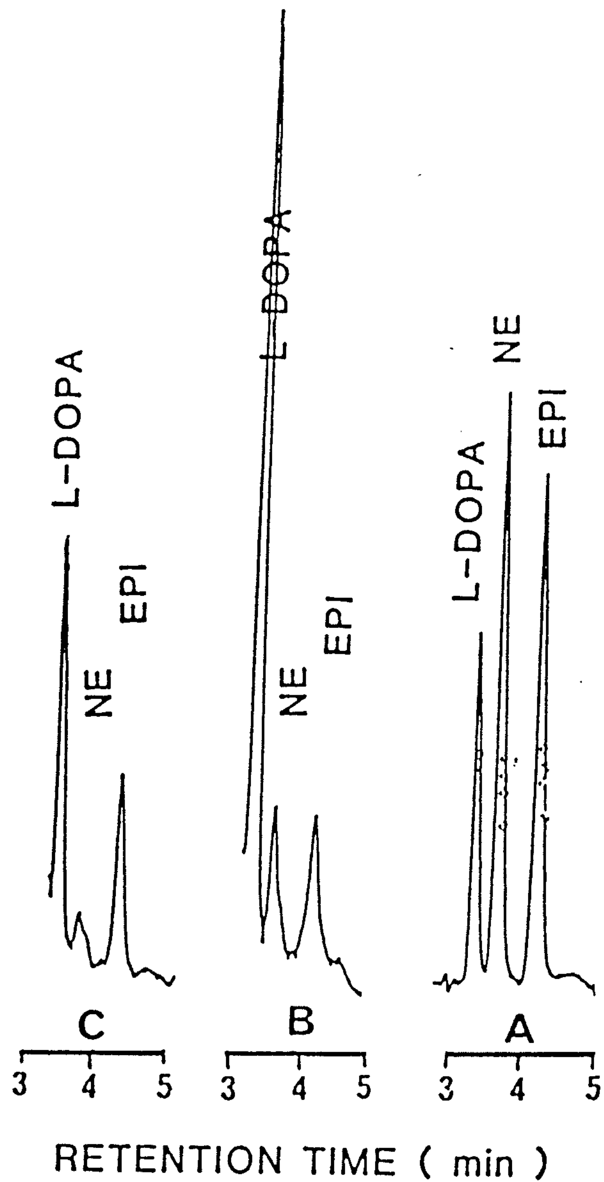

Fig. 29. HPLC chromatograms of L-Dopa in extracts of differentiated and undifferentiated NG108-15 cells. Cells were cultured in medium D containing 10% FCS (undifferentiated) or medium E supplemented with 5 % FCS and 1mM dBcAMP (differentiated). At day 5, cells were extracted with perchloric acid as described in " Materials and Methods" for analysis. Standards of L-Dopa, NE, and EPI were 2 ng of each. (A), standards; (B), differentiated cells; and (C), undifferentiated cells.

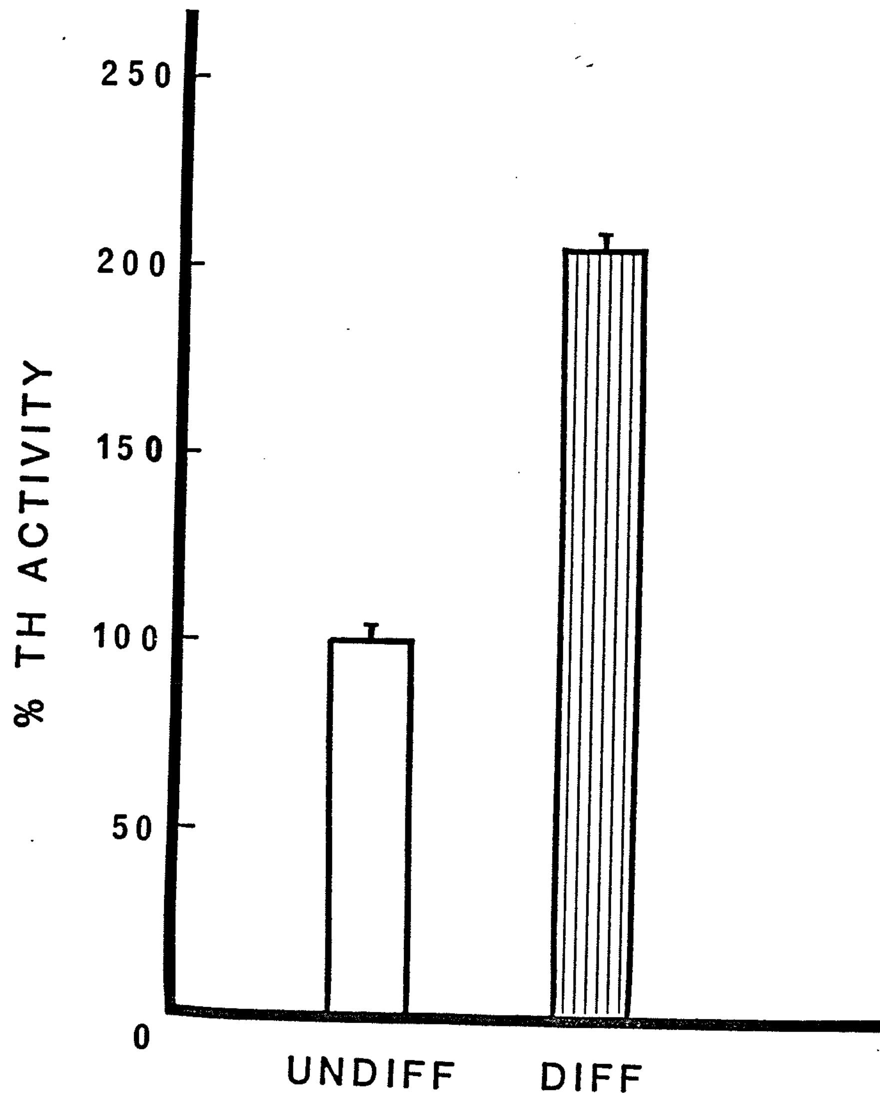

Fig.30. Comparison of tyrosine hydroxylase activity in differentiated and undifferentiated NG108-15 cells. Cells were cultured in either medium E (differentiated) or medium D (undifferentiated) . At day 5, TH activity was determined by measuring the formation of [^{3}H]-OH derived from [^{3}H]-tyrosine as described in "Materials and Methods". TH activity was calculated as fmole /min/ mg protein and expressed in percentage of activity in differentiated as 100 percent.

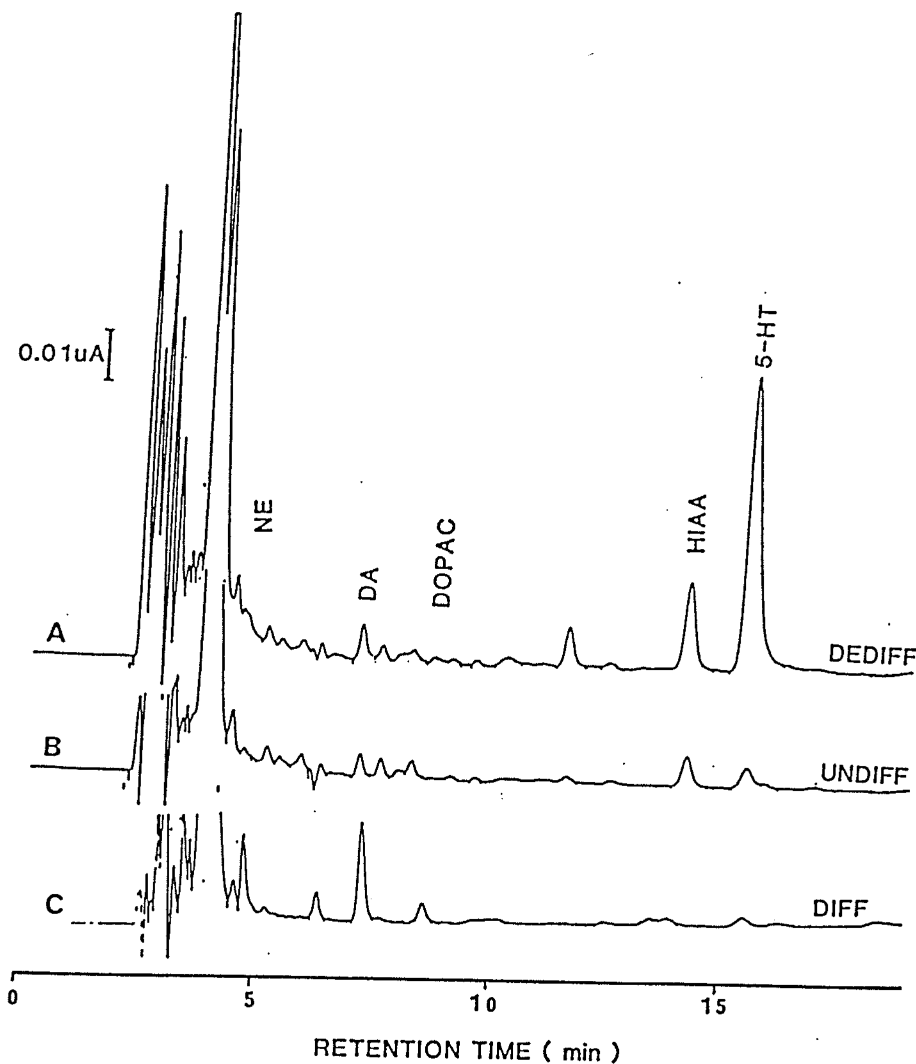

Fig.31. HPLC chromatograms of indolamine in extracts of de-differentiated, undifferentiated and differentiated NG108-15 cells. Cells were cultured in medium E containing 5% FCS plus 1mM dBcAMP (differentiated) or medium D containing 10 % FCS (undifferentiated). To induce de-differentiation of differentiated cells, medium E of the differentiated cells at day 4 was replaced with medium D containing 10% FCS without dBcAMP, and incubated for another 24 hrs. At day 5, Differentiated, undifferentiated and de-differentiated cells were extracted with perchloric acid as described in " Materials and Methods " for indolamine analysis. (A), de-differentiated cells; (B), undifferentiated cells; and (C), differentiated cells.

when the previously differentiated cells became de-differentiated. Fig.31 presents a typical HPLC chromatogram of indolamines in extracts of de-differentiated cells as compared with those in differentiated and undifferentiated cells. The level of 5-HT and its major metabolite, HIAA, increased markedly following morphological changes after removal of dBcAMP from the culture medium. Quantitative measurements of 5-HT and HIAA revealed a 2-fold and 2.7-fold increase of 5-HT and HIAA, respectively, for de-differentiated cells, as compared to the control differentiated cells under normal culture conditions (Fig.31).

To examine the possibility that the changes of indolamine content in NG108-15 cells upon differentiation was not the direct effect of dBcAMP, analysis therefore were carried out on undifferentiated cells grown in suspension culture in medium E containing 1mM dBcAMP. Fig.32 shows chromatograms of indolamines in differentiated cells in medium E, undifferentiated cells in medium D, and undifferentiated cells in suspension culture of medium E. Levels of dopamine and its major metabolite, dopac, in the suspension cells were lower than those of the undifferentiated, but higher than those of the differentiated cells. Fig. 32 shows also that levels of 5-HT in the suspension cells were detectable, though lower than that of undifferentiated cells. These results indicate that changes of contents of neurotransmitters and their major metabolites in NG108-15 cells under differentiated

and undifferentiated conditions are not due to the direct effect of dBcAMP in the culture medium. Fig. 33 shows the chromatograms of alumina extraction of media obtained from differentiated and undifferentiated NG108-15 cells at day 5 of culture. The level of DA was slightly higher in the medium of the differentiated cells. A significant difference in media obtained from differentiated and undifferentiated cells was the presence of Dopac, the major metabolite of dopamine, in the medium of the differentiated cells. This metabolite was not detectable in the medium from undifferentiated cells (Fig.33). However, no significant change in NE level was observed, being 0.055 ng and 0.058 ng/10^6cells/10 ml of medium for undifferentiated and differentiated cells, respectively (Data not shown). As controls, blank medium D and medium E were also analysed under identical experimental conditions, and no detectable level of DA and Dopac was observed, indicating that both dopamine and Dopac in harvested culture media are synthesized and secreted by NG108-15 cells in culture.

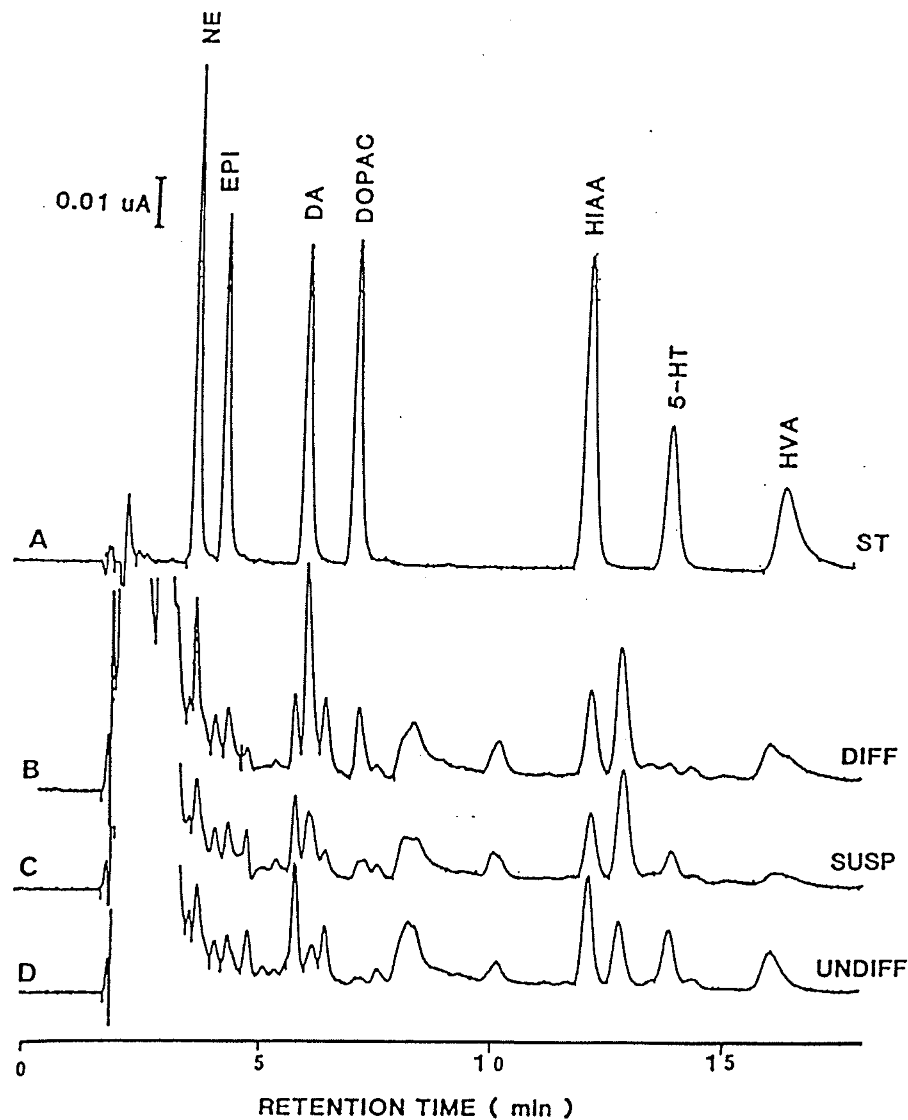

Fig. 32. HPLC chromatograms of indolamine in extracts of cells in monolayer cultures of differentiated and undifferentiated conditions, and suspension culture of undifferentiated conditions. NG108-15 cells were cultured in DMEM containing 5% FCS plus 1mM dBcAMP (differentiated), 10% FCS(undifferentiated), or 5% FCS plus 1mM dBcAMP with constant shaking of suspension condition. At day 5, cells were extracted with perchloric acid for analysis. Indolamine standards were 2 ng of each. (A), standards; (B), differentiated cells; (C), suspension cells; and (D), undifferentiated cells.

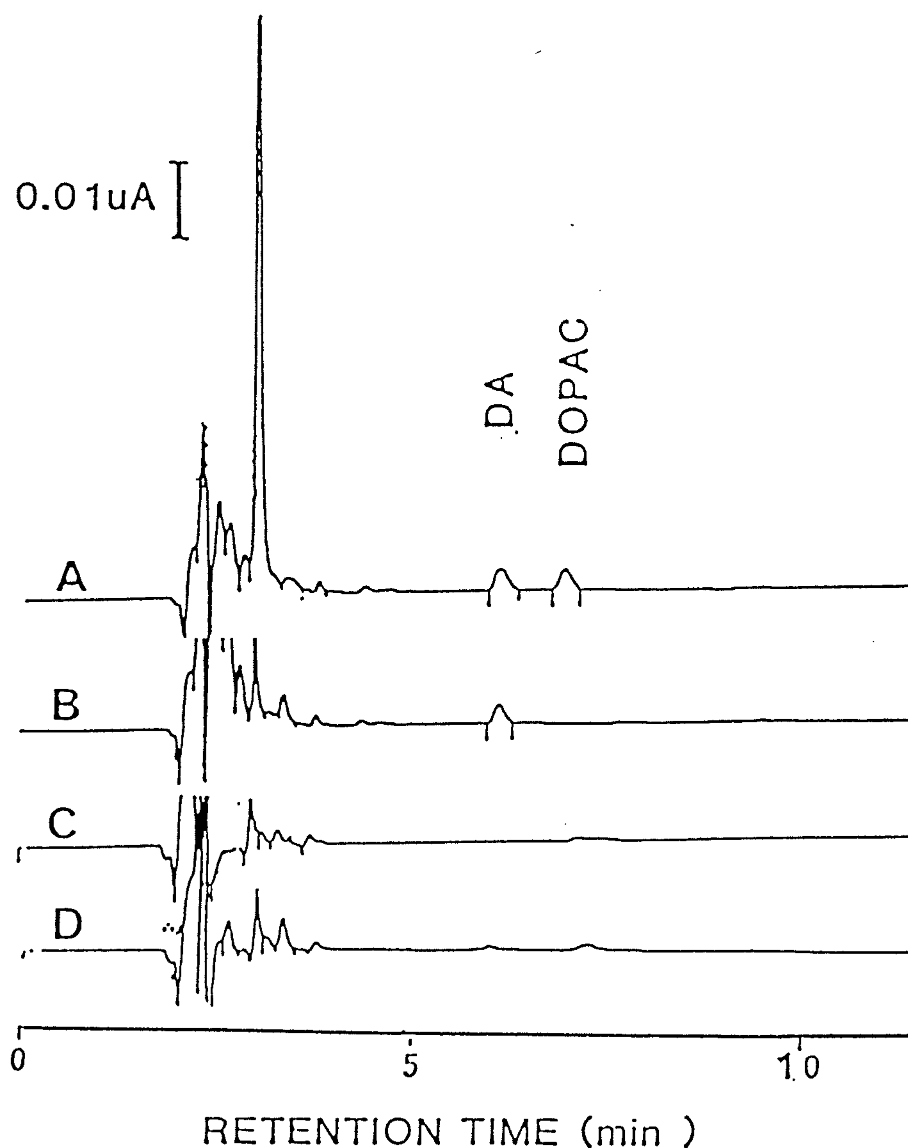

Fig. 33. HPLC chromatograms of alumina extraction of media obtained from differentiated and undifferentiated NG108-15 cells. Cells were cultured in medium D containing 10 % FCS (undifferentiated) or medium E containing 5% FCS plus 1mM dBcAMP (differentiated). At day 5, 10 ml each of the conditioned media from differentiated and undifferentiated cells and control blank medium E and medium D were extracted with acid washed alumina as described in " Materials and Methods ". Aliquots of 20 ul each of the extracts were injected into HPLC for analysis of indolamines. (A), medium from differentiated cells; (B), medium from undifferentiated cells; (C), blank medium E; and (D), blank medium D

Part 2

Serum Factor Causing Neurite Retraction of Differentiated NG108-15 Cells

Neuroblastoma x glioma NG108-15 cells cultured in serum-free medium, containing 1mM dBcAMP, showed extensive neurite outgrowth, mainly from singlets. A majority of the morphologically differentiated cells bore more than 2 long neurites upon treatment of dBcAMP for 48-72 hrs (Fig. 34 A). FCS at a concentration of 10% caused neurite retraction of the morphologically differentiated cells (Fig. 34). The process of retraction was rapid as shortening of neurites was obvious within 1 min after addition of FCS (Fig.34 B) and completed in 5-10 min (Fig.34 D and E). No regeneration of neurites was observed after an additional 18 hrs of culture under these conditions (Fig. 34 F).

Fig.35 indicates the time-dependent nature of neurite retraction. The neurite length of differentiated cells decreased more than 50% in 2 minutes upon addition of 10% FCS and reached its maximum shortening of over 90% in 8 min. The time required for half maximal activity (t 1/2) was estimated to be 1.80 min.

The effect of serum on neurite retraction of differentiated cells was dose-dependent as shown in Fig. 36. FCS at 1% was the lowest dose effective in reducing neurite length in 2 min. No neurite shortening was observed by addition of less than 1% of FCS; whereas, approximatly 5-10% of FCS was needed to reduce the length of neurites of differentiated cells by more than 50% in 2

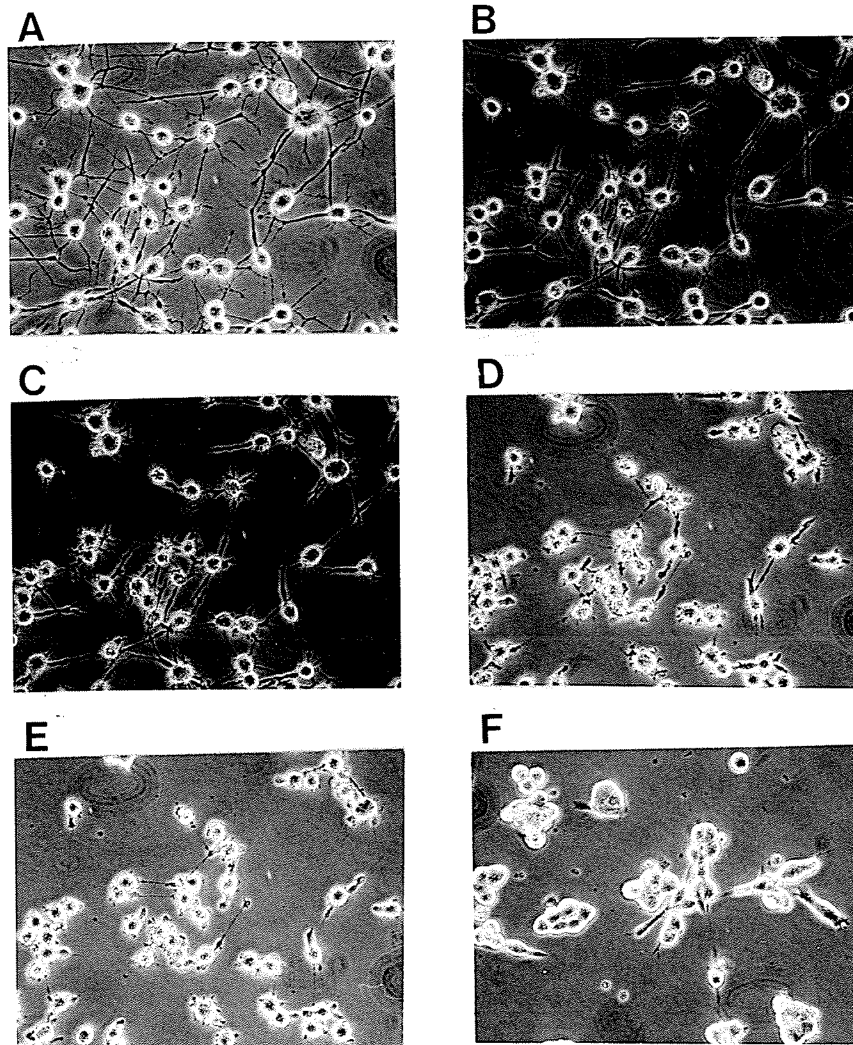

Fig. 34. Neurite retraction of morphologically differentiated NG108-15 Cells. Cells were cultured for 48 hrs in serum-free defined medium, supplemented with 1mM dBcAMP to induce differentiation. FCS was added to the culture to a final concentration of 10%, and photos were taken at various time intervals. A, 0 min; B, 1 min; C, 2 min; D, 4 min; E, 8 min; F, 18 hours (magnification:200 X).

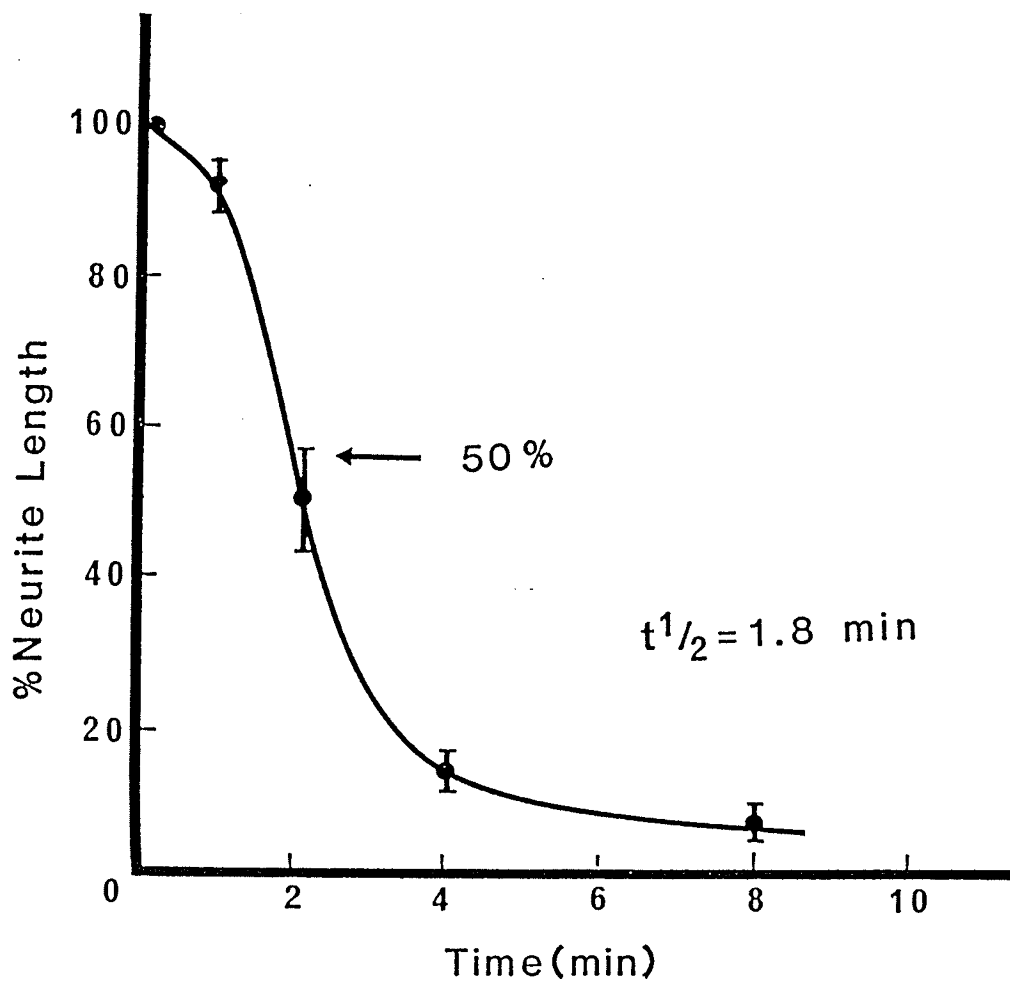

Fig.35 Time course of neurite retraction of differentiated NG108-15 Cells. Neurite retraction in percent is expressed as a function of time upon addition of FCS to a final concentration of 10%. The time required to reach one-half maximal retraction (t 1/2) was estimated to be 1.8 min.

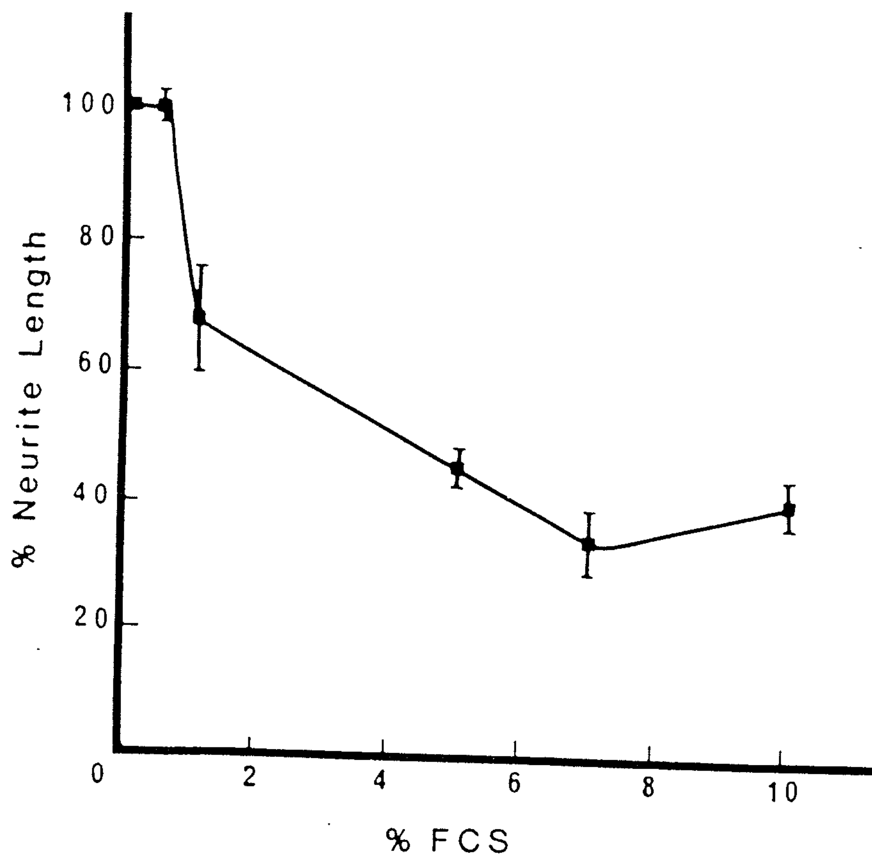

Fig.36. Dose response curve of FCS on neurite retraction. Neurite shortening of morphologically differentiated NG108-15 cells was measured after two min upon addition of various doses of FCS as indicated. Data represent means ± SEM of triplicates.

min (Fig. 36).

No effect on neurite retraction of differentiated NG108-15 cells (Table 3) was observed upon addition of homogenates of rat lung, kidney, heart, skeletal muscle and brain at a protein concentration of 1mg / ml (equivalent to the protein content of 5% FCS). However, spleen and liver homogenates caused slight but significant neurite retraction of 21.9% and 14.3%, respectively (Table 3). Serum samples of various species studied, including rat, sheep, horse and human, showed various degrees of neurite retraction activity. Horse serum appears to possess the highest activity of inducing 73.8% retraction in 2 min. Table 3 also indicates that CSF samples from human and cat studied were void of neurite shortening activity.

It has been reported that NG108-15 cells secrete plasminogen activator (122), which might activate serum plasmin, leading to proteolysis of neurite attachment proteins (123). To examine the possibility of involvment of plasmin and plasminogen activator on the mechanism of neurite retraction, purified plasmin or plasminogen activator at concentration of 10 ug / ml was added to culture medium of morphologically differentiated NG108-15 cells. No neurite shortening response was observed upon addition of either plasmin or plasminogen activator into the culture medium of differentiated cells (data not shown).

The effect of heating and acidification on the serum of the neurite retraction activity in serum is summarized

in Table 4. Acidification of FCS to pH 2.5, followed by reneutralization, reduced significantly (41.7%) the neurite retraction activity. Heating to 56°, 75° and 100°C for 15 min did not affect the retraction activity in serum. The supernatant of boiled FCS was biologically active at 40 ug protein / ml, as compared to 120 ug / ml for untreated control serum samples, an approximate three-fold increase in activity on the basis of protein.

Treatment of the boiled FCS supernatant, after centrifugation, with 2% trypsin for 16 hrs at 37° C markedly reduced (19.3 %) the neurite retraction activity (Table 4). This observation indicates that the serum neurite retraction factor is of protein in nature. Under identical assay conditions, purified preparation of Nerve Growth Factor (NGF) and Platlet Derived Growth Factor (PDGF) did not show any neurite shortening activity on differentiated NG108-15 cells (data not shown).

Upon ultrafiltration by using centricon 10X and immersible CX30 filters, FCS was separated into fractions of molecules of molecular weights less than 10 Kda, 10-30 Kda, and greater than 30 Kda. Neurite retraction activity was found only in the fraction of molecules of MW > 30 Kda, and no activity was detected in fractions of molecules of MW < 10 Kda or 10 - 30 Kda. Fig.37 indicates that the fraction of MW > 30 Kda mimics the effect of FCS in stimulating cell growth. The serum fraction of MW < 30 Kda was not able to stimulate cell growth although the cells survived in culture (data not shown).

The molecular weight of the serum neurite retraction activity was estimated by gel permeation on LKB Ultrogel AcA-44 column, equilibrated in PBS. Neurite retraction activity was eluted in the fractions with an apparent molecular weight of 70 Kda as shown in Fig.38. This fraction was effective at doses of 2-3 ug protein / ml to cause a 50% retraction in neurite length, a 60-fold purification over untreated control serum.

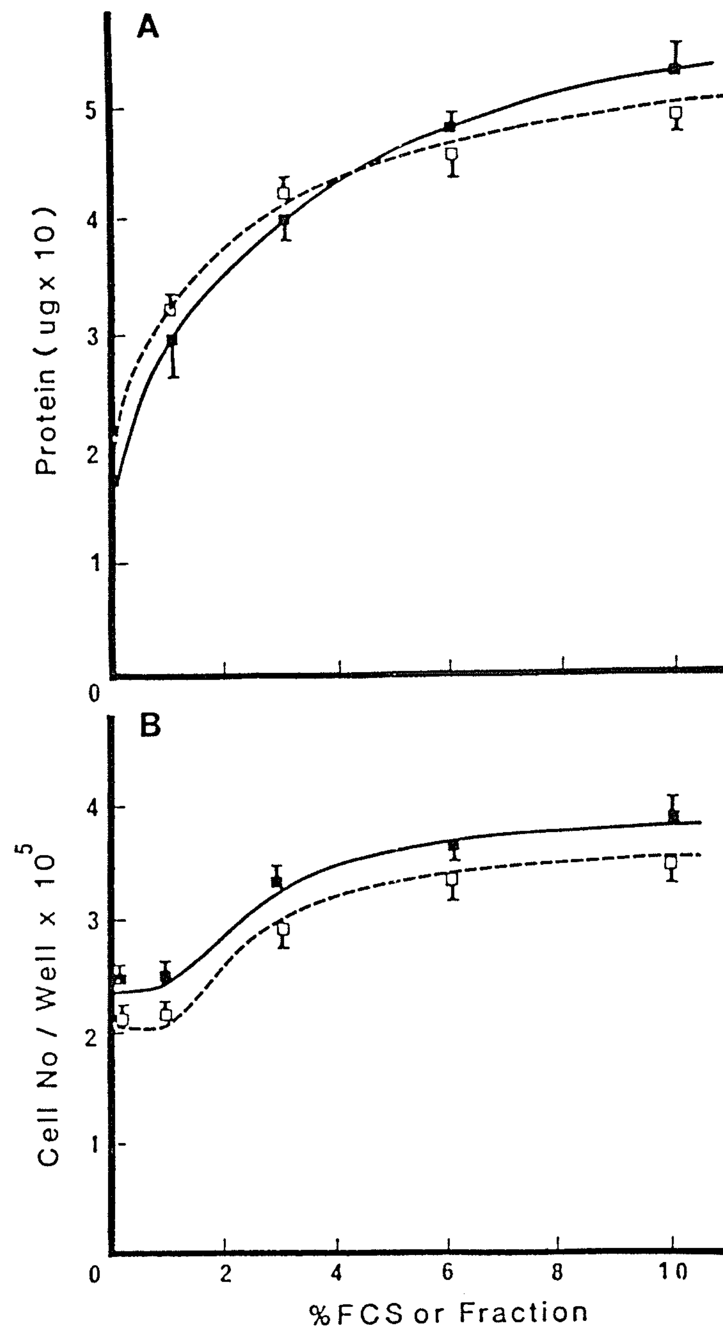

Fig. 37. Growth promoting activity of whole and fractionated FCS. Differentiated NG108-15 cells in serum-free medium were cultured for 3 days after the addition of aliquots of whole FCS (■) or the FCS fraction of MW > 30 Kda (□) to final concentrations of 1 to 10% as indicated. Cell growth was estimated either by (A) protein content or (B) cell number of the culture. Data represent means + SEM in triplicates.

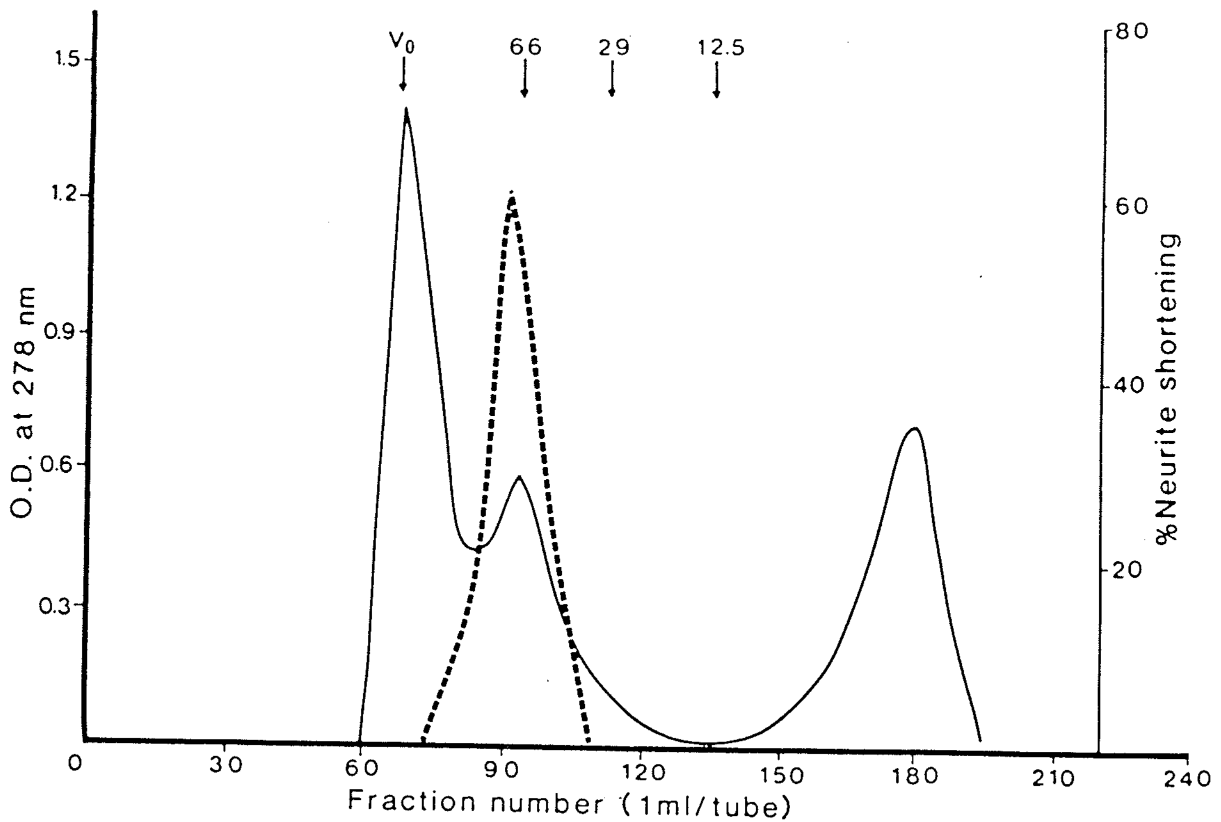

Fig.38. Estimation of molecular weight by gel filtration on Ultrogel AcA-44. The centrifuged supernatant of a boiled FCS sample was applied onto a column of LKB Ultrogel ACA-44 (1.6x110cm) in PBS, pH 7.4, and eluted at 16ml / hr. Fractions of 1 ml / tube were collected and aliquots of 50-100 ul of each fraction were directly assayed for neurite retraction activity. Maximal activity was eluted at tube 90, corresponding to an apparent molecular weight of 70 Kda. Standard protein markers : BSA (66 Kda), carbonic anhydrase (29 Kda) and cytochorome C (12.5 Kda).

Materials	Species/Organs	% Retraction of neurites
Control	PBS	0
Serum	FCS	69.0 ± 4.7
	Rat	53.5 ± 15.3
	Human	65.0 ± 10.9
	Horse	73.8 ± 10.0
	Sheep	60.2 ± 8.2
Tissue	lung	0
	kidney	0
	heart	0
	skeletal muscle	0
	brain	0
	spleen	21.9 ± 4.0
	liver	14.3 ± 3.3
Cerebral Spinal Fluid (CSF)	human	0
	cat	0

Table 3. Neurite-retraction activity in sera, organs and cerebral spinal fluids. Samples of sera and CSF were assayed at a final concentration of 10%, and tissue homogenates of various organs of the rat were tested at a protein concentration of 1mg / ml. Neurite retraction of differentiated NG108-15 cells was measured after 2 min upon addition of testing materials into culture.

Materials	% Retraction of Neurites
PBS	0
Boiled Serum	71.7 ± 3.1
Acidified Serum	41.7 ± 6.4
Boiled serum + PBS	71.8 ± 3.1
Boiled serum + trypsin	19.3 ± 3.5
PBS + trypsin	17.5 ± 5.3

Table 4. Effects of acidification, boiling and trypsin digestion on the neurite retraction activity in serum. Serum samples (FCS) were acidified, boiled or trypsin-digested as described in " Materials and Methods ", and assayed at a final concentration of 10 % . Data represent means ± SEM of triplicates.

DISCUSSION

DISCUSSION

The ability of malignant cells to initiate the normal program of differentiation is of interest in both model system to study development and for insights into the reversible nature of neoplasia (21). It has been demonstrated that many differentiation functions, which are characteristics of mature neurons, are expressed when neuroblastoma cells are induced to differentiate (9) using neurite inducing agents.

In our studies, attempts have been made to induce neuroblastoma cells to become mature and differentiated into ganglion like cells under defined culture conditions (9). Correlation of morphological and biochemical properties upon differentiation of NG108-15 cells has been determined by analyses of several key factors, namely, muscarinic acetylcholine receptors, beta-adrenergic receptors, catecholamines and serotonin and their major metabolites, which are believed to be important in neuronal functions.

1 - Cell Culture

Several well characterized inducers of morphological differentiation of neuroblastoma cells, including dibutyryl cyclic AMP (dBcAMP) (42), PGE 1 (43), theophyline (50) and serum deprivation (51) have been used in this study to induce neurite outgrowth. Comparison of the morphologically differentiated NG108-18 cells, induced by each of these agents (Figs. 1 and 2) indicates that all of these agents

are able to stimulate neurite outgrowth of NG108-15 cells to a variable extent. The number, diameter, density and ramification of neurites emanating from cell body vary markedly depending on the agent used. Serum deprivation, PGE 1 and theophyline (Fig. 2A,B,C) cause neurite outgrowth with a significantly lower density, comparing to that by dBcAMP treatment. However, the presence of dBcAMP in culture medium for cells grown in suspension conditions is not sufficient to stimulate neurite outgrowth (Fig. 2D). This indicates that cell attachment to substratum, as reported by Prasad (43), is required for differentiation. NG108-15 cells grown in DMEM containing 10 % FCS (medium D) are morphologically flat to slightly round with few cells bearing short processes less than the diameter of the cell body (Fig.1A). Since a neurite is generally defined as a process, the length of which equals to or exceeds the diameter of the cell body (47), these cells are therefore defined as morphologically undifferentiated NG108-15 cells. A morphological comparison of differentiated NG108-15 cells induced by various agents (Figs. 1 and 2) reveals also that among the neurite outgrowth inducing agents studied, dBcAMP is the most potent agent of choice to induce differentiation of NG108-15 cells, in terms of length and density of neurites.

2 - Muscarinic Acetylcholine Receptors

To understand the cellular process of differentiation of neuroblastoma cells, the potential ability to dissect

and elucidate the molecular events involved in the various aspects of differentiation is essential (47). One of the molecular events, which occurs during neuronal differentiation, could be the cell surface components, one of which is the neurotransmitter receptor. The present study has elucidated the relationship between the Ach receptor and the degree of neurite extension on intact NG108-15 cells upon induced differentiation. Our data have shown that NG108-15 cells possess approximately 130 % more muscarinic Ach receptors sites upon differentiation (Figs. 3,5 and 8). This increase is a time-dependent process and appears to be parallel to the process of neurite extension (Figs.5 and 6). The initial period for acquisition of additional receptor sites during differentiation appears to be within the first day of culture upon induction by dBcAMP (Fig.5). These data are in good agreement with those reported by Dudai et al. (124) that muscarinic binding sites in rat cerebral cells increases markedly after 5 days of culture when differentiation of cells begins. This increase is a time-dependent course, parallel to synaptogenesis. Similarly, Simantov et al. (125) have shown that the number of Ach receptors increases substantially in mouse neuroblastoma NIE-15 cells after dBcAMP treatment. Our present findings, together with those reported by Ben-Barak et al. (126) that the level of muscarinic binding sites in vertebrate brain coincide with synaptogenesis, suggest that muscarinic receptor sites are regulated during the process of differetiation in both neuroblastoma and neuronal cells.

However, the number of AchR on undifferentiated NG108-15 cells from log to stationary phase in contrast to differentiated cells remains constant (Fig. 5). This observation is in good agreement with the studies on NIE-115 cells by Fakahany et al. (127).

The Kd of 5.6 nM for differentiated cells is slightly higher than that of 4.4 nM for undifferentiated cells (Fig. 8), indicating a lower binding affinity of [^{3}H]-QNB for the Ach receptors on differentiated NG108-15 cells. Our calculated Kd values for undifferentiated and differentiated NG108-15 cells are 9 and 11 times, respectively, higher than the Kd value of 0.5 nM reported by Dudai et al. (124) for homogenates of cultured brain cells. This difference might be due to binding characteristics of muscarinic Ach receptors between brain and neuroblastoma cells, or the presence of binding sites of low affinity and high capacity in intact cells as used in the present studies.

The Hill coefficient (nH) for Ach receptor sites has been estimated to be 0.91 and 1.00 for undifferentiated and differentiated NG108-15 cells, respectively (Fig.9). These values are in complete agreement with nH value of 0.96 for Ach receptors on mouse neuroblastoma cells (106), indicating no negative co-operativity of receptor sites on both differentiated and undifferentiated cells. Computer analyses of these data indicate that there is only one type of binding sites for [^{3}H]- QNB on both differentiated and undifferentiated NG108-15 cells. The Ic50 values of 55 uM

and 259 uM for differentiated and undifferentiated cells, respectively, indicate that the potency of atropine sulfate for muscarinic Ach receptors on differentiated cells is 5 times higher than that on undifferentiated NG108-15 cells (Fig. 10).

Our data indicate that neuroblastoma x glioma NG108-15 cells upon induced differentiation by dBcAMP express neurite extension within 24 hrs of incubation. The regulation of muscarinic Ach receptors is initially associated with the process of neurite extension during neuronal differentiation (Figs.5 and 6). However, the role(s) of the increased binding sites of low affinity for [^{3}H]-QNB and with high affinity for atropine sulfate remains uncertain. The fact that changes in muscarinic Ach receptor sites upon differentiation on intact cells of the neuroblastoma x glioma neuronal model, which offers several advantages over disrupted membrane preparations, similar to those of normal neuronal cells in culture suggests that the process of in vitro neuronal maturation might be resemble to that of in vivo neuronal development.

3 - Beta-adrenergic Receptor

During the last decade, experimental data on biochemical and functional properties of B-adrenergic receptor-adenylate cyclase system have accumulated rapidly. In spite of the success of biochemical dissections, the analysis of the B-adrenergic receptor system on intact

cells has received much less attention (128). The application of ligand binding techniques to study receptors on intact cells involves some methodological difficulties, such as high non-specific binding (128). However, in our present study, we have demonstrated by using [^{3}H]-DHA that intact neuroblastoma x glioma hybrid NG108-15 cells possess a substantial amount (0.23 nM) of specific B-adrenergic receptor binding sites (Fig.14 and 15) and that the non-specific binding of [^{3}H]-DHA is only 17% (Fig. 11 and 12). NG108-15 cells have low non-specific binding, in contrast to reports on several cell lines studied by other investigators (106, 128). A comparison of [^{3}H]-DHA binding to receptors on differentiated (dBcAMP treated) and undifferentiated (untreated) cells (Fig. 13) reveals that the number of DHA binding sites on differentiated NG108-15 cells, induced by 1mM dBcAMP are not significantly different from that of undifferentiated cells. The specific [^{3}H]-DHA binding of beta-adrenergic receptor sites increases only 6% upon differentiation. In spite of an increase of 130% in muscarinic Ach receptor binding on NG108-15 cells (Fig. 3, 5, and 8) upon differentiation, the beta-adrenegic receptors remain relatively unchanged. The binding of [^{3}H]-DHA to B-adrenergic receptor on NG108-15 cells is a saturable process with a Kd of 2.5 nM (Fig. 14), which is very similar to the Kd of 1-2 nM for human neuroblastoma cells (129), but slightly lower than that of 6.7 nM for rat cerebral cortex (130). However, the Kd value of 2.5 nM

for intact NG108-15 cells is markedly higher than those of 60.23 pM, 43.8 pM and 46.7 pM reported for brain cells obtained from chick, rat and mouse, respectively, using cell membrane preparations (131). These discrepancies might be due to differences of β-adrenergic receptors between brain cells and the hybrid NG108-15 cells, or the presence of binding sites of low affinity and high capacity in intact cells as used in the present study versus plasma membranes in other studies.

Analyses of data on saturation experiments (Fig.14) and Scatchard plot (Fig.15) indicate the presence of a single type of binding site for [^{3}H]-DHA on intact NG108-15 cells. In addition, the Hill coefficient (nH) for β-adrenergic receptor sites is estimated to be 1.0 (Fig.16), indicating the absence of co-operativity among [^{3}H]-DHA binding sites. Lack of co-operativity between [^{3}H]-DHA binding sites has been reported for human neuroblastoma cells (129) and homogenates of chick, rat, and mouse cultured brain cells (131). Futhermore, the binding of [^{3}H]-DHA to β-adrenergic receptors on intact NG108-15 cells is a rapid and reversible process with t 1/2 of 1.0 min for association (Fig. 18) and t 1/2 of 3.5 min for dissociation (Fig.17). The Ic50 value for non-radiolabelled ligand to inhibit 50% of [^{3}H]-DHA binding on intact NG108-15 cells has been estimated to be 10 uM (Fig. 19).

To determine the subtype of beta-adrenergic receptors on NG108-15 cells, the rank order potency of agonists of (-) ISO, (+) ISO, EPI and NE to bind beta-adrenergic receptors on

plasma membranes (Fig.20) and intact cells (Fig.21) has been estimated by competition experiments as recommended by Lands et al. (112), to be (-) ISO > (+) ISO > EPI > NE, indicating a type 2 beta-adrenergic receptors. This rank order potency of agonists remains unchanged upon differentiation induced by dBcAMP (Fig.22 and 23). However, the beta 2-adrenergic receptor of both undifferentiated and differentiated cells (Fig.20 and 23) shows stereoselectivity of greater potency (5x) in binding for L-ISO, as compared to D-ISO. However, there are differences between the Ic50 values of various agonists to inhibit 50% of [^{3}H]-NE binding to plasma membranes versus intact cells as well as differentiated versus undifferentiated cells (Table 1). Our data indicate that : 1) almost all agonists possess greater potencies to bind receptors on plasma membranes from either differentiated or undifferentiated cells than on intact cells, and 2) most of the agonists have greater potencies to bind receptors on cells upon differentiation.

Our observations of lower affinities of agonists for receptors on intact cells are in good agreement with those reported by Insel et al.(132). These investigators have shown that agonists initially bound to beta-adrenergic receptors on intact S49 cells with a transient (1.0 min) high affinity, which was reduced upon further incubation. Although the reason for the low affinities of agonists to bind receptors on intact cells remains unknown,the presence of endogenous guanyl nucleotides and, perhaps, the more

efficient coupling between receptors and adenylate cyclase of the intact cells have been suggested (132). Intact cells seem to be able to terminate their response to β-adrenergic agonists by two apparently different mechanisms: i) receptor uncoupling (eg. a rapidly occurring decrease in affinity for agonists) and ii) down regulation, a much later occurring decrease in receptor number. Both decreases appear to be physiological relevant means of desensitizating intact cells to catecholamines (133).

The higher affinity of agonists to bind beta-adrenergic receptors on differentiated cells may be resulted from either synthesis of new receptors with greater affinities or an increase in affinity of the presently available receptors upon dBcAMP treatment. The former speculation is unlikely because the number of beta-adrenergic receptors remain unchanged upon morphological differentiation induced by dBcAMP (Fig.13) and this leaves the latter as the logical possibility.

Since the neuroblastoma x glioma hybrid NG108-15 cells possess substantial amount of β-adrenergic receptors with characteristics similar to those on neuronal cells, this cell line may be a very useful neuronal model for further studies of β-adrenergic receptor. The use of intact cells of a neuronal model may provide information, which is considerably more closely related to receptors on intact neuronal cells than disrupted plasma membrane preparations.

4 - Protein Content

Differences in the poly(A)-containing mRNA in differentiated and undifferentiated mouse cholinergic neuroblastoma cells have been reported (134). It has also been reported (135) that the increment in cellular differentiation of NS20Y cells results in both the disappearance of old and the appearance of new mRNAs in polyribosomes. However, these studies have not revealed the changes of poly(A) mRNA in NS20Y cells at early stages of differentiation.

Prasad et al. (9) have shown that the content of protein per cell in neuroblastoma increased by about two to three folds upon differentiation, induced by neurite enhancing agents, including dBcAMP. Such increase is consistent with observations during the differentiation of mammalian nervous tissue (9). Zisapel et al. (61) have revealed that major changes in exterior surface membrane proteins took place during maturation and differentiation of mouse neuroblastoma cells. When extracts of differentiated and undifferentiated NG108-15 cells are analysed by gel electrophoresis, a protein band of MW of approximately 32 Kda (Fig. 24, lane 3) is strongly stained in dBcAMP-treated cells, and only weakly in DMSO- and DX-treated cells, and undifferentiated cells. Since this protein is present in all treated and untreated cells, it is unlikely

to be a specific protein for differentiation. The strong expression of this protein may be due to a direct effect of dBcAMP. Another protein with MW of approximately 85 Kda (Fig.24, lanes 4 and 5), which is detectable only in DMSO- and DX-treated cells, is also not a differentiation-related protein, because it is not detectable in dBcAMP-treated cells. In undifferentiated cells (Fig.24, lane 2), one protein with MW of 35 Kda , being very weakly expressed, and another of MW of 40 Kda, very strongly stained, are also present at various concentrations in dBcAMP-, DMSO-, and DX-treated cells. Again, these proteins do not appear to be specific initiation of morphological differentiation or neurite formation.

Upon 2-dimensional gel electrophoresis, extracts of $[^{35}S]$-met treated NG108-15 cells show more than 400 protein spots (Fig. 26). However, the protein profile for both differentiated and undifferentiated cells are similar, except differences in concentrations of 12 proteins upon dBcAMP treatment. Differentiation-specific proteins have not been observed in NG108-15 cells upon dBcAMP-treatment. Since $[^{35}S]$-met is incorporated into newly synthesized proteins upon cellular differentiation, it is possible that some of these proteins act as endogenous differentiation factor (DF) for NG108-15 cells. Investigation of factor(s) responsible for early stage of differentiation is apparently important because it is now believed that two factors known as proliferation factor (PF) and differentiation factor (DF) effect each other to

express either of these phenomenon in cancer cells (136). The PF-mediated activity appears to be subjected to regulation of specific exogenous signals, including growth factor (GF) and differentiation factor (DF). The DF at sufficiently high concentration is able to interupt the oncogene-maintained proliferation cycle (137). Endogenous DF is believed to be regulated by exogenous DF, such as dBcAMP or retinoic acid (136). Even though many exogenous differentiation factors are well described (49, 50, 51, 52, 53) no information on endogenous DF is available. In our present study, comparison of autoradiograms of newly synthesized proteins of differentiated and undifferentiated NG108-15 cells (Fig.26) has not revealed such a protein factor. This suggests the possibility that changes in concentration of some of these proteins may act as a signal for differentiation.

5 - Modification of Indolamine Contents

Our data on HPLC analyses of indolamines and their major metabolites indicate that differentiated NG108-15 cells have 40% and 129% more NE and DA, respectively, than those of undifferentiated cells (Table 2). These results are in good agreement with those reported by Richelson (138) and Sandquest et al. (139) that catecholamine synthesis and tyrosine hydroxylase activity increased in differentiated murine neuroblastoma and NIE-115 cells upon treatment with 5-fluorodeoxyuridine and DX respectively. In addition, dopac, the major metabolite of DA, also

increases upon differentiation of NG108-15 cells. There are three possible explanations for the increased levels of DA and dopac:i) increase in neurite extension may facilitate the uptake of DA and dopac from the medium, ii) dBcAMP directly stimulates the synthesis of DA and dopac, and iii) morphological changes,including neurite extension,facilitate the synthesis of DA and dopac. However, our finding that no DA or dopac has been detected in blank medium D for undifferentiated cells or medium E for differentiated cells (Fig.33) exclude the first possibility. Further,the second possibility doesn't seem to be likely because cells grown in suspension culture in the presence of dBcAMP (Fig.32 C) do not contain higher levels of DA or dopac (Fig.32). Our observations suggest the third possibility, that increases of DA and dopac may be due to newly synthesized DA,as synaptosomes have been reported (140) to retain the capacity to synthesize, store,release and metabolize DA. Increases in levels of L-dopa,the DA precursor, (Fig.29)and TH(Fig.30),a rate limiting enzyme in catecholamine biosynthesis, add further support to the conclusion that NG108-15 cells synthesize and release more DA upon differentiation. These observations are in good agreement with those reported by Nicholson et al.(141)that an increase in TH activity occurs after sympathetic neurons in culture ceased dividing. Increase in TH activity appears to reflect the maturation process of the developing brain (138).

The presence of 5-HT in undifferentiated cells, and not in differentiated cells (Fig. 28 and Table 2), indicates a shift of synthesis of neurotransmitters during

differentiation from 5-HT to DA. Similarly, DA and dopac have also been detected in medium obtained from differentiated cells (Fig. 33). Furthermore, data obtained from de-differentiated NG108-15 cells have confirmed that synthesis of 5-TH is regulated during morphological changes (Fig. 31). The fact that no significant difference is observed in NE content in culture media obtained from differentiated and undifferentiated cells suggests that differentiated NG108-15 cells release greater amounts of DA and Dopac, but not NE. These findings indicate that, as neurites develop, the NG108-15 cell begins to acquire the ability to produce and release DA and Dopac, though not all of the catecholamines.

The stimulation of DA synthesis and inhibition of 5-HT synthesis could be interpreted as a result of the dBcAMP-induced differentiation. Studies on " undifferentiated " cells cultured in the presence of dBcAMP in suspension condition (Fig. 32) have revealed that the low level of 5-HT in differentiated cells is at least partially due to morphological changes upon differentiation. Discrepancies in contents of neurotransmitters and their major metabolites (Fig 32) between undifferentiated cells in monolayer culture and " undifferentiated " cells in suspension culture in the presence of dBcAMP could be explained by the fact that, in spite of the suspension condition, small batches of cells become attached and morphologically differentiated (Fig.2D).

It has been reported (135) that more than one neurotransmitter substance and synthesizing enzyme can coexist in the same neuron or nerve ending, such as Ach and catecholamine. The NG108-15 cell has been demonstrated to exhibit both cholinergic and serotonergic properties (142). Our present study has shown that the NG108-15 cell is also dopaminergic (Figs. 27, 28, Table 2) of relatively high levels of DA, and that either DA or 5-HT could be produced, depending on the culture conditions.

6 - Neurite Retraction Factor

It has been established that removal of serum from culture media results in differentiation and neurite outgrowth of neuroblastoma cells (143). Differentiated C 1300 neuroblastoma cells gradually (2-4 hrs) retract their processes prior to mitosis upon replacement of serum (51). The growth promoting capability of serum has been shown non-dializable and heat stable (51). A factor(s) promoting neurite retraction of differentiated neuronal cells has not been specifically identified and characterized.

Our preliminary studies indicate that the neurite retraction activity in serum has a MW of approximately 70 Kda (Fig. 38), and distinguishes itself from PDGF, EGF, FGF, NGF, NSILA, and TGF (144, 145, 146, 147, 148, 149, 150, 151). Furthermore, the fact that the neurite retraction activity is heat and acid stable (Table 4) also distinguishes it from ECGF, FGF, and NGF (148, 152, 153). However, purified NRF is required to clarify

whether or not NRF is directly responsible for the growth promoting capability in serum or is important in preventing neurite outgrowth and indirectly potentiating the effects of other unidentified serum growth factors.

It is noteworthy that NRF is not detectable in both human and cat CSF (Table 3), possibly because the blood-brain barrier restricts the passage of macromolecules from blood circulation into the CSF (154). Peripheral nerves also have a barrier at the endoneurium and the perineurium surrounding the nerve bundles (155). It is conceivable that NRF plays a role in the developing immature neuron before the functional development of the blood-brain barrier. Maturation of the barrier function in relation to parturition is highly variable between species. In sheep the maturation process of the barrier occurs during the entire fetal life; whereas in the rat it takes place mostly after birth (154). It seems likely that accesibility of NRF to neuronal cells during fetal life helps to guide the pathway of proliferation or differentiation of a developing neuron.

It has been demonstrated that NG108-15 cells secreted plasminogen activator (122) which might convert serum plasminogen to plasmin, leading to proteolysis of neurite attachment proteins (123). However, plasminogen is unstable at high temperature (156), and addition of purified plasmin and plasminogen activator to the culture medium of NG108-15 cells has no effect on neurite retraction (Table 4). Thus neurite retraction of morpho-

logically differentiated NG108-15 cells does not act through the plasmin and plasminogen activator system. Addition of high concentration of BSA (3 mg / ml) into culture medium of morphologically differentiated NG108-15 cells does not mimic the action of NRF, indicating that neurite retraction of these cells is not due to hypertonic shock of adding 10% FCS into culture medium. Further, replacement of culture medium with fresh medium prior to the addition of serum factor does not dampen neurite retraction, indicating that NRF does not work through a component in the conditioned medium. The biological role(s) and mechanism(s) of action of serum factor in the maturation and differentiation of neuronal cells remain to be elucidated.

CONCLUSION

The ability of malignant cells to initiate the normal program of differentiation is of wide interest, because the differentiation program is coupled with diminished capacity of cellular proliferation (21). It has been shown (21) that proliferation of neuroblastoma x glioma NG108-15 cells is coincident with the reprogramming for continued cell division and elimination of neurite expression and mature nucleolar feature. In contrast, an elevation of intracellular levels of cAMP in NG108-15 cells induces many differentiated functions characteristic of mature neurons (9). The results of the present study, together with data from other studies on differentiation of neuroblastoma

cells (8, 9, 43, 47, 49, 50, 52, 53, 55, 57, 61, 69, 71, 157) and NG108-15 cells in particular (9, 85, 91, 93, 94, 95, 96, 101, 103, 104, 122, 142) indicate that neuroblastoma cells are capable to initiate the normal program of differentiation, resembling normal neuronal cells during developmental process.

Findings of these studies provide further information on in vitro differentiation to further understand in vivo spontaneous regression of malignant neuroblastoma cells to benign ganglioneuromas. The serum neurite retraction factor is speculatd to be a natural factor to prevent neurite outgrowth of neuroblastoma cells during the early stage of neuronal development. It is hypothesized that accessibility of serum NRF at the early stage of neuronal development may account for neuronal potentiality to proliferate, which is reduced gradually because of the development of the blood-brain barrier at later stages and thus restricting the passage of macromolecules (154), including NRF. This may explain why the NRF is not detectable in CSF (Table 4). However, the exact biological role(s) of NRF remains to be elucidated.

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