THE ROLE OF FOLATE AND VITAMIN B12 IN NEUROTRANSMITTER METABOLISM AND DEGENERATIVE NEUROLOGICAL CHANGES ASSOCIATED WITH AGING

PROCEEDINGS OF A WORKSHOP

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FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB), provides scientific assessments of topics in the biomedical sciences. Comprehensive and quick response reports are based upon literature reviews and the scientific opinions of knowledgeable investigators engaged in work in specific areas of biology and medicine. Proceedings from workshops and symposia contain the data, references, and opinions provided by participants.

These proceedings were developed from a workshop held May 19-20, 1988 on opportunities for research on "The Role of Folate and Vitamin B12 in Neurotransmitter Metabolism and Degenerative Neurologic Changes Associated with Aging" for the National Institute on Aging (NIA) and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The workshop was chaired by Ralph Green, M.D., Chairman, Department of Laboratory Hematology, The Cleveland Clinic Foundation. LSRO wishes to acknowledge Dr. Green's contributions in the conduct of this meeting. This report on the workshop was prepared in accordance with provisions of Conference Grant No. 1-R13-AG07501-01.

The workshop proceedings are presented in two parts: 1) synopses of six invited papers on current understanding and gaps in knowledge, and 2) recommendations on research approaches and priorities derived from working group discussions by workshop attendees.

The invited papers and the working group recommendations were developed independently of NIA, NIDDK, and any other group, governmental or nongovernmental. The speakers, discussion leaders, rapporteurs, and LSRO accept responsibility for the accuracy of their respective portions of these proceedings; however, listing of these individuals does not imply that they specifically endorse each study conclusion. These proceedings were reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent Society of FASEB) under authority delegated by the Executive Committee of the Federation Board. Upon completion of these review procedures, the proceedings were approved and transmitted to NIA and NIDDK by the Executive Director, FASEB.

While this is a report of the Federation of American Societies for Experimental Biology, as the proceedings of a workshop, it reflects the expertise of the participants and does not necessarily reflect the opinion of each individual member of the FASEB constituent Societies.

December 5, 1988
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Kenneth D. Fisher, Ph.D
Director
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SUMMARY

Under the sponsorship of the National Institute on Aging and the National Institute of Diabetes and Digestive and Kidney Diseases, the Life Sciences Research Office of the Federation of American Societies for Experimental Biology held a workshop entitled "The Role of Folate and Vitamin B12 in Neurotransmitter Metabolism and Degenerative Neurological Changes Associated with Aging". The purpose of the May 1988 workshop was to bring together scientists from various disciplines to identify opportunities for research on an important topic interfacing neuroscience, nutrition, and aging.

Fifty-three scientists whose expertise included gerontology, neurology, nutrition, hematology, clinical medicine, epidemiology, cell biology, biochemistry, and physiology developed comprehensive recommendations integrating basic and clinical research on the role of folate and vitamin B12 in neurotransmitter metabolism and neurologic degeneration related to aging.

The workshop included six invited presentations on the current status of knowledge about involvement of folate and vitamin B12 in neurotransmitter metabolism and neurologic degeneration associated with aging. These presentations stimulated discussions in the working group sessions as the invited papers addressed degenerative neurological changes of aging, age-related changes in metabolism of neurotransmitters, experimental approaches for the study of neurological changes, folate and vitamin B12 status of the elderly in the United States, and evidence for a role of vitamin B12 and folate in normal brain function.

In his presentation on degenerative neurological changes of aging, Dr. Donald Price described chemical and structural changes in behavior/brain that occur in aged individuals (using nonhuman primates as a model) and in individuals with Alzheimer's disease. Dr. James Joseph discussed dopaminergic-cholinergic interactions in the striatum, an area of the brain that mediates various simple and complex motor behaviors and shows profound changes during senescence. Use of in vitro systems as a means of exerting greater control over the chemical and cellular complexity of the central nervous system was addressed by Dr. Jean de Vellis. Occurrence and manifestations of folate and vitamin B12 deficiencies in clinic populations in New York City were discussed by Dr. Michael Freedman. Drs. Ralph Green and Sheldon Rothenberg, respectively, identified lines of evidence derived from biochemical studies in animal models and in vitro systems and from clinical observations that support a role for vitamin B12 and folate in normal brain function.

Building upon the ideas presented in the invited papers, five working groups made recommendations about research needs on the role of vitamin B12 and folate in neurotransmitter metabolism and degenerative neurological changes associated with aging.
Key areas of uncertainty regarding determination of the vitamin B12 and folate status of the elderly include (1) the normal ranges of serum vitamin B12 concentration and closer definition of cutoff values; (2) the extent and significance of vitamin B12 malabsorption in the elderly and improved identification of causative factors aside from pernicious anemia; (3) the validity of red cell folate concentrations as a measure of tissue levels; and (4) the neurological significance of subclinical vitamin B12 and/or folate deficiencies in the absence of overt disease.

Elderly populations should be a special focus of studies of nutritional status. Because of age-related changes in sensitivity to such agents as drugs, neurotransmitters, hormones, and vitamins, even marginal deficiencies could have greater effects in elderly individuals than in younger persons or alter manifestations of vitamin B12 or folate deficiency.

Specific recommendations were made for assessment of vitamin B12 and folate status by means of population surveys and clinical studies. For example, folate status should be studied more comprehensively in elderly population groups at risk of deficiency, such as those with low socioeconomic status, who have been reported to have low serum folate and/or RBC values. Useful populations for clinical studies might include patients with inborn errors of metabolism involving vitamin B12 or folate, patients with depression or other putative neuropsychological manifestations that might be related to vitamin B12 or folate deficiency, and patients with certain hematologic disorders who might be considered as a subgroup for study.

The significance of "subclinical deficiencies" of both vitamin B12 and folate remains to be determined. Effects of aging on the parameters measured to assess vitamin B12 and folate status should be examined more extensively. There is a need to determine whether cutoffs derived on the basis of hematological disease are appropriate for neurological disease.

The usefulness of and correlation among indicators of vitamin B12 and folate status should be studied further. Additional measures of status should be developed and evaluated.

A well controlled collaborative study is needed to evaluate multiple measures of both vitamin B12 and folate concentrations as a means of determining relationships among measurements of status (serum and RBC folate, serum vitamin B12, methylmalonic acid, homocysteine, etc.).

Therapeutic trials of folate and vitamin B12 in population groups with suspected inadequacy should be conducted. The appropriate dosage and length of time for treatment need to be determined.
Factors that decrease vitamin B12 and folate absorption in elderly persons should be studied. Additional information is needed on folate content of foods and bioavailability.

Regardless of the public health significance of impaired folate and/or vitamin B12 status in the elderly, there are large gaps in basic knowledge of the effects of folate and vitamin B12 on the nervous system.

Folic acid and vitamin B12 are probably not key cofactors in the metabolism and synthesis of neurotransmitters including dopamine, norepinephrine, tryptophan, epinephrine, and possibly acetylcholine with respect to synthesis from choline. Basic data are needed on possible neurochemical mechanisms whereby deficiency of vitamin B12 and/or folate could impair neurologic function.

Evidence for an effect of vitamin B12 deficiency on the nervous system is generally convincing but uncertainty exists as to whether folate deficiency results in structural or functional disturbances in the nervous system in the elderly. Important gaps exist in the knowledge of possible mechanisms of action of both vitamin B12 and folate in the metabolic processes of the nervous system.

Better methods are needed for assessing neurologic manifestations of vitamin B12 deficiency. Concentrations of various metabolites in cerebrospinal fluid may be better indicators of deficiency than serum or urinary levels. The usefulness of various tests of evoked neurologic responses needs further evaluation.

Modern techniques used in neuroscience should be applied to study the effects of vitamin B12 and/or folate deficiency on the aging nervous system. More current and emerging research-related methods could be useful in multidisciplinary studies including participation by hematologists, psychiatrists, neurophysiologists, and neurologists to determine the most appropriate methods and research approaches. These techniques include CAT scanning, NMR scanning, PET scanning, DNA probing, and electroencephalography, brain-evoked potential responses, and nerve conduction measurements. Cultures of CNS cells and tissue explants also offer valuable approaches for in vitro studies of effects of folate and vitamin B12 in neurologic structure and function.
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DEGENERATIVE CHANGES IN NEURONS OF AGED NONHUMAN PRIMATES
AND INDIVIDUALS WITH ALZHEIMER'S DISEASE

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INTRODUCTION

In primates, age-associated changes occur in behavior and in brain structure/chemistry. It has been suggested that alterations in the expression of certain genes associated with aging may be important in the development of brain abnormalities. This review describes some of the alterations in behavior/brain that occur in aged individuals (using nonhuman primates as a model) and in individuals with Alzheimer's disease (AD).

AGED PRIMATES

Aged humans and rhesus monkeys (potential lifespan >35 years) develop age-associated deficits in cognition and memory. These impairments, which become evident after 20 years of age, are detected by behavioral tasks that assess the integrity of specific regions of brain. Among subjects of the same age, significant variability exists in performance of specific tasks (Presty et al., 1987). Our studies of aged nonhuman primates are based on the hypothesis that animals with impairments of different tasks have age-related alterations in specific regions of brain.

What kinds of structural and chemical changes could contribute to these behavioral deficits? Aged monkeys exhibit several types of structural/chemical changes in brain that are

* Presented as "Degenerative Neurological Changes of Aging" by Donald L. Price, M.D.
similar to those that appear in aged humans and, to a greater extent, in individuals with AD. In animals of the same age, there are differences in the distribution and severity of lesions. Older primates show age-related reductions in sizes of cortical neurons (Applegate et al., 1987; Terry et al., 1987), and some cortical neurons exhibit immunoreactivities for A68, a protein enriched in brains of humans with AD (Wolozin and Davies, 1987). At the end of the second decade of life (equivalent to humans of 50-60 years of age), axonal pathology (enlargements, tortuosity, etc.) appears in cortex; some abnormal axons are not associated with deposits of amyloid, but others appear as abnormal neuritic components surrounding senile plaques. These neurites are enlarged filaments containing axons, mover terminals and dendrites. They are derived from a variety of neurotransmitter systems (i.e., cholinergic, monoaminergic, and peptidergic neurons) (Kitt et al., 1984; Struble et al., 1984; Walker et al., 1985; Walker et al., 1988). Relationships may exist between the presence of plaques and alterations in a variety of neurotransmitter markers. In addition to neurites, senile plaques also contain deposits of amyloid. Amyloid is composed of a unique 4 kilodalton (kD) β-protein (A4) (Masters et al., 1985; Selkoe et al., 1987; Wong et al., 1985) that appears to be derived from a much larger precursor polypeptide (APP), coded for by a gene on the long arm of human chromosome 21. The APP gene transcript is most abundant in neurons, whereas other cells in brain appear to express lower levels of this gene (Bahmanyar et al., 1987; Cohen et al., 1988; Goedert, 1987; Higgins et al., 1988; Koo et al., 1988). Coexisting with the β-protein in amyloid deposits are sulfated glycosaminoglycans (Cork et al., 1988) and α-antichymotrypsin (α-ACT) (Abraham et al., 1988a) a serine protease inhibitor. Possible interactions between these various components in deposits of amyloid are not well understood. However, it is clear that age alone is an important factor in the development of these types of pathologies in brain.

Although clinical-pathological correlations have not yet been published to explain age-associated deficits that occur in primates, it is likely that impairments in task performance of older individuals are due to some of the structural/chemical abnormalities in brain.

ALZHEIMER'S DISEASE

Structural and chemical abnormalities that occur in brains of aged primates are similar to those that occur, to a much greater extent, in AD. The clinical syndrome of AD (i.e., progressive impairments in memory, language, visuospatial perceptions, etc.) is due to a variety of abnormalities in brain. In this degenerative disease, populations of nerve cells (defined,
in part, by their locations, circuitries, and transmitter specificities) in brainstem nuclei, basal forebrain, amygdala, hippocampus, and neocortex exhibit a variety of abnormalities (for review, see Price, 1986). These include alterations of cytoskeletal elements and the appearance of unusual constituents in perikarya of vulnerable cells. Moreover, senile plaques, composed of neurites associated with extracellular deposits of β-protein (A4), are a cytological hallmark of AD.

Some of the circuits affected in AD have been identified. Cholinergic neurons, located in the medial septum, diagonal band, and nucleus basalis develop neurofibrillary tangles (NFT) (Candy et al., 1983; Whitehouse et al., 1982); distal axons/nerve terminals of these cells form neurites in some plaques. As these neurons degenerate, cholinergic markers, including muscarinic (M2) receptors and nicotinic receptors, are reduced in amygdala, hippocampus, and neocortex (Francis et al., 1985; Kellar et al., 1987). Involvement of brainstem monoaminergic systems is more variable. Serotoninergic neurons of the raphe complex show NFT, the number of cells may be reduced, and changes may also occur in serotoninergic markers in target fields (Curcio and Kemper, 1984; D'Amato et al., 1987). Catecholaminergic neurons of the locus coeruleus may show NFT, and the number of nerve cells may be reduced. Noradrenergic innervation in targets is decreased, and noradrenergic axons/terminals may constitute neurites in plaques (Powers et al., 1987). Hippocampal pyramidal neurons are severely affected in AD; these cells develop NFT, Hirano bodies, and granulovacular degeneration (Ball, 1977; Kemper, 1983). Involvement is not equal in all zones of the hippocampus. NFT and loss of pyramidal neurons appear to be greatest in CA1 and CA2 (Jamada and Mahraein, 1968; Kemper, 1983). Because these cells probably use excitatory amino acids as transmitters and because, at present, we do not have satisfactory markers for these systems, structural abnormalities and loss of these neurons are not reflected in neurotransmitter assays. In neocortex, NFT and reductions in numbers of neurons are observed consistently (Hansen et al., 1988; Kemper, 1983). Pyramidal neurons of cortex, which appear to use excitatory amino acids as transmitters, are affected as are smaller cortical neurons using peptidergic transmitters (i.e., coricotenin-releasing factor (CRF) or somatostatin) (Davies et al., 1980; De Souza et al., 1986; Rossor et al., 1980). Both types of peptidergic neurons develop NFT and give rise to neurites in plaques (Powers et al., 1987; Struble et al., 1987). Interestingly, the numbers of somatostatin receptors are reduced (Beal et al., 1985), but CRF receptors are increased in cortex (De Souza et al., 1986). Although levels of a variety of other peptides do not appear to be altered significantly in neocortex, some of these peptidergic systems contribute neurites to plaques (Struble et al., 1987).
In AD, affected neurons show NFT, which contain paired helical filaments (PHF) associated with 10- and 15-nm straight filaments. NFT exhibit immunoreactivity for a variety of constituents, including microtubule-associated proteins (tau and MAP2), phosphorylated epitopes of the neurofilament protein, A68 (a 68-kD protein enriched in AD brain) (Anderton et al., 1982; Brion et al., 1985; Cork et al., 1986; Joachim et al., 1987; Perry et al., 1985; Wolozin and Davies, 1987) and ubiquitin, a protein required for ATP-dependent proteolytic degradation of intracellular proteins (Mori et al., 1987; Perry et al., 1987). NFT-containing neurons eventually die, leaving extracellular accumulations of PHF as "ghosts" or "tombstones." Other cytoskeletal elements are also abnormal in these cells. Rod-shaped cytoplasmic Hirano bodies (paracrystalline arrays of actin containing microfilaments (Goldman, 1983) and granulovacuolar degeneration (clusters of electron-dense granules enriched in tubulin-like immunoreactivity) (Ball, 1977; Price et al., 1986) are common in pyramidal cells of hippocampus.

In AD, senile plaques, which are a hallmark of this disease, appear to be remarkably similar to plaques in aged monkeys. Amyloid appears to be identical. Although available evidence suggests that some cases of familial AD are linked to a gene on chromosome 21 (St. George-Hyslop et al., 1987a), the APP gene is not linked to the presence of disease in these cases (St. George-Hyslop et al., 1987a,b; Van Broeckhoven et al., 1987). It is likely that another gene, located on chromosome 21, plays a more central role in initiating a cascade of events, the eventual result of which is the complex pathology that occurs in AD. This cascade of events leads to the development of senile plaques with deposition of β-amyloid in sulfated glycosaminoglycans (Snow et al., 1987) and α-ACT (Abraham et al., 1988b) as well as the development, in neurons, of NFT, Hirano bodies, and granulovacuolar degeneration.

Clinical-pathological correlations suggest that certain clinical signs may reflect, in part, lesions in specific transmitter circuits. For example, reductions in numbers of neurons in the locus ceruleus may be most conspicuous in depressed patients with AD (Zweig et al., 1988). The combination of cholinergic deafferentation of hippocampus, intrinsic hippocampal pathology, and lesions of entorhinal cortex are believed to be major factors in memory deficits that occur in this disease. Disorders of language, visuospatial skills, praxis, etc., reflect dysfunction of cortical circuits due to deafferentation, disconnection, and pathology intrinsic to cortical neurons.

CONCLUSION

Two factors, age and genes, appear to be very significant in the development of age-associated structural/chemical changes that occur in brains of aged primates. Although aged monkeys do
not develop AD, recent investigations suggest that these animals provide a useful model to study some of the abnormalities that occur in aged humans, and to a greater extent, in individuals with AD.

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LITERATURE CITED


ALtered striatal cholinergic-dopaminergic reciprocal inhibitory control in senescence


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While the subject of neurotransmitter metabolism in aging is both interesting and timely, it is such a broad area that the space limitations of this report and the time limitations of the talk preclude discussions of the entire area. What I have chosen to do for this presentation is to limit the discussion to that concerned with dopaminergic-cholinergic interactions in the striatum. This is an area of the brain that mediates various simple and complex motor behaviors, and it shows profound changes in senescence.

Evidence derived from numerous sources indicates that there are very significant changes occurring within both the striatal dopamine (DA) and acetylcholine (ACh) systems during aging. The exact specifications of these alterations is dependent upon both the species examined and the conditions of the experiment, but indications are that some of the most striking changes take place in the various populations of receptors from these systems. In the case of the striatal DA system, the receptors were originally divided into two subtypes (D₁ and D₂) (Kebabian and Calne, 1979), and subsequent findings indicated that each receptor subtype showed a different pattern of change with age. It appeared that while the D₂ receptor showed remarkably consistent age-related declines in concentration, the changes occurring in the concentration of striatal D₁ receptors were much more variable. Initial assessments of D₂ receptor loss with age indicated a 35% decrease in binding with no loss of binding affinity in the aged rat. Subsequent observations have indicated that the degree of D₂ loss is dependent upon the assay conditions, the strain or species of animal tested, and the particular dopamine-sensitive ligand that is utilized, but this decline generally ranges from about 36 to 66% (Henry et al., 1986, Joseph et al., 1978, Severson and Finch, 1980, Severson et al., 1982).

* Presented as "Age-Related Changes in Metabolism of Acetylcholine and Dopamine" by James A. Joseph, Ph.D.
If age-related changes in the Bmax or Kd of the striatal D₁ receptor are considered, the results from at least one laboratory show parallel losses between D₁ receptor concentration and DA-stimulated adenylate cyclase activity that are the greatest between 3 and 12 months of age in the rat (Henry et al., 1986). However, findings from laboratories using other strains of rats (e.g., Sprague-Dawley) (O'Boyle and Waddington, 1984) or mice (e.g., C57BL/6) (O'Boyle and Waddington, 1984) indicate no change in the concentration of striatal D₁ receptors with age. Examination of postmortem human material indicates an actual increase in D₁ receptors with age (Morgan et al., 1984). Since the changes in the concentration of striatal D₂ receptors seem to follow a progressive decline throughout the lifespan of the organism, while changes in the concentration of striatal D₁ receptors do not, these results suggest that there may be increases in the striatal D₁/D₂ receptor ratio in senescence, a finding which has been confirmed in humans (Morgan et al., 1984) and rats (Henry et al., 1986).

Assessments of age-related differences in striatal muscarinic cholinergic receptor binding indicate that there are decreases in the number of these binding sites in the striatum as a function of age. Declines in the concentration of striatal muscarinic receptors of about 25-29% in the rodent have been reported in studies using the ligand, (-) [³H]-quinuclidinyl benzilate (³H-QNB) (Morin and Wasterlain, 1980). These declines were seen primarily in the medial and caudal portions of the striatum (Strong et al., 1984), a pattern that approximates that seen with respect to loss of D₂ receptors.

One question that arises from these findings is that since there appears to be a great deal of interactive modulation that takes place between the striatal DA and ACh systems, how do age-related declines in their concentrations alter these respective interactions? This question has not received a great deal of systematic study, but there are indications that there may be age-related declines in the normal reciprocal inhibitory control (RIC) that is exerted between these two systems. Recent studies have shown that while striatal DA-stimulated adenylate cyclase activity is inhibited by muscarinic agonists such as oxotremorine or carbachol in striatal broken cell preparations prepared from young rats (6 mo), these agents were ineffective in inhibiting this activity in senescent rats (24 mo) (Coupet et al., 1985).

Additional assessments carried out to examine DA modulation of ³H-ACh release from striatal slices from mature, middle-aged and senescent rats indicated that the DA agonist, apomorphine,
was effective in inhibiting $K^+$-evoked release of $^3$H-ACh from superfused striatal slices obtained from mature and middle-aged rats but not from those prepared from old rats (Thompson et al., 1984).

These findings, when considered in light of those discussed above indicate that as receptors from both of these transmitter systems are lost with age, alterations in one may profoundly affect the functioning of the other, and RIC that is normally exerted between these two systems is significantly diminished. Recent experiments in our laboratory were directed toward further examination of age-related alterations in cholinergic regulation of striatal DA autoreceptors. It is now widely accepted that striatal DA release is under the control of a group of inhibitory DA autoreceptors (Cubeddu and Hoffman, 1982; Cubeddu and Hoffman, 1983; Cubeddu et al., 1983; Farnebo and Hamberger, 1971; Hoffman and Cubeddu, 1982; James and Cubeddu, 1984; Kamal et al., 1981; Kelly, 1981; Parker and Cubeddu, 1985; Sakurai et al., 1982; Starke et al., 1978, Westfall et al., 1976).

If these autoreceptors are stimulated via DA agonists or inhibited with DA antagonists, $K^+$-evoked release of DA will be respectively inhibited or enhanced. Normally, this control is in turn mediated through inhibitory muscarinic and/or nicotinic heteroreceptors (Lehmann and Langer, 1982; Raiteri et al., 1982; Raiteri et al., 1984; Sakurai et al., 1982; Westfall et al., 1983) presumably located on the same terminals as the autoreceptors. Either muscarinic (e.g., oxotremorine, carbachol, bethanecol) or nicotinic (e.g., nicotine) agonists can activate the heteroreceptors, which inhibit the DA autoreceptor and potentiate the $K^+$-evoked release of DA from the striatum (Parker and Cubeddu, 1985; Plotsky et al., 1977; Raiteri et al., 1984). The actions of the muscarinic agonists on DA release can be attenuated by both atropine as well as the more selective $M_1$ muscarinic antagonist pirenzepine (Plotsky et al., 1977).

Thus, in one set of experiments (Joseph et al., 1988a), enhancement of DA release was assessed in superfused striatal slices obtained from young, middle-aged, and old rats following application of muscarinic or nicotinic agonists and muscarinic antagonists. Additionally, in order to examine any age-related alterations in DA autoreceptor function directly, the potentiation of $K^+$-evoked DA release by haloperidol was also determined.

The procedure was carried out by obtaining striatal tissue from mature (6 mo), middle-aged (12 and 18 mo), and aged (24 mo) Wistar rats. Cross-cut striatal slices (300 μm) were prepared
using a McIlwain tissue chopper. Slices from equal numbers of animals were pooled for each age group and placed in small glass vials containing a modified Krebs-Ringer basal release medium (2.5 mM KCl) that had been bubbled for 30 minutes with 95% O₂/5% CO₂. One hundred and fifty µl aliquots (approximately 1.1-1.4 mg tissue/chamber) were placed in a superfusion apparatus containing 16 parallel chambers. Typically, pooled tissue from two animals was used to fill four of the parallel chambers. The tissue and buffer media were maintained at 37°C throughout the course of the experiment. Following placement into the superfusion chambers, the tissue was allowed to equilibrate for 30 minutes. It was continuously perfused with the oxygenated basal release medium at the rate of 124 µl/min. After the equilibration period, a 5 min baseline fraction was collected, and the medium was then switched to one containing the release medium (30 mM KCl). Following the switch, 5 min fractions continued to be collected for 30 minutes. To this oxygenated release medium was added one of four concentrations of the agent under study [(0 µM, 100 µM, 500 µM, and 1 mM of oxotremorine, carbachol, pilocarpine, bethanecol, or nicotine); (haloperidol, 0 µM, 10 µM, 100 µM, 500 µM)]. In addition, the specificity of any muscarinic agonist enhancement of KCl-induced release of DA was examined following application of 500 µM oxotremorine in the presence or absence of graded concentrations of atropine (100 µM, 500 µM, 1000 µM) or pirenzepine (500 µM, 1000 µM, 2000 µM). DA release was assessed using high performance liquid chromatography (HPLC) coupled to electrochemical detection.

The results indicated that although the release of DA in striatal slices obtained from all of the age groups was increased over basal release when a depolarizing concentration of KCl (30 mM) was introduced into the superfusion medium, the muscarinic enhancement of such K⁺-evoked release was significantly blunted in the various ages. The pattern of this deficit tended to be dependent upon the particular agent that was utilized for enhancement. Lowered enhancement of K⁺-evoked release of DA was not observed until 18 to 24 months of age with oxotremorine or pilocarpine but occurred as early as 12 months of age with carbachol or bethanecol. These deficits seemed to be confined to the muscarinic portion of this system, since no age-related differences in enhancement of K⁺-evoked release of DA were observed when nicotine or haloperidol were added to the release medium.

The exact locus of the deficit within the muscarinic system, however, remained to be specified. Previous research (see Roth, 1988 for review) had suggested that post-receptor changes that
occur from neurotransmitter/receptor interactions involve the mobilization of Ca\(^{++}\) and that age-related dysfunctions that occur appear to be the result of impaired Ca\(^{++}\) mobilization. An enormous amount of data has accumulated in recent years which has shown that ligand activation of mAChR's results in Ca\(^{++}\) mobilization which is brought about through a signal transduction process involving ligand-induced increases in the turnover of phosphatidylinositol (PI), a product which may be further phosphorylated to form phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). The activated ligand-receptor complex cleaves PIP\(_2\), and one product resulting from this cleavage is 1,4,5-inositol trisphosphate (IP\(_3\)) which diffuses into the cytoplasm, releases calcium from storage, and ultimately induces a physiological response (see Fisher and Agranoff, 1987 for review). Any age-related alterations at any point in this pathway would lead to reduced Ca\(^{++}\) mobilization and a lowered ACh responsivity.

The initial step in this pathway, of course, is the binding of the ligand to the receptor and the coupling of the receptor to the effector. It has been shown (Lippa et al., 1985) that beginning with middle age, the proportion of mAChR existing in desensitized, decoupled (from the effector), conformational/orientational states increases, and muscarinic agonists introduced into the system are more likely to encounter AChR in the desensitized states. Physiological responses resulting from these encounters are likely to be blunted. Response decline to oxotremorine and pilocarpine was not observed prior to 18-24 months of age, while responses to carbachol and bethanecol appeared as early as 12 months of age. It has been postulated previously that full agonists such as carbachol maximally stimulate PI and allow a change in the conformation/orientation of the mAChR to a sensitized, decoupled state while partial (B) agonists such as oxotremorine only poorly stimulate PI turnover (Fisher et al., 1983; Fisher et al., 1984), and the receptor-effector coupling is not altered. Thus, in some cases muscarinic agonists may actually induce a change in the conformational state of the receptor to one that is decoupled from the effector. In such instances, response decrements are more likely to appear even earlier in the life-span. Since B agonists induce no further decoupling of the receptor from the effector, age deficits are seen later with oxotremorine or pilocarpine than with carbachol. Therefore, age-related deficits in AChR-D\(A\) autoreceptor interactions may begin at the ligand-AChR interface. If this is true, then one test of this hypothesis might be to "bypass" the AChR, induce Ca\(^{++}\) mobilization from the membrane more directly, and determine if age-deficits in AChR inhibition of striatal DA autoreceptors continue to be observed. These tests were carried
out in the next set of experiments (Joseph et al., 1988b) in which either the Ca$^{++}$ ionophore A23187 of IP$_3$ was added to the release medium to enhance the K$^+$-evoked release of DA from striatal slices of young and old animals. Again, the results pointed to a deficit at the mAChR-ligand interface as being responsible for the response blunting in senescence since there were no age-related differences in enhancement observed when these agents were used to enhance the K$^+$-evoked release of DA from the striatal slices.

We realize that we cannot as yet rule out such factors as age-related alterations in G-proteins or deficits in IP$_3$ formation as being responsible for the response blunting in this system and experiments to address these issues are being carried out, but for the remainder of this discussion we have chosen to focus on the possible mechanisms that may be responsible for the alterations in the changes of the mAChR in senescence.

In order to determine where to begin to search for these mechanisms, we hypothesized that perhaps the putative age-related alterations in mAChR which result in changes in their conformation/orientation states and decreases in their responsivity may occur from membrane changes taking place as a result of lipid peroxidation from accumulated free radical damage. Previous research has indicated that the nigrostriatal system may be particularly susceptible to oxidative damage from free radicals. There are at least three sources of evidence for this suggestion. First, persons who have intravenously self-administered a meperidine analog contaminated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) develop severe Parkinsonian-like movement aberrations. MPTP is known to be a neurotoxin which selectively destroys cells in the substantia nigra presumably by inducing the formation of free radicals. Other areas of the brain are spared from any alterations by MPTP. Second, if buthionine sulfoxine, an inhibitor of glutathione (a potent free radical scavenger) is administered to mice, both the resultant motor behavioral deficits and the nigrostriatal damage resemble those seen with MPTP. Third, during aging it has been postulated that the organism is less able to deal with the life-long accumulated damage from free radicals, and again one central neuronal system profoundly affected is the nigrostriatal system with the accompanying motor behavior deficits.

Thus, we believed that if we could mimic the effects seen in mAChR responsivity by using a procedure that would generate free radicals in young animals, it would lend some support for the contention that these age-related changes are the result of oxidative damage.
Since it is known that various sources of radiation are potent generators of free radicals, we examined the muscarinic (oxotremorine - 0, 500 µM) enhancement of K\(^+\)-evoked release of DA from striatal slices obtained from young rats irradiated with 0-5 Gy of \(^{56}\)Fe irradiation, and pretested on a wire suspension task (3-14 post irr.)(Joseph et al., 1988c). The results indicated that both the oxotremorine enhancement of K\(^+\)-evoked release of DA and the performance on the motor task were lowered in all the treated groups to the same degree as that seen previously in old animals.

While further experiments are needed to test this hypothesis, these findings suggest that the mAChR membrane alterations that occur during aging may involve damage by free radicals. We are presently carrying out additional studies to make these determinations.

LITERATURE CITED


EXPERIMENTAL APPROACHES FOR THE STUDY OF NEUROLOGICAL CHANGES

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Because of the cellular diversity and complex cytoarchitecture of the central nervous system as well as the existence of the blood-brain barrier and the production of regulatory molecules within the brain itself, it is difficult to analyze the molecular mechanisms involved in cellular differentiation and aging by animal experimentation. The development of in vitro systems has enabled researchers to exert greater control over the chemical and cellular complexity of the nervous system (Saneto and de Vellis, 1987). Many vertebrate and invertebrate preparations of neural cells are now available. As a result, the molecular dissection of cell lineages and cellular differentiation has greatly improved and has revealed a growing spectrum of regulatory molecules and mechanisms that participate in neural cell proliferation, generation of cell lineages, and phenotypic differentiation (Arenander and de Vellis, 1989).

Cultures which are prepared directly from the tissues of an organism are called primary cultures (Saneto and de Vellis, 1987). The first system consists of placing a fragment of nervous tissue, either developing or adult, in an in vitro situation. This is known as the explant culture, which can be maintained live for months or even years. It allows for the control of the chemical and physical environment of the cells. Substances can be added or withdrawn from the culture medium, allowing a precise temporal analysis of the sequence of events which occur, for instance, in development or in hormone action. It removes the problem of the blood-brain barrier and the influence of homeostatic mechanisms and other organs. It also allows for the study of a discrete nervous area in isolation from the rest of the organism. Explants of developing nervous tissue display a cellular development, synaptogenesis, myelination, and biochemical differentiation similar to the in vivo situation. This system should be useful for the study of age-related changes in aging research.

The second cell culture system, dissociated cell culture (Saneto and de Vellis, 1987), consists of mechanically or enzymatically dissociating the tissue to a suspension of single cells. The dispersed cells are usually cultured on the pretreated surface of a culture dish. Dissociated cell cultures allow the visualization of individual living cells which can be monitored morphologically and electrophysiologically. It is possible to obtain and correlate biochemical, morphological, and electrophysiological data from a single cell. An alternative to observing and quantitating parameters at a single cell level
is to separate the cell types either prior to culturing, for example, by cell sorting using appropriate cell surface markers, or after the culture is established. Ingenious methods developed in several laboratories now make it possible to obtain cultures enriched 90 to 99% in one type of the following cells: cerebral neurons (Morrison et al., 1986), cerebellar granule cells, oligodendrocytes (Saneto and de Vellis, 1985), astrocytes (McCarthy and de Vellis, 1980; Morrison and de Vellis, 1981), Schwann cells, neurons of dorsal root, sympathetic, ciliary, nodose, cervical, or myenteric ganglia, glial progenitor cells, chromaffin cells, neurons of discrete central nervous system (CNS) areas, etc. These cultures are the system of choice for the biochemist (Saneto and de Vellis, 1987).

The third system consists of placing the dispersed cells in a rotating flask. Within a few hours, cell aggregates form. This system provides a more structured, three-dimensional, extracellular space which approximates the in vivo conditions for cell growth and development. Such conditions reduce the dilution of secreted cellular factors and increase the opportunity for morphological and biochemical differentiation to proceed more like in vivo events. The cells inside the aggregate are first distributed at random. Then they sort out into patterns often resembling the organization seen in vivo (Lu et al., 1980).

The culture media for the growth of mammalian cells were chemically defined and optimized in the 1960s for amino acids, vitamins, salts, and metabolites in the presence of added sera, usually fetal calf serum. During the last 10 years, the uncontrollable, undefined nature of sera has been circumvented by replacing serum with pure growth factors, hormones, and adhesion molecules (Morrison and de Vellis, 1981; Saneto and de Vellis, 1985, 1987). This has led not only to the discovery of the various target cells for different factors but also to the discovery of many new target cell-derived factors (Arendander and de Vellis, 1989).

The inadequacies of morphological criteria to identify population subsets have led neurobiologists to turn to immunological approaches to develop cell markers. There are now immunologic markers for the major neural cell types (Arendander and de Vellis, 1989). These have begun to be available commercially and through the American Type Culture Collection (ATCC) as hybridoma cell lines producing monoclonal antibodies. For some cell types, an array of appropriately restricted markers have been developed to define functional or developmental subsets of a specific cell type. For example, the oligodendrocyte cell lineage is marked by the sequential appearance of myelin-associated antigens allowing the distinction of three developmental stages which arise from bipotential progenitor cells identified by the monoclonal antibody A2B5 to cell surface gangliosides. These progenitor cells have been found in the adult rat brain and, therefore, may be used for renewal of oligodendrocytes.
The next stage, called pre-oligodendrocyte, is marked by the appearance of sulfatides (04 antibody), transferrin, and glycerol phosphate dehydrogenase, and the decline of A2B5, Vimentin, and ganglioside GD3. The immature oligodendrocytes display the myelin lipid, galactocerebroside, and the complete loss of Vimentin, GD3, and A2B5. The third stage, mature oligodendrocytes, express myelin basic protein, proteolipid protein, and begin to make myelin membrane. This model system has allowed the identification of regulatory molecules which control the proliferation of specific developmental subsets and their progression through the three stages (Arenander and de Vellis, 1989). The cells remain plastic for quite some time and can be reversed by the presence and/or absence of certain signals.

With respect to dissociated cell cultures from the adult nervous system, there are well characterized culture systems established for oligodendrocytes, astrocytes, chromaffin cells, and dorsal root ganglion cells. There is a need to develop CNS neuronal cultures.

Analysis of changes with time in primary cultures composed of an homogeneous cell population can easily be conducted by using current biochemical assays (Edmond et al., 1987) and molecular biology techniques (Holmes et al., 1988; Kumar et al., 1986). In cultures of mixed cell types one can envision the use of computerized, quantitative image analysis at the cellular and subcellular levels, of molecules visualized by specific radioactive precursors and ligands, labelled recombinant DNA probes (Holmes et al., 1988), and antibodies (immunocytochemical detection) (Espinosa de los Monteros and de Vellis, 1988).

LITERATURE CITED


VITAMIN B12 AND FOLATE STATUS OF THE ELDERLY IN THE UNITED STATES

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Both vitamin B12 and folic acid deficiency states were first described as hematological diseases with florid megaloblastic anemia (Beck, 1988). As time has progressed it has become clear that in addition to megaloblastic anemia vitamin B12 deficiency causes neurological damage in the posterior-lateral columns with loss of vibratory sense, joint position sense, and balance. Patients with B12 deficiency have also been reported to have a wide variety of psychiatric problems from mania ("megaloblastic madness") to dementia (Crantz, 1985). Folic acid deficiency is thought also to cause neurological disease, a wide variety of psychiatric problems including dementia (Grinblat, 1985) as well as the identical hematological disease as B12 deficiency.

There are case reports in the medical literature and other "anecdotal" evidence that the psychiatric manifestations of vitamin B12 or folate deficiency can occur without any hematologic manifestations (Crantz, 1985; Grinblat, 1985). Similarly, the neurological signs can occur without either the hematologic or psychiatric problems (Crantz, 1985; Grinblat, 1985). Since both of these deficiency states were initially recognized as hematologic problems, physicians often do not consider them as possibilities unless there are some hematologic abnormalities.

Dementia is one of the major problems affecting our elderly population. It is estimated that 10% of all people over age 65, 20% of people over age 75, and 30% of people over age 85 have some variety of dementia (Berk and Freedman, 1987). As people live longer and the elderly make up a greater percentage of the population, dementia has, thereby, emerged as one of the greatest health problems in the USA. The question of how many of these demented people suffer from B12 and/or folate deficiency, two potentially reversible forms of dementia, therefore, is of major clinical significance. Unfortunately, we do not yet have the answer.

VITAMIN B12

Vitamin B12 is the term used to describe the cobalamin molecules. The four cobalamins of importance in animal cell metabolism are cyanocobalamin, hydroxocobalamin, and two coenzyme forms adenosylcobalamin and methylcobalamin. Adenosylcobalamin is the coenzyme of methylmalonyl-CoA mutase, an enzyme catalyzing the first step in the pathway of propionic acid. Methylcobalamin is the coenzyme for the methyltransferase enzyme needed for the
conversion of homocysteine to methionine. This step interacts with folic acid metabolism as it serves primarily as a means for converting $\text{N}^+$-methyltetrahydrofolate to tetrahydrofolate. Adenosylcobalamin is mainly found in tissues and methylcobalamin is the major cobalamin in serum. A deficiency of vitamin B12 will, therefore, lead to an increase in both methylmalonic acid and homocysteine (Beck, 1988).

**B12 DEFICIENCY**

Pernicious anemia is the most common illness known to cause vitamin B12 deficiency. This illness is caused by a lack of gastric intrinsic factor necessary for the absorption of B12 in the distal ileum. Clinically, this illness presents with a macrocytic anemia, thrombocytopenia, leukopenia with hypersegmented neutrophils, ineffective erythropoiesis, as well as neuro-psychiatric problems. This illness appears to be an "autoimmune" illness with an atrophic gastritis and the hematologic manifestations are easily reversed by administration of vitamin B12 (Beck, 1988; Crantz, 1985). The manic state ("megaloblastic madness") usually is reversed but the dementia and neurological signs are less often and incompletely reversed by vitamin B12 administration.

In addition to pernicious anemia (atrophic gastritis), other causes of B12 deficiency include gastrectomy, malabsorption syndromes, bacterial or parasitic overgrowth in the intestines, pancreatic disease and perhaps a strict vegetarian diet. A Schilling test is used to differentiate between these states. In pernicious anemia one often sees autoantibodies against intrinsic factor and/or parietal cells. Antibodies against intrinsic factor are much more specific for the diagnosis (Beck, 1988; Crantz, 1985).

Currently, in clinical practice one uses a serum vitamin B12 to screen for patients with deficiency. We initially screened 378 elderly patients who presented to the NYU-Bellevue Geriatric Comprehensive Care Clinic (73 men and 305 women) (Marcus et al., 1987). The mean age was 77 and the patients showed no signs of megaloblastic anemia, their red cell mean corpuscular volumes (MCV) were within the normal range and there were no hypersegmented neutrophils identified on their peripheral smears (the mean lobe count of the neutrophils was also normal). Furthermore, there was no endemic for infective erythropoiesis.

Twenty-six (7%) of these patients were found to have a low (<200 pg/ml) serum B12 level. Two of these patients were shown on Schilling test to have pernicious anemia and three had a malabsorption syndrome. Two patients had iron deficiency which falsely lowers the serum B12. Anti-intrinsic factor antibodies were negative in the remaining nineteen patients.
The nineteen patients with low serum B12 and no other evidence of hematologic disease were studied by examining the amount of vitamin B12 bound to the transcobalamin proteins. Vitamin B12 in plasma is bound to three transport proteins - the transcobalamins (TCs). TC I and II function as storage proteins, are of rapid (R) electrophoretic mobility and are synthesized in granulocytes. TC II is of slow (S) mobility, is synthesized in the liver and is responsible for transport and cellular uptake of cobalamin. Congenital absence of TC I is apparently harmless while severe to lethal megaloblastic anemia occurs in infants lacking TC II. We found that about 17% of the total B12 was carried on TC II in young, normal controls. In contrast, in both the low B12 elderly and the normal B12 elderly (B12 level >200 pg/ml) only about 4% of the B12 was bound to TC II. This would suggest that the elderly in general have a borderline B12 status. Even the normal B12 elderly had on the average lower levels of B12 than the young. In addition, the amount of B12 in storage (TC I and TC III) was also reduced in the elderly with this decrease being more marked in the low B12 elderly. Of note, is that the number of binding sites on TC II was normal in the elderly suggesting that there is no qualitative abnormality in TC II (Marcus et al., 1987).

Several reports in the literature have suggested that there is an increased prevalence of low B12 assays in the demented elderly when compared to the normal elderly (Babitz et al., 1986; Cole and Prohal, 1984). We found about a 20% prevalence of lower serum B12 in a group of elderly presenting with complaints of memory loss (Babitz et al., 1988). We attempted a small randomized prospective double-blinded, placebo-controlled study to investigate if B12 could reverse cognitive loss. The subjects were fourteen elderly patients with low serum B12 but normal Schilling tests and no signs or symptoms of B12 deficiency except for mild to moderate cognitive defects. They were randomly assigned to receive either cyanocobalamin (1000 μg) or normal saline placebo as four weekly intramuscular injections. There were no statistically significant improvements on neuropsychological testing even after crossover of the placebo patients to receive B12 in a double-blinded manner. However, even though the group did not reach statistical significance, several patients seemed to have a dramatic improvement. While we realize that this is anecdotal it certainly suggests that further studies are necessary as this study was very small and the length of treatment of B12 dosage was perhaps too short.

Also of note is that in these fourteen patients, two were subsequently shown to have pernicious anemia by Schilling test even though they were hematologically perfectly normal and their vitamin B12 levels were "borderline" (150-200 pg/ml) or on repeated examination were "low-normal" (200-250 pg/ml) (Babitz et al., 1988).
This phenomenon of low serum B12 also exists in a hospitalized younger population and particularly in patients with AIDS (Thompson et al., 1987). The significance of these findings is also not clear.

We have also tried to determine if red cell distribution width (RDW) elevation would identify these patients (Thompson et al., 1988a). This test is not as sensitive as presence of hypersegmented neutrophils and was of no added value. Even hypersegmentation will miss patients with malabsorption due to lack of intrinsic factor (pernicious anemia) shown on Schilling test (Thompson et al., 1988b).

FOLIC ACID

In human metabolism (Marcus and Freedman, 1985) tetrahydrofolate is a catalytic self-regenerating acceptor-donor of one-carbon units in anabolic and catabolic reactions involving one-carbon transfers. A number of metabolic systems in animal tissues are known to require folate coenzymes with impairment of thymidylate synthesis being the key event in folate deficiency that produces clinical manifestations. Since folic acid and B12 metabolisms interact at the methyltransferase steps, a deficiency of folic acid will result in an increase in homocysteine (but not methylmalonic acid).

Green vegetables are the richest sources of folate in human diets but organ meat, yeast and mushrooms also contain it. There are small body stores of folate and people can become deficient in one to four months (Marcus and Freedman, 1985).

FOLIC ACID DEFICIENCY

Humans can become folate-deficient during growth, pregnancy and in a wide variety of diseases. The lack of a balanced diet can lead to a folic acid deficiency.

We screened 326 geriatric patients for folate deficiency measuring fasting serum and red cell folate levels (Grinblat et al., 1986). We compared two methods, a microbiologic (MBA) and radioassay (RA). With the MBA, 6.8% of the patients and 12.2% of young controls had low levels of red cell folate and 1.8% of patients and 4.8% of controls had low serum levels. In the RA, all of the patients had normal serum and red cell folates. One control had a low serum folate. In fact, the elderly had higher levels of measurable folic acid than the young. In this group we also found that ten of the patients had low levels of serum B12 with normal hematologic values and Schilling tests. In this group the red cell folate was higher than control or the non-low B12 elderly. Forty percent of those patients with low B12 had macular degeneration of the eyes which raises the possibility of an association between this illness and B12 deficiency.
Nutritional assessment of folate intake in this elderly population with normal serum folates showed that many people were ingesting fewer than 0.4 mg/day (RDA), as low as 0.2 mg/day. These data are consistent with the idea that the minimal daily requirement of folate is actually much less than the RDA (Grinblat, et al., 1986). We have also surveyed many older ambulatory people and found that in the absence of alcoholism, depression, and severe illnesses, the elderly are eating a balanced diet in New York City.

CONCLUSIONS

1) A significant proportion of the elderly population (10%) has a low serum B12 level which includes a low percentage bound to the physiological transport protein TC II as well as to the storage proteins TC I and III. This suggests an early deficiency state of B12 and the dependence upon nutritional uptake as a source of vitamin B12.

2) A greater proportion of patients with cognitive defects have low serum B12 levels (20% in our series).

3) A low serum B12 is also seen in hospitalized sick, young people, particularly those with AIDS.

4) Serum B12 levels seem to be more sensitive than any hematologic abnormality including MCV, RDW, hyper-segmented neutrophils or evidence for ineffective erythropoiesis in identifying patients with B12 deficiency.

5) On repeated examination some of the "low" B12 patients had B12 serum values in the normal range, even when they subsequently were found to have pernicious anemia on Schilling test. Thus, we do not know how many patients with low normal serum B12 levels are deficient.

6) Being borderline "low" or very low in serum B12 (<100 pg/ml) did not differentiate between having an abnormal Schilling test or not. These results and those in #5 point to the importance of measuring levels of metabolites such as homocysteine and methylmalonic acid (Lindenbaum et al., 1988) as well as the serum B12.

7) Very few elderly ambulatory patients were found to be folate-deficient by serum and RBC folate determinations. Nutritional assessment showed that the RDA of 0.4 mg/day may be set too high and that 0.2 mg/day is sufficient to protect against deficiency of this vitamin.
8) Folate deficiency has been reported to cause a wide variety of neuropsychological symptoms. Since in most series these are either patients with alcoholism or severe psychiatric illness, it is hard to evaluate how frequent folate deficiency is in the elderly and how much it contributes to dementia.

9) Either the active metabolites of folic acid or homocysteine should be measured to determine if indeed there is a deficiency state of this vitamin in the elderly with dementia.

10) Large randomized long-term trials of B12 and folic acid treatment will be necessary to answer the question of whether or not some dementia in the elderly is potentially reversible in treatment with these vitamins.

LITERATURE CITED


EVIDENCE FOR A ROLE OF VITAMIN B12 IN NORMAL BRAIN FUNCTION

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The evidence that vitamin B12 plays a role in normal brain function comes from several sources. First, there have been a number of case reports describing cerebral complications in patients with vitamin B12 deficiency (Anonymous, 1965; Evans et al., 1983; Herman et al., 1937; Isaaksson et al., 1971; Shovron et al., 1980; Woltman, 1924). Second, a variety of neurologic, including mental, manifestations have been reported in inborn metabolic errors involving cobalamin-dependent enzymes, cobalamin co-enzyme biosynthesis or cobalamin-binding plasma proteins (Cooper and Rosenblatt, 1987). Third, there is evidence obtained from a variety of vitamin B12-deficient animal models to support a role for vitamin B12 in the nervous system (Agamanolis et al., 1976; Green et al., 1975; Kark et al., 1974; Metz and Van der Westhuyzen, 1987; Oxnard and Smith, 1966; Siddons, 1974). These three sources of evidence for a role of vitamin B12 in brain function will be examined.

The effects of vitamin B12 deficiency on the nervous system have been extensively reviewed (Agamanolis et al., 1983; Kunze and Leitenmaier, 1977; Pant et al., 1968; Richmond and Davidson, 1958). A variety of neurological complications has been described. A survey of the literature (Agamanolis et al., 1983) reveals that nervous system symptoms occur in 75-90% of patients with pernicious anemia. Moreover, nervous system symptoms are the presenting complaint in 25% of patients with B12 deficiency and the onset and severity of neurological manifestations do not correlate with the presence or severity of anemia. The clinical features of B12-deficient myeloneuropathy are summarized in Table 1.

Mental and psychiatric disorders, which have been described in 4-16% of patients in some reports (Herman, 1937) and in an even higher proportion in others (Anonymous, 1965), range from nonspecific disturbances such as anxiety, irritability, confusion, apathy and fluctuations of mood to intellectual impairment, depression with suicidal tendencies and paranoid psychosis (Woltman, 1924).

Although electrophysiologic studies have been inconclusive, there are reports of slowed nerve conduction (Kayser-Gatchalian and Neundorfer, 1977), abnormal electroencephalogram tracing (Walton et al., 1954), delayed visual evoked responses (Troncoso et al., 1979) and abnormal brain stem auditory evoked responses and sensory evoked responses (Fine and Hallet, 1980; Krumholz et al., 1980). This suggests that mild or subclinical lesions may be more frequent than has previously been recognized.
Table 1. Clinical Features of B12-Deficient Myeloneuropathy

1. Early myelopathy
   Weakness and paresthesiae, particularly of the distal parts of the lower extremities

2. Progressive Myelopathy
   Spasticity, loss of position- and vibration-sense and unsteadiness of gait

3. Advanced Myelopathy
   Spastic paraplegia, contractures, ataxia and other sensory losses

4. Other Neurological Complications
   Various mental and psychiatric disorders, visual impairment, peripheral neuropathy, autonomic involvement

The neuropathological features of B12-deficient myeloneuropathy are summarized in Table 2. With respect to the brain, confluent foci of spongy change have been demonstrated in the central and convolutional white matter, but only few patients with brain lesions have been described and the clinico-pathological correlations are not detailed.

Table 2. Neuropathological Features of B12-Deficient Myeloneuropathy

1. Early Myelopathy
   Distention of myelin sheaths (patchy "spongy" change), particularly in posterior and lateral columns of the upper thoracic and lower cervical cord

2. Progressive and Advanced Myelopathy
   Disintegration of myelin and removal by macrophages
   Coalescence of spongy foci
   Some axonal loss

3. Confluent foci of spongy change in the central and convolutional white matter
   Optic nerve lesions
   Axonal degeneration of peripheral nerves
The neurologic manifestations of vitamin B12 deficiency in infants and children differ somewhat from those of adults. Most cases of vitamin B12 deficiency in infancy and childhood are due to inborn errors of metabolism involving the enzymes or binding proteins for vitamin B12 (Cooper and Rosenblatt, 1987). However, a syndrome of B12 deficiency in breast-fed infants of vegetarian mothers has been described which includes neurological features of apathy, developmental regression, and involuntary movements of the head, trunk, and limbs (Higginbottom et al., 1978). Neurological manifestations in the inborn errors of metabolism involving vitamin B12 include seizures, mental retardation and abnormal cerebellar and spinal cord function (Rosenberg, 1983). Inborn errors of cobalamin metabolism have recently been reviewed (Cooper and Rosenblatt, 1987). In general, it appears that the central nervous system is affected more frequently in vitamin B12-deficient infants and children than in adults. This suggests that the nervous system is much more susceptible to the damaging effects of vitamin B12 deficiency during the active period of development and myelogenesis. Furthermore, central nervous system damage in younger individuals does not have the same selective localization seen in adults but appears to result in a more diffuse involvement of the nervous system.

It is useful to consider what is known of the biochemical functions of vitamin B12 in eukaryotic cells. At least two enzyme reactions in mammalian cells are known to require cobalamin coenzymes. One of these, methionine synthase uses methylcobalamin (MeCbl) and also involves folate (Weisbach and Taylor, 1968). The other, methylmalonyl-CoA mutase which converts methylmalonate to succinate requires adenosylcobalamin (Ado-Cbl) and folate is not involved in this reaction (Smith and Monty, 1959; Stadtman et al., 1960). These reactions are shown in Table 3.

<table>
<thead>
<tr>
<th>Table 3. Cobalamin-Dependent Enzyme Reactions</th>
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<tr>
<td>1. Methionine Synthase</td>
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<tr>
<td>Me-Cbl</td>
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<tr>
<td>Homocysteine + methyl THFA ----&gt; methionine + THFA</td>
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<tr>
<td>2. Methylmalonyl CoA mutase</td>
</tr>
<tr>
<td>Ado-Cbl</td>
</tr>
<tr>
<td>Methylmalonyl-CoA &lt;-&gt; Succinyl-CoA</td>
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35
Cellular uptake of vitamin B12 takes place through receptor-mediated endocytosis of the plasma vitamin B12 binding protein transcobalamin II. Cobalamin is released from the TCII-Cbl complex and the TCII is broken down intracellularly (Youngdahl-Turner et al., 1979). Cbl is then either converted to Me-Cbl for participation in the methionine synthase reaction, which is cytosolic, or is transported into the mitochondria where it is reduced and adenosylated to Ado-Cbl for participation in the methylmalonyl-CoA mutase reaction (Rosenberg, 1983).

Progress in the understanding of the mechanism of neurologic damage induced by vitamin B12 deficiency was, for a long time, hampered by the lack of suitable experimental animal models. Although a number of attempts were made to render conventional small laboratory animals vitamin B12 deficient by dietary means, these failed because of the habit of coprophagia in small rodents. If they did succeed, they did not result in neurologic complications such as those encountered in vitamin B12-deficient humans (Agamanolis et al., 1983). However, a limited number of successful animal models have been described. These include nonhuman primates (Agamanolis et al., 1976; Kark et al., 1974; Oxnard and Smith, 1966; Siddons, 1974) and fruit eating bats (Green et al. 1975; Metz et al., 1987). In addition it has been demonstrated that the anesthetic gas nitrous oxide interferes with cobalamin metabolism (Amess et al., 1978; Banks et al., 1968) and chronic administration of this gas has been used successfully to chemically induce cobalamin deficiency in a number of species (Deacon et al., 1978; Reed et al., 1979). Additionally, several analogues of cobalamin (Siddons, et al., 1975) and the amino acid analogue cycloleucine (Jacobsen et al., 1973) have been used to induce cobalamin-deficient-like states in experimental animal models.

Concerning nonhuman primates, the induction of vitamin B12 deficiency and the development of neurologic sequelae require a period of 4-5 years (Kark et al., 1974). In contrast, nutritional deficiency of vitamin B12 can be induced in the fruit bat Rousettus aegyptiacus in 9-12 months and is associated with the development of neurologic but not hematological complications (Green et al., 1975; Metz and Van der Westhuizen, 1987). The neurological manifestations displayed by vitamin B12-deficient fruit bats include abnormalities in locomotion involving both flight and climbing (Green et al., 1975).

The precise mechanism whereby vitamin B12 deficiency results in neurological damage is not known. Several hypotheses have been put forward to explain the pathogenesis of vitamin B12-deficient neurological complications. These are listed in Table 4 and will be discussed.
Table 4. Pathogenesis of Vitamin B12-Deficient Neurological Complications

1. Methylmalonate hypothesis
2. Methylation hypothesis
3. Cyanide hypothesis
4. Analogue hypothesis

In the methylmalonate hypothesis, it is proposed that both methylmalonate and its precursor propionate, which accumulate in vitamin B12 deficiency, provide abnormal substrates for fatty acid synthesis. These are incorporated into myelin lipid (Frenkel, 1973; Vivacqua et al., 1966). In normal fatty acid synthesis, the anchor end of the fatty acid backbone is the two-carbon compound acetate. By additions of malonyl-CoA, a series of straight-chain, even-carbon fatty acids is produced. In vitamin B12 deficiency, the propionate (three-carbon) which accumulates can replace acetate. This gives rise to a series of odd-carbon fatty acids. In addition, methylmalonate can replace malonate as the chain elongator, giving rise to a series of branched-chain fatty acids. Evidence from several sources supports the methylmalonate hypothesis. Frenkel (1973) studied incorporation of 14C-propionate in cultures of peripheral nerves obtained from patients with pernicious anemia and demonstrated the occurrence of abnormal fatty acids as predicted by the hypothesis. We have measured methylmalonyl-CoA mutase levels in vitamin B12-deficient fruit bats and have found extremely low levels in brain, with undetectable levels in the spinal cord of severely deficient animals (Green et al., 1983). A compelling argument implicating the mutase reaction rather than the methionine synthase reaction in the neurological complications of cobalamin deficiency is the clinical observation that neurological complications are infrequent in clinical folate deficiency and that of the two known cobalamin-requiring enzyme reactions in man, methylmalonyl-CoA mutase is the one that does not require folate.

On the other hand, there is some evidence against the methylmalonate hypothesis. Urine and plasma methylmalonate levels show a poor correlation with the occurrence of neurological complications (Brozovic et al., 1967). More compellingly, neurological complications are not consistently seen in the inborn errors of metabolism involving biosynthesis of adenosyl-cobalamin and resulting in congenital methylmalonic-aciduria (Carmel and Goodman, 1982; Rosenberg, 1983). Moreover, neurological complications are indeed more frequently and consistently encountered in the inborn errors of metabolism involving the formation of methyl-Cbl (Cooper and Rosenblatt, 1987).
The methylation hypothesis implicates interference with the methionine synthase reaction as the cause of neurological complications in vitamin B12 deficiency. Interruption of this metabolic pathway results in a limiting supply of methionine and consequently of the important methyl-donor S-adenosyl methionine. This results in impairment of methylation reactions including the methylation of myelin (Dinn, et al., 1980; Scott and Weir, 1981). It has been further speculated that the clinical observation that administration of folate to vitamin B12-deficient patients results in aggravation of myelin damage occurs because folate stimulates cell division which consumes more of the available methionine (Scott et al., 1981). Again, there is experimental evidence to support the methylation hypothesis. It has been shown that dietary supplementation of methyl groups retards or prevents the neurological complications of cobalamin deficiency in vitamin B12-deficient fruit bats (Van der Westhuizen and Metz, 1984). Nitrous oxide, which primarily impairs the generation of methyl-Cbl and therefore the methionine synthase reaction, produces neurological complications both in humans (Layzer, 1978; Layzer et al., 1978) and in experimental animals (Reed et al., 1979). However, it should be pointed out that nitrous oxide ultimately results in interference with the methylmalonyl CoA mutase reaction as well (Chanarin, 1980). Further support for the methylation hypothesis comes from the observation that the valine analogue cycloleucine, which inhibits the conversion of methionine to S-adenosyl methionine, produces a myeloneuropathy in rodents which is similar to the neurological complications of vitamin B12 deficiency (Jacobsen et al., 1973). However, it is curious that rodents are resistant to the direct effects of vitamin B12 deficiency. Methylation of myelin basic protein is not affected in vitamin B12-deficient fruit bats (Deacon et al., 1986; McLoughlin and Cantrill, 1986).

The cyanide hypothesis is based on the fact that cyanide, which can arise either from dietary thiocyante or directly from inhalation of tobacco smoke, has an extremely high affinity for the upper axial ligand position of cobalt in the cobalamin molecule. Consequently, in the presence of cyanide, other forms of cobalamin are rapidly and irreversibly converted to cyanocobalamin, which is physiologically inert, unless it becomes reconverted through hydroxocobalamin to the coenzyme forms methyl-Cbl and adenosyl-Cbl. Support for this hypothesis came from the clinical observations of various toxic neuropathies, confusional states and ambylopia in West Africans and West Indians who consume diets rich in cyanogenetic glycosides such as cassava (Matthews and Wilson, 1971; Victor, 1970). However, this hypothesis has not received experimental support, and epidemiologic evidence seeking to explain the occurrence of neurological complications in patients with vitamin B12 deficiency as a function of thiocyante intake or smoking habits has not been convincing.

The cobalamin analogue hypothesis is based on the premise that the variable occurrence of myeloneuropathy in vitamin B12 deficiency may depend on the presence or absence of various
cobalamin analogues. It has been shown that cobalamin analogues are present in human and animal sera and tissues (Kolhouse et al., 1980; Kondo, et al., 1980). These analogues could theoretically inhibit the two cobalamin-requiring enzymes. There is some experimental evidence to support this hypothesis. Cobalamin analogues administered to vitamin B12-deficient baboons resulted in the formation of abnormal fatty acids (Siddons, et al. 1975). It was also suggested that the amounts of cobalamin analogue measured by a differential radioassay, which were higher in the plasma of vitamin B12-deficient patients with myeloneuropathy, would correlate with the occurrence of such complications (Kolhouse et al., 1980). On the other hand, we have not been able to demonstrate the presence of cobalamin analogues using a differential radioassay in the plasma of cobalamin-deficient fruit bats with myeloneuropathy (Green and Jacobsen, 1980), and others have failed to detect analogues by this method in the serum of nitrous oxide-treated bats (Van der Westhuyzen et al., 1982). Moreover, using a sensitive HPLC method to separate cobalamin congeners, we were unable to identify any abnormal cobalamins in organ extracts from vitamin B12-deficient fruit bats treated with nitrous oxide that had been previously given high specific activity radiolabeled cobalamin parenterally (Green et al., 1982).

Currently, none of the above hypotheses have been proven. Further experiments with available animal models need to be carried out. Even if the biochemical role of vitamin B12 in the nervous system is elucidated by the use of these model systems, this would not necessarily shed light on the reasons for the variable occurrence of nervous system complications in humans with vitamin B12 deficiency, or the mechanism whereby vitamin B12 deficiency results in an interference with more subtle and complex brain functions. In humans it is necessary to demonstrate a cause and effect relationship between vitamin B12 deficiency and cerebral complications. To prove such a relationship it must ultimately be shown that the cerebral manifestations are reversed, in whole or in part by administration of vitamin B12.

LITERATURE CITED


EVIDENCE FOR A ROLE OF FOLATE IN NORMAL BRAIN FUNCTION

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Folic acid, (pteroylglutamic acid, PteGlu), is a water-soluble molecule with a molecular weight of 441 and composed, as shown below (Pike and Brown, 1975), of three structural domains, a pteridine, p-aminobenzoic acid (PABA), and glutamic acid. Whereas some bacteria synthesize PteGlu from PABA, mammalian cells cannot, and require an exogenous nutritional source of the vitamin.

Structure of Folates:

\[
\text{Pteridinenucleus} \quad \text{Ptericoid} \quad \text{Glutamic acid} \quad \text{PteGlu (monopteroylglutamic acid)}
\]

\[
\text{NH}_2\text{C}_2\text{N}_2\text{N}_2\text{N}\text{CH}_2\text{NH}^\bullet\text{H}_2\text{COOH} \quad \text{OH} \quad \text{COOH} \quad \text{CH}_2
\]

\[
\text{NH}_2\text{C}_2\text{N}_2\text{N}_2\text{N}\text{CH}_2\text{NH}^\bullet\text{H}_2\text{COOH} \quad \text{OH} \quad \text{COOH} \quad \text{CH}_2
\]

PteGlu, per se, is the oxidized form of the vitamin, and in order to become biologically active, the 5, 6, 7, and 8 atoms of the pteridine ring must first be reduced to tetrahydrofolate (\(\text{H}_4\text{PteGlu}\)). This reduction is mediated by the enzyme, dihydrofolate reductase (EC 1.5.1.3). \(\text{H}_4\text{PteGlu}\) is the reduced pteridine from which all the biologically active folate cofactors are derived.

Another intracellular modification of the reduced folate is the enzymatic addition of glutamic acid residues to produce the polyglutamate forms of this cofactor. Polyglutamation of folate serves to retain folate in the cell and to increase its affinity as a cofactor for some enzymatic pathways. Actually, the nutritional sources of folate (meats and vegetables) contain folate in this reduced state since food is, in fact, intracellular folate (Cossins, 1984).
The biological function of folate is to provide carbon atoms for a variety of biosynthetic and metabolic pathways. These include the biosynthesis of purines; a cofactor for a number of transmethylation, i.e., methylation of deoxyuridine monophosphate (dUMP) to form deoxythymidylate monophosphate (dTMP), methylation of homocysteine to form methionine; and a carrier of a one-carbon unit for the interconversion of some amino acids. These include the serine-glycine pathway and the conversion of histidine to glutamic acid. The carbon units are carried on the reduced folate molecule in the following oxidation states:

<table>
<thead>
<tr>
<th>Methanol:</th>
<th>N5-methyl(5-CH$_3$): methylation of homocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde:</td>
<td>N5N10-methylene(5,10-CH$_2$-): methylation of dUMP</td>
</tr>
<tr>
<td>Formate:</td>
<td>N5N10-methenyl(5,10-CH$_2$-): biosynthesis of purines</td>
</tr>
<tr>
<td>Formate:</td>
<td>N10-formal(10-CHO-): biosynthesis of purines and for protein synthesis.</td>
</tr>
<tr>
<td>Formate:</td>
<td>N5-formimino(5 CH=NH-): metabolism of histidine</td>
</tr>
</tbody>
</table>

Other biochemical pathways are also necessary for the biosynthesis and interconversion of these folate cofactors. One source of 5,10-CH$_2$-H$_4$PteGlu is by the transfer of the carbon unit from serine (serine transhydroxymethyltransferase) as it is converted to glycine (Schirch, 1984). 5,10-CH$_2$-H$_4$PteGlu is reduced to 5-CH$_3$-H$_4$PteGlu by the flavin enzyme, 5,10-CH$_2$-H$_4$PteGlu reductase with NADPH as cofactor (Matthews, 1984).

Eucaryotic cells contain a multifunctional enzyme which interconverts 10-CHO-H$_4$PteGlu, 5,10-CH-H$_4$PteGlu, and 5,10-CH$_2$-H$_4$PteGlu (Villar et al., 1985). The source of the carbon unit for this exchangeable pool is the serine to glycine conversion.

The enzyme, dihydrofolate reductase (DHFR), plays a critical role in the intracellular metabolism of folate because it reduces dihydrofolate (H$_2$PteGlu) to H$_4$PteGlu. H$_2$PteGlu is generated from only one biochemical step in mammalian cells, the transfer of the methyl group from 5,10-CH$_2$-H$_4$PteGlu to dUMP to form dTMP with oxidation of the pteridine moiety. Thus, replicating cells accumulate H$_2$PteGlu which is rapidly reduced to H$_4$PteGlu by DHFR. Normally H$_2$PteGlu does not accumulate unless DHFR is lacking or inhibited by folate antagonists (e.g., methotrexate). Inactivation of DHFR will lead to a depletion of H$_4$PteGlu and this in turn leads to depletion of the other folate cofactors which are derived from this parent molecule (Blakley, 1984).
DHFR can also catalyze the reduction of 7,8-dihydrobiopterin to tetrahydrobiopterin (Kaufman, 1967). Normally, this is not likely to be an important pathway for pterin metabolism in vivo because the cofactor function of tetrahydrobiopterin in hydroxylase reactions results in the formation of quinonoid 7,8-dihydrobiopterin for which there is normally sufficient dihydropteridine reductase (and NADH) for its reduction to tetrahydrobiopterin. Only the nonquinonoid isomerization product, 7,8-dihydrobiopterin is the substrate for DHFR. In the absence of dihydropteridine reductase the brain contains too little DHFR to maintain a sufficient pool of tetrahydrobiopterin.

In addition to these enzymes which are required for the utilization and interconversion of folate cofactors, there are now a number of folate binding proteins (FBP) which appear to play a role in folate metabolism. Mammalian cells and biological fluids contain both hydrophobic membrane-associated and soluble hydrophilic FBPs (Wagner, 1984). In brain, the membrane-associated protein has been identified in the choroid plexus (Spector, 1977) and it is likely, although not unambiguously established, that this membrane FBP functions in the transport of folate into the cerebrospinal fluid (CSF) (Chen and Wagner, 1975; Spector and Lorenzo, 1975). This role for the FBP in the choroid plexus provides the mechanism by which $5\text{-CH}_3\text{-H}_4\text{PteGlu}$ accumulates in the CSF to a concentration three to four fold greater than plasma.

The evidence for the role of folate in brain metabolism is derived from biochemical requirements and clinical observation. The biochemical evidence stems from a number of established facts:

1) Neurons synthesize complex proteins (structural and cytosolic) which require messenger ribonucleic acid (mRNA) and transfer RNA (tRNA) and the formyl derivatives of folate are required for purine synthesis and translation of mRNA.

2) A number of folate-dependent biochemical pathways have been identified in brain tissue. Homocysteine-methionine methyl transferase (methionine synthetase) (Spector et al., 1980) is an essential pathway and the cofactor for this reaction is cobalamin (as methyl cobalamin). This pathway is important for two reasons. First, it is required for supplementing the dietary source of methionine which is enzymatically converted to S-adenosylmethionine (SAM), the carrier of methyl
groups for a variety of transmethylations. Second, the methylation of homocysteine generates tetrahydrofolate from $5\text{-CH}_3\text{-H}_4\text{PteGlu}$ and this maintains the important pool of the other reduced folate cofactors.

Other enzymes required for folate cofactor metabolism have also been found in brain tissue (McClain, 1979). These include serine transhydroxymethylase, methylene dehydrogenase which generates the methenyl derivative from $5,10\text{-CH}_2\text{-H}_4\text{PteGlu}$, and methylene reductase which reduces $5,10\text{-CH}_2\text{-H}_4\text{PteGlu}$ to $5\text{-CH}_3\text{-H}_4\text{PteGlu}$.

DHFR, though once thought to be absent from mature brain tissue, is present but at only 10% or less of that contained in liver (Spector et al., 1977). Fetal and growing brain understandably contain one more DHFR than mature brain because there is rapid cellular replication which requires the synthesis of dTMP. $5,10\text{-CH}_2\text{-H}_4\text{PteGlu}$ provides the methyl group and is oxidized to $\text{H}_4\text{PteGlu}$ which must be reduced to $\text{H}_4\text{PteGlu}$ to maintain the pool of reduced folates. The role of DHFR in adult brain is less apparent since there is little replication of mature neurons. However, unlike tissues in which $5\text{-CH}_3\text{-H}_4\text{PteGlu}$ is the predominant intracellular folate, $\text{H}_4\text{PteGlu}$ is the major folate in brain tissue (Brody et al., 1976). Since $\text{H}_4\text{PteGlu}$ is a labile folate, non-enzymatic oxidation may occur generating some $\text{H}_2\text{PteGlu}$ which would then require DHFR for reduction. DHFR may also function to reduce 7,8-dihydrobiopterin, but the concentration of this pterin isomer in normal brain is negligible.

A recent observation in our laboratory has been the identification by radioimmunoassay (Iqbal et al., 1986) and Western blotting of a high molecular protein in mouse brain which cross-reacts with DHFR against antiserum raised to the purified enzyme. The precise structure of this protein and its relationship to DHFR are presently under active investigation.

Folate probably does not have a direct cofactor function in neurotransmitter metabolism as once reported for $5\text{-CH}_3\text{-H}_4\text{PteGlu}$ (Laduron, 1972). In vitro, this folate cofactor may indirectly provide a carbon unit, as formaldehyde, after oxidation to $5,10\text{-CH}_2\text{-H}_4\text{PteGlu}$ in the presence of an electron acceptor and methylene tetrahydrofolate reductase (Stebbins et al., 1976). $5,10\text{-CH}_2\text{-H}_4\text{PteGlu}$ then spontaneously dissociates into formaldehyde and $\text{H}_4\text{PteGlu}$. The formaldehyde can then nonenzymatically methylate biogenic amines (Hsu and Mandell, 1975). Though theoretically possible, it is dubious whether this reversal of direction of methylene tetrahydrofolate reductase occurs normally in cellular
folate metabolism. However, with deficiency of cobalamin, 5-CH$_3$-H$_4$PteGlu may accumulate (i.e., "methyl trap") and possibly change the kinetics of this pathway especially because SAM, an allosteric inhibitor of this enzyme, is low when there is diminished methylation of homocysteine. In this regard, we reported several years ago that the urine of some patients with pernicious anemia contained a folate which could be reduced to 5-CH$_2$-H$_4$PteGlu by borohydride (Rothenberg et al., 1978). This is a property of 5,10-CH$_2$-H$_2$PteGlu and it was not observed with a urine sample from a folate-deficient patient.

The clinical evidence for a role of folate in brain metabolism is substantial. First, congenital methylene tetrahydrofolate reductase deficiency and congenital malabsorption of folate are accompanied by functional and structural disorders of the brain (Erbe, 1975). In the former disorder, there is deficient generation of 5-CH$_3$-H$_4$PteGlu from 5,10-CH$_2$-H$_4$PteGlu and this results in a low methionine pool with deficiency of SAM. In addition, there may be impaired transport of folate into the brain in this disorder because CSF folate is low. A folate-responsive schizophrenia has also been reported in an adolescent with this defect (Freeman et al., 1975).

The congenital disorder of impaired intestinal absorption of folate is accompanied by low CSF folate indicating that the choroid plexus shares in this defect of folate transport (Freeman et al., 1975). These patients also had mental retardation, seizures and ataxia. The status of the membrane-associated folate-binding protein is unknown in this disorder.

Congenital deficiency of formimino transferase, the enzyme which catalyzes the transfer of the formimino group of formiminoglutamic acid to H$_4$PteGlu, has also been reported to be associated with mental retardation and structural brain abnormalities in some patients (Arakawa et al., 1963) but not in others (Niederwieser et al., 1974).

Another clinical observation indicating a role for folate in brain metabolism is the observation that chronic intrathecal administration of methotrexate to children for prophylaxis against cranial leukemia may have profound neurotoxicity which may be due to secondary folate deficiency (Kamen, 1986).

Other clinical observations which appear to support a role for folate in normal neuronal metabolism have been the association of an organic brain syndrome in the elderly with folate deficiency, rarely with demyelinating disorders, and
sometimes with polyneuropathies. However, though there is an inconsistency in the precise pathway perturbed, some patients have responded to folate replacement (Carney and Sheffield, 1970). The literature is replete with evidence based on case reports for a relationship of impaired neural function with folate deficiency, but most students of this subject can only speculate on the precise biochemical perturbation which is responsible for these disturbances in these patients.

A congenital defect in the methylation of homocysteine has been reported with the primary deficiency of the enzyme homocysteine-methionine methyltransferase (methionine synthetase) (Arakawa et al., 1967). The same biochemical perturbation occurs with impaired synthesis of methylcobalamin (Mudd et al., 1970). The consequence of these disorders would be diminished methionine associated with homocystinemia and homocystinuria, "trapping" of $5\text{-CH}_3\text{-H}_4\text{PteGlu}$ and deficiency of $\text{H}_4\text{PteGlu}$. In these infants mental retardation and seizures indicate impaired cerebral development and function.

Studies from this laboratory in collaboration with Allen and coworkers (Allen et al., 1983) revealed a defect in CSF folate transport in a young woman with the Kearns-Sayre syndrome who had progressive central nervous system deterioration, bilateral cerebral calcification and reduced muscle carnitine. A similar case without carnitine deficiency has been reported by Dougados, et al. (1980) so that folate deficiency in the brain may be an important contributing factor in this disorder.

Dr. Ian Butler and I were unable to find a quantitative abnormality of norepinephrine, dopamine, or 5-hydroxy-tryptamine in brain, or their respective metabolites, 3-methoxy-4-hydroxy-phenylethylene glycol, homovanillic acid, and 5-hydroxyindoleacetic acid in brain and CSF in rats after 10 weeks on a folate-deficient diet (Butler and Rothenberg, 1987). However, a similar study in rats both deficient or after excess folic acid, demonstrated lower levels of 5-hydroxy-tryptamine than control rats (Bachevalier and Botez, 1979). In man, there has been an association between folate deficiency, neuropsychological behavior, and neurotransmitters (Botez et al., 1979). In some patients with vitamin B12 deficiency, and in folate-deficient patients with neuropsychiatric symptoms which improved with folate therapy, there was low CSF 5-hydroxyindoleacetic acid (Botez et al., 1982).
The future holds substantial promise for defining the precise biochemical defect which impairs cerebral function in folate deficiency because of new and more sensitive methods in analytical biochemistry and molecular biology. My own laboratory is now focusing in two areas: 1) to define the significance of the high molecular protein(s) in the brain which has immunologic homology to DHFR; and 2) to determine if there is a decrease in the expression of the folate-binding protein in the choroid plexus and neuronal cells in aging. Toward this end we have cloned the complementary DNA of a human folate binding protein (Sadasivan and Rothenberg, 1988) which should provide the means to study the expression of this gene.

LITERATURE CITED


EXPERIMENTAL APPROACHES TO MEASURE AGE-RELATED CHANGES IN METABOLISM OF NEUROTRANSMITTERS

Working Group 1

Discussion Leader: George Roth, Ph.D.
Rapporteur: David Morgan, Ph.D.

This Working Group identified two key problems that impeded discussion of this topic: first, difficulty in identifying a neurochemical connection between deficiencies of vitamin B12 and folate and neurologic dysfunction; and, second, uncertainty about incidence and prevalence of deficiencies of vitamin B12 and folate in the elderly. In attempting to identify neurochemical mechanisms that might be influenced by deficiencies of vitamin B12 and folate, the Working Group discussed such topics as conversion of phosphatidylethanolamine to phosphatidylcholine, changes in lysine methylation and DNA methylation, synthesis of epinephrine and acetylcholine and the effect of S-adenosylmethionine. Possible changes in membrane fluidity with aging could also be of interest, in particular, increases in rigidity of central nervous system (CNS) cell membranes. Differences in odd- and even-number fatty acids might influence membrane fluidity. In the opinion of the Working Group, folic acid and vitamin B12 are probably not key cofactors in the metabolism and synthesis of dopamine, norepinephrine, tryptophan, epinephrine, and possibly acetylcholine with respect to synthesis from choline.

With regard to possible deficiencies of vitamin B12 and folate in the elderly, the Working Group concluded that population studies do not provide convincing evidence to document deficiencies in a substantially higher proportion of the elderly compared with younger people. However, based on evidence of changes in sensitivity to such agents as drugs, neurotransmitters, hormones, and vitamins, the Working Group noted the possibility that even a marginal deficiency could have greater effects in elderly individuals than in younger persons. Finally, it was suggested that other B vitamins such as thiamin and pyridoxine might have more direct effects on neurotransmitter metabolism and synthesis than folate and vitamin B12.

NEEDED INFORMATION AND SUGGESTED APPROACHES

Reliable data are needed on the prevalence of deficiencies of vitamin B12 and folate in the elderly. Suggested approaches for obtaining these data are presented in the report of Working Group 3.
Basic data are needed on possible neurochemical mechanisms whereby deficiency of vitamin B12 and/or folate could impair neurologic function. Suggested approaches included the following:

- A clinical trial of vitamin B12 and folate supplementation in dementia patients should be considered (note: this was not a unanimous suggestion of all members of the Working Group).

- The effects of vitamin B12 and folate deficiencies in cultures of neurologic cells should be studied in vitro (see synopsis of Dr. de Vellis' presentation).

- The methods for depleting vitamin B12 and folate in animal models by means of vitamin antagonists should be explored.

- The use of such scanning techniques as positron emission tomography (PET) and nuclear magnetic resonance (NMR) imaging should be investigated to establish, initially at a very descriptive level, the brain regions that show dysfunction in the presence of vitamin B12 and/or folate depletion.

- The use of animal models with relatively short lifespans, such as rodents, should be explored in investigations involving aging. The use of wire-bottom cages to prevent coprophagia and the depletion of vitamin concentrations via vitamin antagonists are possible approaches.

- Additional data are needed on the effects of aging on fluidity of membranes of central nervous system cells. Such data may yield insights on possible links between vitamin B12 and folate deficiencies and neurologic dysfunctions. Of associated interest is the possibility that differences in even- and odd-number fatty acids might affect membrane fluidity.
EXPERIMENTAL APPROACHES FOR THE STUDY OF NEUROLOGICAL CHANGES

Working Group 2

Discussion Leader: Paul Coleman, Ph.D.
Rapporteur: Andrew Monjan, Ph.D.

It was not clear to this Working Group that vitamin B12 and/or folate deficiencies in the elderly, short of pernicious anemia, represent a problem deserving high-priority attention. Members of the Working Group were not aware of data to demonstrate a need for high-priority attention to this subject. However, regardless of the public health significance in the elderly, there are large gaps in basic knowledge of the effects of folate and vitamin B12 on the nervous system. For example, folate is known to be vital for the developing CNS, but its role in the function of the aged CNS is unknown.

Neurologic and neuropsychiatric evaluation of persons with high methylmalonic acid (MMA) concentrations may indicate whether elevated MMA is associated with increased risk of neurologic and neuropsychiatric deficits and may define pathologies associated with deficiencies of vitamin B12 and folate.

There appears to be very little work going on in which modern techniques of neuroscience are used to study the effects of vitamin B12 and/or folate deficiency on the aging nervous system.

NEEDED INFORMATION AND SUGGESTED APPROACHES

- Double-blind clinical trials are needed to establish cognitive deficits in the elderly before and after treatment with supplemental vitamin B12 and folate.

- When pernicious anemia is present, vitamin B12 deficiency leads to a variety of disturbances of the nervous system including peripheral neuropathies, cerebrospinal tract involvement, and movement disturbances. These deficits could be the starting points for investigating possible CNS involvement in cases of vitamin B12 deficiency in the absence of anemia. Associations of vitamin B12 with affective disorders and dementias prior to pernicious anemia are less well established.

- Modern techniques of neuroscience that should be considered in studies of vitamin B12 and/or folate relationships with neurological changes include quantitative morphology, immunohistochemistry, evoked response kindling and long-term potentiation, biochemistry of transmitters, structural and metabolic systems, and molecular biochemical studies. For
example, studies which define chemical classes of neurons to determine whether there is a chemically defined class that is more susceptible to deficiencies of vitamin B12 or folate might offer new insights.

- There is a clinical impression that the older patient with pernicious anemia is more vulnerable to dementia than the younger patient. This impression needs to be critically evaluated.

- There appears to be no work on vitamin B12 or folate deficiency in aged animals. Differences in responses to vitamin B12 and folate deficiencies and the associated mechanisms need to be clearly defined among animal models.

**Cell and Tissue Culture Techniques**

- Varying the levels of vitamin B12 and/or folate in the media of cell and tissue cultures could be a means of investigating a number of possible effects of these nutrients on neurological function. An example is evaluating responses of different types of nervous system cells at different developmental stages to determine possible cell-specific or cell stage-specific action of vitamin B12 and/or folate. During the processes of neurogenesis, biogenesis, or phenotypic expression, are there some stages that are more particularly sensitive to requirements for vitamins? Isolated cells and micro-explants may be used to study the physiology of neurons and the glia as it may be affected by manipulation of vitamin B12 and folate levels. Are possible effects mediated via the glia or are there direct effects on neurons? The in vitro systems also offer the possibility of studying renewal of molecules within cells or renewal of cell populations, which is relevant to the problem of aging.

- One question that arose was whether vitamin B12 or folate deficiency may render the organism less able to cope with stress. Are remyelination, recovery from stroke, or repair of other neurological damage retarded in the deficient organism? Does vitamin B12 or folate deficiency place the aged organism at greater risk for decreased function? Will such deficiency accelerate biomarkers of aging?

**Animal Models**

- Rats and small rodents may be too short-lived to show the neurologic deficits seen in longer-lived species. Moreover, rats may be too behaviorally adaptive to
show the subtle changes potentially present in vitamin B12 or folate deficiency. Although rats do not develop pernicious anemia in vitamin B12 deficiency, they may show bone marrow changes similar to those seen in human subjects. Folate deficient rats also show increased susceptibility to audiogenic seizures. Does the available information indicate that the rodent may be a model of the effects of vitamin B12 or folate deficiency that are not dependent on pernicious anemia and that may differ from effects in the fruit bat and the subhuman primate? The Working Group suggested that it would be advisable to establish a human neurological syndrome of vitamin B12 and/or folate deficiency before attempting to develop an improved animal model.

If it appears worthwhile to study the effects of vitamin B12 or folate deficiency on the aging nervous system, regions of the nervous system that merit investigation should be determined. Suggestions of the Working Group included taking clues from behavioral/clinical signs and symptoms, 14C 2-deoxyglucose localizing studies in animal models, and perhaps PET scan data.
APPROACHES TO DETERMINE THE FOLATE AND VITAMIN B12 STATUS OF THE ELDERLY POPULATION

Working Group 3

Discussion Leader: Lynn Bailey, Ph.D.
Rapporteur: Susan M. Pilch, Ph.D.

The Working Group reviewed the strengths and shortcomings of available methods of assessing vitamin B12 and folate nutriture and prepared suggestions for research and development to improve and/or validate techniques and their interpretation. Key areas of uncertainty include: (1) the normal ranges of serum vitamin B12 concentration and closer definition of cutoff values; (2) the extent and significance of vitamin B12 malabsorption in the elderly and better identification of causative factors aside from pernicious anemia; (3) the meaning of red cell folate concentrations as a measure of tissue levels; and, (4) the neurological significance of subclinical vitamin B12/folate deficiency in the absence of overt disease.

This Working Group considered use of clinical and population studies as approaches to investigate questions concerning folate and vitamin B12 status. The Working Group recognized a need for funding of large-scale, collaborative studies in which batteries of laboratory tests would be employed to refine available data on vitamin B12 and folate nutriture in the elderly.

NEEDED INFORMATION AND SUGGESTED APPROACHES

Vitamin B12 Status

- The significance of subclinical deficiency (low serum B12 or elevated metabolites in the absence of overt disease) needs to be determined. What are the functional consequences of such "deficiency"?

- Further study of the usefulness of measuring adenosylcobalamin in serum is needed. Serum methylcobalamin is the more variable component in serum. Adenosylcobalamin is the primary intracellular form and its level in serum may be more closely related to the other clinical indicators of deficiency than the total vitamin B12. Further development and standardization of the methodology for its measurement are needed to make the assay convenient because the compound currently measured is photolabile.

- Further examination of the correlation among the various tests (i.e., serum vitamin B12 and MMA, dU suppression, etc.) is needed. The correlations within different population groups require study (i.e., free-living, ambulatory elderly; persons in nursing homes; persons about to enter nursing homes).
More research is needed on absorption of cobalamin from foods in the elderly because conditions such as achlorhydria may influence nutrient absorption.

The value of measuring holotranscobalamin II levels as an indicator of vitamin B12 deficiency should be determined.

Research involving therapeutic trials of vitamin B12 in population groups with suspected B12 inadequacy should be conducted.

Effects of aging on the parameters measured to assess vitamin B12 status should be examined.

Recommendations for clinical assessment of vitamin B12 status

- Serum vitamin B12 is the most sensitive technique but it is most useful as a screening tool. In the clinical setting, it is preferable to cast a wide net to ensure that all patients with disease will be identified, even at the risk of identifying false positives. The current normal range for serum vitamin B12 concentration is stated to be 200-900 ng/ml, but the normal range needs to be defined because patients with values between 200 and 300 ng/ml are often found, on further tests, to be deficient. When liberal cutoffs are used the number of false low values may be high, but this is preferable to overlooking individuals who are deficient. This initial screen needs to be followed up with additional tests.

- Both serum and urinary MMA are elevated in vitamin B12 deficiency. Recent gas chromatography/mass spectroscopy (GC/MS) methodology allows detection of MMA in both serum and spot urine samples. The MMA in the urine is considerably more concentrated than in plasma and can be expressed on a per mg creatinine basis. Therefore, a 24-hr urine collection is not necessary. The urinary MMA is considered to have a high sensitivity and specificity and to be a useful test for identifying vitamin B12 deficiency when values rise above 5 µg/mg creatinine. It is recognized that the glomerular filtration rate is reduced in the elderly and that urinary MMA is elevated in cases of renal insufficiency; however, this is not considered to be a factor that would interfere with the assessment of MMA.
• Serum homocysteine is a stable and easily measured compound that is elevated in both vitamin B12 and folate deficiencies. It is considered a good indicator used in conjunction with serum MMA, in that most vitamin B12-responsive patients have been found to have abnormal values for at least one of these measures.

• The intrinsic factor antibodies test is often used clinically to diagnose the presence of pernicious anemia prior to a Schilling test.

• The Schilling test is used not so much to identify vitamin B12 deficiency as to characterize the reason for deficiency. A normal Schilling test does not rule out deficiency of the vitamin. If the Schilling test is positive when the serum B12 concentration is borderline or equivocal, then the test may be considered diagnostic for vitamin B12 deficiency. It should be noted that there are differences between the test as originally designed, in terms of the cobalt isotope and the amount of cold cobalamin used, and the test as it is conducted now. Standardization of the method is required.

• Vitamin B12 absorption from foods represents another test to determine the reason for B12 deficiency. Absorption tests would be desirable in the elderly because they have a higher incidence of malabsorption syndromes than the younger population; however, they are difficult to perform.

• The deoxyuridine (dU) suppression test, which can distinguish between vitamin B12 and folate deficiencies, is considered to be a very sensitive indicator of B12 deficiency. In many cases, it indicates B12 deficiency before changes occur in other parameters. The method has been developed for whole blood samples in addition to bone marrow samples, but it is quite costly. It may be appropriate in a clinical research setting.

• Examination of mean cell volume (MCV) may result in erroneous conclusions in diagnosing vitamin B12 deficiency. MCV increases with age, and factors other than vitamin B12 deficiency may cause increased MCV in the elderly.

• Holotranscobalamin II (a measure of physiological transport) decreases in deficiency in parallel with serum vitamin B12.
• Measurement of vitamin B12 concentrations in cerebrospinal fluid needs to be explored.

• Dietary assessment represents a means of evaluating vitamin B12 intake. Food analyses for B12 content were considered adequate by members of the Working Group. If food consumption data are collected, a minimum of three days of data collection is suggested. It should be recognized that problems may exist with dietary recall in the elderly.

• The final criterion to establish vitamin B12 deficiency is a positive response to a therapeutic trial of vitamin B12 administration.

Recommendations for Assessment of Vitamin B12 Status in Population Surveys

• The serum levels of vitamin B12 should be measured. It is a sensitive, convenient, and inexpensive assay. The measure is useful as a screening method and for population studies.

• Spot urine samples would be easy to collect in population studies for measurement of serum and urinary MMA. Large numbers of serum samples can now be measured with the new GC/MS methodology.

• Elevations in serum MMA and/or serum homocysteine have been found to occur in most vitamin B12-responsive subjects. Serum homocysteine is now measured clinically and might be used in population studies in conjunction with other measures.

Folate Status

• The significance of subclinical deficiency should be determined.

• More research is needed on methodology and criteria. For example, standardization of serum and RBC folate methods and definition of age-specific significance of low values are needed.

• Cooperative studies with several labs performing various tests are needed to determine correlations among various parameters. Emphasis on collaborative studies is needed to enable all assessment tools to be examined in one study.
• The potential of folate metabolites (including intermediates in purine synthesis such as GAR and ACAR) to assess folate status should be investigated.

• The determination of hypersegmentation should be better defined and standardized; its relationship to other indicators of folate status should be examined and an automated method for assessment of hypersegmentation should be developed.

• A more sensitive assay for formiminoglutamic acid (FIGLU) which would eliminate the necessity for a loading dose of histidine is needed. A high pressure liquid chromatography method might be developed.

• The relationship of white blood cell size (automated method) and folate status should be investigated.

• Possibilities for a more practical dU suppression test should be studied.

• Folate status should be studied more comprehensively in elderly population groups at risk of deficiency (such as those with low socioeconomic status) who have been reported to have low serum folate and/or RBC values.

• Additional followup of persons identified as possibly deficient by initial screens should be pursued. Such followup studies may permit clarification of the criteria for deficiency.

Recommendations for clinical assessment of folate status

• Although it is a less specific measure than serum vitamin B12, serum folate reflects recent folate intake and can be used as a screening tool. Samples must be collected from fasting patients. Values may be affected by alcohol consumption and smoking. Patients with levels above an appropriate cutoff need not be examined further for hematological disease resulting from folate deficiency; however, cutoffs established for hematological disease may not be appropriate for neurological disease. An effect of aging on cellular uptake of folate has been suggested by studies showing higher serum folate values and impaired uptake of supplemental folate by elderly persons. Each lab must develop its own criteria for adequacy, as experience suggests that no single "normal" range is applicable to all methods in all labs. Quality control and quality assurance must be maintained.
• Classically, RBC folate concentration has been thought to reflect tissue stores and to decline with declining folate concentrations in the liver; however, this conclusion is based on little experimental data. RBC concentration reflects long-term status in contrast to serum and, therefore, would not be influenced by fasting. The sensitivity and specificity of the assay may be problematic. Difficulties in interpretation of results are introduced when RBC values are determined on whole blood hemolysates with correction for hematocrit applied afterwards, rather than standardizing the sample to a given hematocrit before preparation of the hemolysate.

• Neutrophil hypersegmentation is the single hematological variable of choice (better than elevated MCV) for detecting folate deficiency. However, it does not distinguish between folate and vitamin B12 deficiency and hypersegmentation may be caused by other conditions (i.e., myelodysplastic syndrome). The definition of hypersegmentation, the number of segments required, and the criteria for the percent of cells affected differ from individual to individual.

• The dU suppression test is the most sensitive and specific test for folate deficiency. The pattern of response can differentiate between folate and B12 deficiency. Use of bone marrow samples for the test may limit the use of this test outside a clinical research setting. A method has been developed for use with whole blood samples; however, it is expensive.

• The formiminoglutamic acid excretion test (FIGLU) is useful but cumbersome. Methods are needed that would eliminate the requirement for histidine loading and collection of a 24-hour urine sample. Newer methods are being standardized following a histidine loading dose, including use of an enzymatic assay for FIGLU, divided doses of histidine, and 24-hr urine collection. However, the distinction between vitamin B12 and folate deficiencies is not clear.

• Measures of MMA and serum homocysteine levels can be used to distinguish between folate and vitamin B12 deficiencies. (See discussion of MMA above.) Serum homocysteine levels are elevated in folate deficiency. Both metabolites are stable.
The final criterion to establish folate deficiency is a positive response to a therapeutic trial of folate administration.

Recommendations for assessment of folate status in population surveys

- Serum folate
- RBC folate
- Hypersegmentation
- Homocysteine elevation (may be useful)
- Therapeutic trial

Vitamin B12 and Folate Status

- Multiple measures of both vitamin B12 and folate concentrations in a well controlled, collaborative study are needed to determine relationships among measurements of status (serum and RBC folate, serum B12, MMA, homocysteine, etc.). Studies with elderly populations should be a special focus.

- The utility of measures of holotranscobalamin II and methyl-, hydroxyl-, and adenosylcobalamin as assessment tools should be examined. Is there a shift in the methyl:adenosylcobalamin ratio in deficiency?

- Additional information on folate content of foods and bioavailability is needed.

- A battery of neuropsychiatric tests should be developed to relate to serum folate and vitamin B12 concentrations and other status assessments and to assess response to intervention. These include the following:
  - global assessments of memory function and motor control;
  - tactile thresholds and temperature sensitivity;
  - simple neurological tests or questionnaires that can be administered in the home; and,
  - reports of relatives.

- Vitamin B12 and folate levels in the central nervous system in control subjects and dementia patients in various age groups should be quantified. The various one-carbon forms of folate (including the number of glutamate residues) and folate metabolites should be measured in the CNS and periphery.

- The use of MMA as a population assessment tool should be investigated. Other clinical conditions that may affect MMA need to be determined.
• Factors that decrease vitamin B12 absorption with age and folate absorption in a large number of elderly subjects should be studied.

• Changes in dU suppression with aging need to be investigated.

• Cell culture techniques to examine dU suppression in glial cells should be developed.

• Effects of drug interactions on folate status and cellular folate uptake need to be evaluated. Are drug effects more detrimental in the aged?

• The significance of "subclinical deficiencies" of both vitamin B12 and folate remains to be determined. Are cutoffs derived on the basis of hematological disease appropriate for neurological disease? In therapeutic trials, what is the appropriate dosage and length of time for treatment?
APPROACHES TO DETERMINE RELATIONSHIPS OF VITAMIN B12 AND FOLATE NUTRITURE TO NEUROTRANSMITTER METABOLISM

Working Group 4

Discussion Leader: Ian Butler, FRCP
Rapporteur: Kenneth D. Fisher, Ph.D.

The outline below includes the topics, research needs, approaches, and methods considered by the Working Group. More detailed comments of the Working Group follow the outline.

NEEDED INFORMATION AND SUGGESTED APPROACHES

I. Key Questions for Consideration

A. Evidence for vitamin B12 and/or folate effects on nervous system?
   1. vitamin B12 - strong evidence but mechanism unknown
   2. folate - less compelling evidence and mechanism unknown

B. Time course of neurologic deficits and nutrient deficiency
   1. folate - weeks
      vitamin B12 - months
   2. repletion effects

C. Multiple impacts
   1. nutrient deficiencies (e.g., pyridoxine, thiamin, carnitine?)
   2. indirect or additive effects of drugs (e.g., phenytoin, alcohol)
   3. polygenic effects (e.g., neurotransmitter systems, DNA transcription)

II. Research Approaches

A. Clinical
   1. Study Populations
      a. normal subjects - effects of aging
      b. aged population
      c. patients with defined inborn errors of metabolism
      d. patients with psychiatric disorders
      e. patients with hematologic disorders
2. Study Methods
   
a. development of standardized methodology
b. specific tissues of interest (e.g., serum, RBC, CSF, cultured fibroblasts, etc.)
c. neuroradiology (e.g., CAT, MRI)
d. electrophysiology (e.g., EEG, brain evoked responses, nerve conduction studies)
e. neuropsychology (e.g., before and after treatment)
f. future techniques (e.g., MR-Spectroscopy, PET, DNA probes)

B. Nonclinical

1. Human Models
   
a. inborn errors of metabolism
b. defined populations (e.g., hematologic, psychiatric, neurologic, pediatric, gerontologic, nutritional criteria)

2. Animal Models
   
a. fruit bat
b. monkey

3. Tissue Cultures
   
a. cell type, (e.g., neurons, fibroblasts, astrocytes)
b. function studied, (e.g., nutrient effects, biogenic amine synthesis)
Several types of human populations should be studied including normal elderly subjects, for whom the Working Group recognized a lack of sufficient data on vitamin B12 and folate nutrure. When possible, concentrations of these nutrients should be measured in other body compartments (for example, cerebrospinal fluid) in addition to the genitourinary and hematopoietic systems.

Responses of patients with defined inborn errors of metabolism involving vitamin B12 or folate or their metabolites tend to be amplified, offering opportunities to refine techniques of investigation and therapy. Other useful population subgroups for study might include psychiatric patients with depression or other putative neuropsychological manifestations that may be related to vitamin B12 and folate deficiency. In addition, patients with certain hematologic disorders should be considered as a subgroup for study, for example, those with the hematologic stigmata of pernicious anemia such as macrocytic anemia, thrombocytopenia, and leukopenia with hypersegmented neutrophils. With regard to study methods, the Working Group suggested that more of the research-related methods currently in use or emerging in some laboratories be used in future investigations.

The selection of body fluids or tissues for measurement is clearly important. Serum folate concentrations, for instance, probably are limited in terms of estimating folate nutrure, and their correlations with tissue levels of folate, including CNS tissues, have not been elaborated. Cerebrospinal fluid may be the most feasible body fluid for estimating CNS levels of B12 and folate. Cultures of CNS cells and tissue explants offer valuable approaches for in vitro studies.

Computerized axial tomographic (CAT) scanning is a technique widely used by neuroradiologists and nuclear magnetic resonance (NMR) scanning is a superior technique for examining CNS tissues. NMR may be particularly appropriate for studying disturbances of vitamin B12 metabolism in white matter. This is one example of the importance of acquiring more normative data in elderly subjects for comparative purposes.

With respect to electrophysiological study methods, the Working Group emphasized the importance of electroencephalography, brain evoked potentials responses, and nerve conduction measurements, all of which are quantifiable and may be performed before and after treatment. Hematologists, psychiatrists, neurophysiologists, and neurologists should participate in joint discussions and coordinated research planning to identify the most appropriate electrophysiological methods and research approaches for examining central and peripheral nervous system function and responses to deficiencies of vitamin B12 and folate.
• Future research may profitably include PET scanning and DNA probing. In addition, NMR spectroscopy offers potential advantages in measuring biological chemicals such as fatty acids and other metabolites in the gray and white matter of selected regions of the brain, with a fair degree of tissue specificity.

• Animal models may offer means of learning more about storage locations of vitamin B12 and folate in the brain and how quickly the brain concentrations can be depleted or repleted. Limited evidence suggests folate depletion is much more rapid than with vitamin B12 (note: vitamin B12 depletion in the fruit bat model may take a year). Very little is known about the time courses for such depletions in the CNS of animals as well as human subjects.

• "Multiple impacts" were discussed in terms of possible neurologic effects of simultaneous deficiencies of thiamin and pyridoxine. Problems with elevated MMA levels, particularly in vitamin B12 deficiency and potential depletion of carnitine, were also considered.

• Drug effects may be significant in terms of manifestations that may distort or mask responses to vitamin B12 or folate deficiency. For instance, with an underlying, subtle neurologic effect, would the addition of phenytoin or alcohol affect the parameters under investigation?

• "Polygenic effects," though poorly understood, presumably exert significant genetic influence on individual responses to deficiencies of such nutrients as vitamin B12 and folate. Are there other neurotransmitter systems that impact on one another, possibly leading to multiple effects?

• With regard to effects on the nervous system, the Working Group considered the evidence generally convincing for an effect of vitamin B12 deficiency, but equivocal for folate deficiency. There are important gaps in knowledge of possible mechanisms of action of both vitamin B12 and folate in the metabolic processes of the nervous system.
APPROACHES TO DETERMINE RELATIONSHIPS OF VITAMIN B12 AND FOLATE 
NUTRITURE TO AGE-RELATED CHANGES IN NEUROLOGICAL DEGENERATION 

Working Group 5

Discussion Leader: John Lindenbaum, M.D.
Rapporteur: Susan Ettinger, Ph.D.

In addressing their assignment, the Working Group emphasized two fundamental considerations. First is the need to define the clinical significance of low serum cobalamin levels in some subgroups of the elderly. The size of these subgroups is thought to be small. Determining the clinical significance will require objective, quantitative, controlled assessment of cognitive and other neurologic functions before and after cobalamin therapy. The therapy should be intensive, with generous doses of vitamin B12 for sufficiently long periods of both treatment and followup because of the recognized delay in neurologic recovery in some patients with florid, obvious, neurologic cobalamin deficiency of the nervous system. While some patients in such a clinical trial may show dramatic, unequivocal reversal of cognitive deficits, others may have combined CNS deficits in which cobalamin deficiency is only one of the involved factors; hence, measurement of partial improvements would be an essential part of the evaluations.

Second, aside from the substantial problem of alcoholism in the elderly, there is a lack of conviction that a public health problem exists that urgently requires a lot of study regarding the prevalence of folate deficiency in the elderly. Uncertainty still exists as to whether folate deficiency in the elderly results in structural or functional disturbances in the nervous system. This remains to be shown in carefully controlled studies.

NEEDED INFORMATION AND SUGGESTED APPROACHES

Vitamin B12

- There is a need to define the clinical significance of the high prevalence of low serum cobalamin levels in some subgroups of the elderly population and the reported association of low serum cobalamin levels with impaired cerebral function.

- The functional significance of low serum cobalamin concentrations as well as low normal serum cobalamin concentrations (e.g., 200-300 pg/ml) should be assessed in population or clinical studies. Suggested approaches include assessing cobalamin enzyme function by measuring metabolite levels (MMA and homocysteine in serum or urine); documenting the response of metabolite levels to vitamin B12 treatment; and conducting careful, objective,
quantified, and placebo-controlled assessment of cerebral and other neurologic functions before and after intensive cobalamin therapy for sufficient periods of followup (at least 6-12 months). Hematologic followup may prove useful even in patients with initially normal MCVs which may decline within the normal range after treatment.

- The likelihood that vitamin B12 therapy will produce only partial improvement or a change in the rate of progressive impairment in cerebral function should be anticipated in some patients because cobalamin deficiency may be superimposed on other primary causes of dementia.

- Because some individuals are unable to assimilate dietary cobalamin, the Schilling test will not identify all patients who may develop vitamin B12 deficiency. Tests of absorption of cobalamin from foods need to be further evaluated as to their usefulness in detecting elderly patients who develop clinically significant vitamin B12 deficiency. Patients who present with dementia as well as elderly patients without obvious mental deterioration would be suitable subjects for a controlled study.

- There is need for better methods of assessing neurologic manifestations of vitamin B12 deficiency. Cerebrospinal fluid levels of metabolites such as MMA may be better indicators of deficiency than serum or urine levels of metabolites.

- It would be useful to develop imaging techniques to define nervous system sites of localization and impairment in patients, for example, PET scan study of transcobalamin II-vitamin B12 uptake in the brain or radioactive folate uptake or utilization.

- The usefulness of various tests of evoked neurologic responses needs further evaluation.

- Several unanswered questions could be addressed with current or future technologies. Are there differences in vitamin B12 coenzyme function in the brain of young and old animals? Do nervous system cell lines differ at varying ages? Can the impairment in some cell function (for example, a step in myelin synthesis) be demonstrated in cell lines deprived of vitamin B12 or folate or subjected to vitamin B12 inactivation by nitrous oxide exposure? Does the myelin of elderly animals differ in structure or function from that of young animals?

**Folate**

- Whether folate deficiency causes structural or functional disturbances in the adult or aged human nervous system remains a clinically unproven suspicion.
- More controlled observations, such as may be provided by ongoing studies of methyffolate therapy of depression, are needed.

- Mechanisms of transport of folate into the nervous system require better definition.
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