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EFFECTS OF ANTIEPILEPTIC DRUGS ON IMMUNE FUNCTION
IN HUMAN SUBJECTS AND MICE

by

Nadine C. Margaretten

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Toxicology
(Immunotoxicology)

Approved:

UTAH STATE UNIVERSITY

Logan, Utah

1985

DEDICATION AND ACKNOWLEDGEMENTS

This dissertation is dedicated to my husband Glen and daughter Nicole for their love, and understanding and support of my work. I am also grateful to our families for their encouragements.

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Nadine C. Margaretten

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ABSTRACT

Effects of Antiepileptic Drugs on Immune Function
in Human Subjects and Mice

by

Nadine C. Margaretten, Doctor of Philosophy
Utah State University, 1985

Major Professor: Dr. Reed P. Warren
Department: Interdepartmental Program in Toxicology

A number of immune abnormalities have been found in epileptic patients treated with antiepileptic drugs (AED). The alterations seen range from mild suppression of immunoglobulins to severely impaired humoral and cellular immunities. There is evidence for both drug effects and genetic or acquired factors as contributors to these abnormalities. In order to examine the basis for immune abnormalities in patients with epilepsy, a number of experimental designs were employed: clinical studies, *in vitro* studies, and use of an animal model.

Peripheral blood mononuclear cells (PBMC) isolated from epileptic patients currently receiving AED were found to have a reduced OKT4+/OKT8+ ratio. A reduced natural killer (NK) cell activity was found which may be due to a low proportion of Leu 11+ cells. A reduced NK cell activity was also found in healthy siblings of the patients, indicating a possible genetic basis for the level of this activity. Antibody-dependent cell-mediated cytotoxicity (ADCC), mitogenic responses, and total rosette-forming cells of PBMC isolated from patients were found to be normal.

The AED phenytoin has been associated with a variety of immune function alterations and lymphoma. In this study, phenytoin was found to depress basal and augmented NK cell activity of human cells in a dose-dependent manner in vitro. This depression was reversible following short-term exposure and at levels considered therapeutic. Phenytoin also depressed ADCC, thus one mechanism by which phenytoin alters immune function is by its depression of cell-mediated cytotoxicity. In contrast to results obtained with phenytoin, the AED carbamazepine did not significantly alter NK cell activity, but the diluent propylene glycol depressed activity.

NFS mice given phenytoin produced lower specific antibody titers following antigen challenge. Body weights, specific organ weights for thymus, spleen, and liver, and blood cell counts were normal in these mice. The protocol was well tolerated by the animals at phenytoin dosages ranging from therapeutic to neurotoxic. Susceptibility to murine hepatitis virus was found to be increased in mice given a high dose of phenytoin. This animal model should allow investigations into toxic dose levels and mechanisms by which phenytoin and other AED alter immune function.

(106 pages)

CHAPTER I
INTRODUCTION

The epilepsies are a group of chronic central nervous system disorders having in common the occurrence of sudden and transitory episodes (seizures) of abnormal phenomenon of motor (convulsions), sensory, autonomic, or psychic origin. Seizures are usually correlated with abnormal and excessive electroencephalogram discharges (Rall and Schleifer, 1980). The occurrence of epilepsy in the general population is between 3 and 6 per 1000 people (Hauser, 1978). There are approximately twenty two thousand patients with epilepsy in the state of Utah and two million in the United States. The majority of patients with epilepsy receive antiepileptic medication for the control of seizures on a long term basis. The object in the treatment of seizure disorders is to suppress seizure activity while keeping side effects at a minimum. Often this is a difficult ideal to achieve due to the nature of the disorder and the treatment regimen itself; that is by definition epilepsy is a chronic condition and treatment is long-term. Multiple therapy is often necessary due to the presence of more than one seizure type in a patient. Other factors are thought to contribute to long-term side effects of antiepileptic drugs: these include treatment at a young age, periods of acute toxicity, and the presence of other conditions such as mental retardation.

Many of the toxicities associated with the long-term use of antiepileptic drugs have recently been reviewed including their effects on the immune system (Oxley et al 1983). There appears to be a different individual susceptibility to the immunosuppressive effects of

antiepileptic drugs with the majority of patients being unaffected. Certain individuals may be dramatically affected however, and display complex immunodeficiencies as either a primary or secondary reaction to a drug or combination of drugs. Patients on multiple therapy have been found to have decreases in specific antibody titers and increases in frequency of respiratory infections. Long-term use of phenytoin, a commonly used antiepileptic drug (AED), has been most notably associated with decreases in IgA but both humoral and cellular immunity can be affected and to a significant degree.

The intent of this study was to further the understanding of how the immune system is affected by AED. Three approaches were employed: clinical studies, *in vitro* studies, and use of an animal model. In the clinical studies, peripheral blood mononuclear cells were isolated from patients currently receiving AED to determine any long-term effects by these drugs on function and relative numbers. Cells from healthy siblings were also studied as a means to assess a genetic role in immune function.

Epilepsy itself has been found to be associated with immune system abnormalities in some cases. In order to investigate effects of AED on certain lymphocyte activities without the complication of epilepsy, several *in vitro* studies were done using cells isolated from healthy donors. The AED phenytoin and carbamazepine were studied for effects on killer cell activities.

A murine model to study effects of AED on immune function was developed to bypass the complications and limitations of clinical and *in vitro* studies. Mice were given phenytoin long-term in studies of immune responses which represent a cooperation of different cell types

within the immune system. The effects of phenytoin on specific antibody production and susceptibility to murine hepatitis virus infection were ascertained.

CHAPTER II
REVIEW OF THE LITERATURE

Description of Epilepsy

Epileptic attacks have been described as electrical explosions of the brain. In the normal brain, a balance of excitation and inhibition allows relevant information to be processed while preventing an overload of information and uncontrolled neuronal activity. Electroencephalogram (EEG) studies have indicated that there is usually a focus or general area which is the source of epileptogenic activity. These foci are inherently more excitable, and discharge more frequently than normal. If not held in check by sufficient inhibitory mechanisms, the excitation spreads from the focus to nearby normal tissue. A seizure then occurs, the manifestations of which are dependent upon the extent of spread of excitation and the anatomical location of the high activity area (motor areas, sensory areas, or reticular activating system, etc.) It is thought that a seizure is maintained by a positive feedback mechanism of neurons and that several factors come into play to stop an epileptic attack. These include post-synaptic inhibition by hyperactive neurons, active inhibition by areas of the brain outside of the seizure focus, and local metabolic changes in the seizure focus such as depletion of high energy substrates and a buildup of metabolic waste products (Rall and Schleifer, 1980, Guyton, 1981).

Seizures in a patient are categorized after a careful case history, a neurological exam, and an EEG in an effort to localize the lesion, find the etiology, and initiate the correct therapy. The importance of correct diagnosis of seizure type lies in the therapy.

Various AED are useful in controlling different types of seizures. A proper match of drug with seizure type will likely reduce or abolish seizures.

The International Classification of Epileptic Seizures lists 2 main classes of seizures: focal (partial) and general (bilateral/symmetrical) (Gastaut, 1970). Partial seizures are further divided into those with elementary (cortical focal) and complex (temporal lobe and psychomotor) symptomatology.

Characteristics of partial seizures include localized convulsions or sensory disturbances, and for the latter, confused behavior with impairment of consciousness and anterior temporal lobe focal abnormalities. Focal epilepsy is usually a result of local organic lesions or functional abnormalities, such as a scar or tumor, a destroyed area of the brain, or congenitally abnormal circuits in the brain. It can involve almost any part of the brain, either localized regions of the cortex or deeper structures of the cerebrum or brain stem. These brain lesions promote rapid discharges in local neurons. A discharge rate greater than 1000 per second results in a spread of synchronous waves over adjacent cortical regions. The waves are presumably from localized reverberating circuits that gradually recruit adjacent areas of the cortex into the discharge zone. When an excitation wave spreads over the motor cortex, a progressive march of contractions occurs on the opposite side of the body, usually starting at the mouth and progressing toward the legs. If the progression is in the opposite direction, the name Jacksonian is used. A focal attack can be either confined to a specific area of the brain, or if strong signals from the focus can excite the mesencephalic portion of the

reticular activating system, a grand mal attack can occur. Manifestations of psychomotor seizures include a short period of amnesia, a moment of incoherent speech, an attack of rage, sudden anxiety or fear, or a moment of motor activity. These types of attacks characteristically involve a part of the limbic portion of the brain (Guyton, 1981).

Generalized seizures are divided into several classifications. The absence or petit mal seizures are characterized by a brief and abrupt loss of consciousness associated with high-voltage, bilateral synchronous, 3/second spike and wave pattern in an EEG. These seizures are thought to involve the thalamocortical portion of the reticular activating system. Attacks usually last between 3 and 30 seconds during which time the patient is unconscious and twitchlike contractions of muscles, usually in the head region (such as eye blinking) occur. Return to consciousness and resumption of activities follows (Guyton, 1981).

Tonic-clonic or grand mal seizures are major convulsions with extreme neuronal discharges in all areas of the brain; cortex, cerebrum and the reticular activating system. Discharges are transmitted from the reticular formation into the spinal cord resulting in generalized 'tonic convulsions' of the entire body. Tonic-clonic convulsions (alternating muscular contractions) follow toward the end of the attack. This type of seizure lasts for a few seconds to many minutes and is followed by a severe fatigue lasting for several hours with a prolonged depression of all central nervous system functions. The EEG taken during a grand mal seizure reveals high voltage, synchronous discharges over the entire cortex, with both sides of the brain giving

the same type of discharge with the periodicity like that of normal alpha waves. It is thought that a grand mal seizure is caused from synchronous discharges from the reticular activating system via local reverberating circuits, or reverberation back and forth between the reticular activating system and the cortex (Rall and Schleifer, 1980; Guyton, 1981).

Bilateral massive epileptic myoclonus seizures are isolated clonic jerks associated with brief bursts of multiple spikes in the EEG. Infantile spasms are a progressive disorder with convulsive spasms leading to mental deterioration. Clonic seizures occur in young children and are associated with rhythmic clonic contractions of all muscles, loss of consciousness, and marked autonomic manifestations. Atonic seizures are associated with loss of postural tone. Akinetic seizures are characterized by impairment of consciousness and a complete relaxation of all musculature, secondary to excessive inhibitory discharge (Gastaut, 1970).

A certain percentage of seizure disorders appears to be genetic in origin. However, most seizures are a result of acquired nervous system lesions, either at the time of birth or afterwards. Primary or idiopathic epilepsy denotes cases where no cause of the seizures can be identified, whereas with secondary or symptomatic epilepsy various conditions are thought to contribute to the etiology. These factors include congenitally malformed circuits in the brain, neoplasm and other space-occupying lesions, head trauma, hydrocephalus, hematomas, cerebrovascular changes subsequent to childhood infection, and infarctions from various causes. Damage to neurons from a variety of sources can cause a epileptic foci to develop. Other conditions which

by themselves do not normally cause epileptic seizures in normal neuronal tissue can precipitate or potentiate an epileptic attack given an excitable focus. These conditions include abnormal pH, osmolarity, gas concentrations (O_2 , CO_2) or glucose concentrations in blood, certain drugs, blood pressure changes, certain mental states and metabolic diseases, or other stressor factors (Rall and Schleifer, 1980). Fever, loud noises, or flashing lights may elicit a seizure in patients with a hereditary predisposition to grand mal attacks (Guyton, 1981).

Description of Antiepileptic Drugs (AED)

The commonly used AED are summarized by Rall and Schleifer (1980). Several of the barbituates, long used as sedative-hypnotic drugs, are currently used as AED. It is thought that while the sedative properties of phenobarbital are a result of generalized stimulation of receptors for the inhibitory neurotransmitter gamma aminobutyric acid, its anticonvulsant properties are more related to its potentiation of inhibitory pathways that are recruited during discharge of epileptogenic foci.

Oral absorption of phenobarbital is slow but complete. It is about 50% bound to plasma proteins and is evenly distributed over the body. Its major metabolite, parahydroxyphenobarbital, is inactive and excreted in urine. Phenobarbital is used for controlling generalized tonic-clonic and cortical seizures. Other barbituates in use as AED are mephobarbital and metharbital.

Phenytoin is a widely used and relatively well studied AED. It is the 5,5-diphenyl non-sedative derivative of phenobarbital. Phenytoin

reduces the development of maximal seizure activity and the spread of the seizure process from an active focus without causing central nervous system depression. Phenytoin restores abnormally increased excitability toward normal, modifies the pattern of maximal electroshock seizures and reduces the duration of neuronal afterdischarge. Phenytoin affects the movement of ions across cell membranes; it decreases resting Na^+ fluxes and the Na^+ current, decreases the Ca^{2+} influx depolarization, and delays the K^+ outward current during an action potential. This leads to an increased refractory period and a decrease in repetitive firing.

Phenytoin is a weak acid (pK_a 8.3) with limited aqueous solubility and dose-dependent elimination. It is extensively bound to plasma proteins and is widely distributed to all tissues. Its major metabolite, the parahydroxyphenyl derivative, is inactive and excreted in bile and urine.

Phenytoin is effective in controlling most forms of epilepsy except absence seizures and is also used in the treatment of psychosis and cardiac arrhythmias. Other hydantoin derivatives used to control seizures are mephenytoin and ethosuximide.

Primidone, a deoxybarbiturate, is a cogener of phenobarbital. It resembles phenobarbital in its anticonvulsant properties but is more selective in modifying electroshock seizure patterns. Primidone is metabolized into two active products: phenobarbital and phenylethylmalonamide, and is effective in all but absence seizures.

Carbamazepine, an iminostilbene, is related to the tricyclic antidepressant drugs. Its anticonvulsant effects are similar to phenytoin but its mechanism is unknown. Carbamazepine is rapidly absorbed after

oral administration and is approximately 80% bound to plasma proteins. Its 10,11-epoxide metabolite is also active. It is used for temporal lobe epilepsy alone or in combination with generalized tonic-clonic seizures.

Ethosuximide, a succinimide, is used primarily for controlling absence seizures. Both ethosuximide and trimethadione (to be discussed) offer protection against the convulsant action of pentylene-tetrazol. Ethosuximide elevates the threshold for electroshock seizures and blocks spiking activity in primary and secondary foci and the associated clonic seizure activity produced experimentally in the rat.

Ethosuximide is evenly distributed to all tissues and is inactivated by hepatic microsomal enzymes into several inactive metabolites. Other succinimides in use are methsuximide and phensuximide.

Valproic acid is a simple branched-chain carboxylic acid used against a variety of seizures with minimum sedative and other central nervous system effects. The mechanism of action of valproic acid is thought to be an interaction with gamma-amino butyric acid (GABA) metabolism and/or augmentation of GABA-mediated postsynaptic inhibition.

Valproate is rapidly and almost completely absorbed after oral administration, is approximately 87% bound to plasma proteins, and is equilibrated mainly in the extracellular space. It is metabolized to several products and may inhibit hepatic enzymes as indicated by its interaction with phenobarbital.

The oxazolidineone drugs are also used as AED. Paramethadione and trimethadione are used for controlling absence seizures. Dimethadione,

the N-demethyl metabolite of trimethadione, is also active.

Diazepam, a benzodiazepine sedative-antianxiety drug, is valuable in the management of status epilepticus. Diazepam suppresses the spread of seizure activity produced by epileptogenic foci in the cortex, thalamus, and limbic structures and facilitates GABA-mediated synaptic systems. It is extensively metabolized to a variety of products by reduction of the 7-nitro group to an inactive amino derivative, and to an active N-demethyl derivative.

Other AED less commonly in use are phenacemide (phenylacetylurea), a straight chain analog of phenytoin; phenacemide, and acetazolamine, a carbonic anhydrase inhibitor.

Chronic Toxicities Associated with Antiepileptic Drugs

A review of many of the chronic toxicities resulting from the use of AED is available (Oxley et al, 1983). The disorders encountered are in the general categories of hepatic, hematological, connective tissue, copper and ceruloplasmin, calcium and bone, motor and cerebellar, and immunological.

Hepatic disorders. Severe hepatic damage is rarely encountered with any of the AED currently in use. However, numerous cases of hepatic toxicity have been reported especially with phenytoin, valproic acid and phenobarbital. Only a few cases of hepatic damage following therapy with .pa trimethiadone, methoin, and the benzodiazepines have been reported (Jeavons, 1983).

Drugs may cause hepatic damage by several mechanisms. Hepatotoxic drugs cause direct damage to the liver; the effects are predictable and

dose-dependent. In contrast, unpredictable, dose-independent effects result from hypersensitivity or host idiosyncratic reactions, such as metabolic abnormalities. Metabolic aberrations may have a genetic basis or be the result of exposure to other agents which cause alteration of drug metabolizing pathways (Zimmerman, 1978). Hypersensitivity and idiosyncrasy can cause either necrosis or cholestasis. Hypersensitivity responses, frequently associated with rash, fever, and eosinophilia, generally appear from 1-5 weeks after initiation of treatment and there is a prompt response to a challenge dose. In contrast, a toxic response from a metabolite may become evident from one week to 12 months after initiation of therapy.

In most cases where AED are the cause of liver damage, evidence points to hypersensitivity or drug idiosyncrasy as the etiology. Given the possibility of hepatic disorders due to AED, it is advisable that patients as well as clinicians be informed of possible side effects and that the patients be seen frequently, especially during the first 6 months of therapy. Warning signs for phenytoin, phenobarbital, and carbamazepine toxicities include skin rashes, fever, lymphadenopathy, and jaundice. Those for valproic acid include malaise, anorexia, vomiting, recurrence of seizures after initial control and jaundice. It has been recommended that a monitoring scheme be set up to more closely guard against liver damage (Loyning et al, 1983). It is thought that multiple drug therapy plays an important role in hepatotoxicity (Jeavons, 1983).

Copper and ceruloplasmin levels. Various anticonvulsant medications have been found to alter plasma copper and ceruloplasmin levels. Phenytoin monotherapy has been found to increase copper but not ceruloplasmin concentrations while valproic acid and ethosuximide decreased both. Primidone or carbamazepine monotherapy have not been found to alter either copper or ceruloplasmin levels. In a recent study, untreated epileptic children did not have significant changes in either copper or ceruloplasmin concentrations as compared with normal, age-matched control subjects (Fichsel et al, 1983).

Hematological disorders. Several of the AED drugs have been associated with marrow suppression (Reynolds, 1983a). In a number of cases, carbamazepine was the probable cause of aplastic anemia (Pisciotta, 1975). Phenytoin may cause aplastic anemia especially when given in combination with other drugs (Best, 1963). Phenytoin has also been associated with leucopenia, agranulocytosis, and thrombocytopenia. In one case of erythroid aplasia, phenytoin appeared to inhibited DNA synthesis in erythroid cells, specifically, the step of deoxyribotide formation (Reynolds, 1983a).

Valproic acid has been associated with platelet dysfunction, thrombocytopenia, and bleeding. Suggested mechanisms have been postulated (Reynolds, 1983a).

Several cases of neonatal coagulation defects have been reported in infants born to mothers treated with phenobarbital and/or phenytoin. The bleeding, due to a depression of vitamin K-dependent coagulation factors (II, VII, IX, and X) is correctable or preventable with vitamin K therapy. Phenytoin has also been shown to have this effect experimentally (Reynolds, 1983a).

Several of the AED have been associated with megaloblastic anemia due to folate deficiency, reflected by subnormal serum and red blood cell (RBC) folate levels. Serum vitamin B12 levels may also be abnormal. Both folate and vitamin B12 (cobalamine) are necessary for the maturation of the RBC (Guyton, 1981). Macrocytosis has been seen in a large percentage of patients on combined phenytoin and primidone therapy (Chanarin et al, 1976).

In non-anemic patients, folate and vitamin B12 levels may still be subnormal. Low folate levels have been found in serum, most commonly

in patients receiving multiple AED therapy, and also in red blood cells, and cerebrospinal fluid (Reynolds, 1983a). There is evidence that phenytoin, phenobarbital, primidone, carbamazepine, and valproic acid exert some effect on folate metabolism but possibly by different mechanisms. Several hypothesis have been postulated, including enzyme induction by phenytoin, phenobarbital and carbamazepine; however valproic acid is not an enzyme inducer. The other hypotheses are: competitive interaction between folate coenzymes and the drugs, malabsorption of folic acid, and increased demand for folic acid as a coenzyme for AED-hydroxylation or for other enzymes induced by the drugs (Reynolds, 1983a).

Children treated with valproic acid have shown several hematological disorders: thrombocytopenia, prolonged bleeding time and hypofibrinogenemia. A dose relationship to the drug and possible relationship to its hepatotoxic effects have been suggested (Rochel and Ehrenthal, 1983).

A deoxyuridine suppression test has been developed to assess folate deficiency in patients on long-term AED therapy. This test measures the ability of bone marrow cells to convert deoxyuridine to thymidine for DNA synthesis, a reaction specifically disordered in megaloblastic anemia. Although results have been conflicting, a recent study has shown that the test is clinically useful in detecting significant folate deficiency (Burman, 1983) and concluded that megaloblastic anemia in patients on long-term AED therapy is due to folate deficiency.

Connective tissue disorders. A variety of connective tissue disorders have been seen in patients receiving AED. Gingival hyperplasia, a proliferation of the gums and sometimes other tissues, is thought to be caused from stimulation of fibroblasts due to folate deficiency. It occurs in about one out of three epileptic patients on phenytoin therapy. The severity of this condition varies proportionally with levels of phenytoin in the serum and saliva. Poor oral hygiene aggravates this condition. Other unknown factors are thought to be involved in the etiology of gingival hyperplasia, such as the epileptic condition itself, and age (Schmidt, 1983a).

Facial changes occur in about two thirds of all institutionalized, severe epileptic patients. The coarsened features seen include thickening of the lips and subcutaneous facial tissues, and a wide, club-shaped nose such that the face develops an acromegalic appearance. Most often, this condition occurs in patients given multiple drug therapy; however this condition has not been well studied (Schmidt, 1983a).

Dupuytren's contracture, a proliferation of elastic fibers and thickening of the palmar collagen fiber bundles, is found in one third of all male and one fourth of all female epileptic patients. Ledderhouse syndrome, fibromas of the mucous membrane of the mouth and of the plantar fascia of the foot, may then develop in these patients. Phenobarbital and primidone treatment have been linked to these syndromes (Schmidt, 1983a). In a recent study (Schmidt et al, 1983c) 4 patients on phenobarbital therapy for either epilepsy (3) or migraine headaches developed Dupuytren's contracture and Ledderhouse syndrome. Discontinuance of phenobarbital in the non-epileptic patient resulted

in a regression of this condition. Froscher and Hoffman (1983) have shown that discontinuance of phenobarbital in the majority of cases with Dupuytren's contracture results in an improvement of the condition. A number of other factors are thought to be involved in the etiology of the above disorders including chronic trauma, and duration and dose of AED treatment.

A frozen shoulder syndrome, a non-rheumatic stiffness in the shoulders and other joints may develop in patients on phenobarbital or primidone therapy. Other connective tissue disorders may accompany this condition. This syndrome may be reversed with physical therapy without drug discontinuance (Schmidt, 1983a, Janz and Piltz, 1983).

In contrast to the above disorders, AED have been shown to be beneficial for wound repair and the healing of ulcers (Schmidt, 1983a). In a recent study of children on AED therapy (Trimble and Corbett, 1983) hirsuties, gum hypertrophy and low weights were noted. High mean levels of serum phenytoin were associated with gum hypertrophy while high levels of phenobarbital were associated with low relative height. Hirsuties and height and weight abnormalities were associated with increased frequency of seizures but not with gum hypertrophy.

Lyell's syndrome, a toxic epidermal necrosis, has occurred in a number of patients receiving AED. This is a severe adverse reaction to a drug with a 22% mortality rate, and any re-exposure to a drug or related drug to a patient that has shown a hypersensitivity to a particular drug is dangerous. This syndrome has been found with carbamazepine, mephenytoin, phenytoin, phenobarbital, and primidone (Schmidt, 1983a).

Disorders of mineral metabolism. Osteomalacia and rickets have been attributed to AED therapy with vitamin D deficiency being a common feature. Long-term and high dosage (usually multiple) drug therapy are thought to contribute to these disorders. An increased frequency of hypocalcemia and raised alkaline phosphatase levels have been found. Under controlled conditions, AED have been found to induce a mild biochemically detectable vitamin D deficiency in spite of criticisms that various factors, namely outdoor activities and nutrition, affect serum 25-hydroxyvitamin D levels (Offerman, 1983).

The ability of several of the AED to induce microsomal mixed-function oxidase activity and to stimulate biliary excretion is thought to be responsible for the development of vitamin D deficiency: twenty five-hydroxylation of vitamin D and various steroids is metabolized by mixed function oxidase enzymes. Although twenty five-hydroxy vitamin D is still active, it could be further oxidized to inactive metabolites. It is known that vitamin D and its metabolites are biotransformed and excreted in bile as acid, neutral, glucuronide or sulfate forms. Enterohepatic recirculation has been demonstrated to be important for at least the 1,25-dihydroxy active metabolite (Offerman, 1983). These factors are thought to play a role in the development of vitamin D deficiency.

Christiansen and Tjellesen (1983) have presented evidence that drug-induced osteomalacia is somewhat unlike that of classical osteomalacia. Vitamin D₂ but not D₃ is effective as a treatment. There is evidence for drug-induced liver enzyme induction as a cause of vitamin D deficiency, however, other mechanisms are also thought to be involved.

In a recent study of institutionalized epileptic patients on AED therapy (Berry et al, 1983) serum twenty five-hydroxy vitamin D, calcium and alkaline phosphatase levels were evaluated. The authors concluded that drug-induced hypocalcemia was exacerbated but not caused by vitamin D deficiency, which in turn is primarily induced by insufficient exposure to sunlight. Vitamin D therapy does correct levels toward normal but does not correct the concomitant hypocalcemia indicating that these two deficiencies are not parallel. In another group of epileptic patients (Krause et al, 1983) there was evidence that disturbance in bone metabolism is a problem after initiation of AED treatment, particularly in children, followed by a normalization of metabolism. Long-term therapy (over 10 years) may again be a sensitive period of drug-induced bone metabolism imbalance. Several patients on carbamazepine monotherapy had hypocalcemia, hypophosphataemia and elevated alkaline phosphatase levels, suggesting that carbamazepine alone may disturb bone mineral metabolism (Keränen et al, 1983).

A recent survey of institutionalized epileptic patients indicated that the type of seizure may be more related to the number of sustained fractures incurred rather than drug treatment; namely tonic seizures in which the patients fall rigidly to the ground increase fracture risk (Allen and Oxley, 1983).

Motor and cerebellar function alterations. Cerebellar dysfunction (reversible and permanent) has occurred with phenobarbital, primidone and phenytoin therapy. However, Dam (1983) has shown that in patients with a low density of Purkinje cells, there is a greater correlation of degeneration of Purkinje cells to severe epilepsy (frequent

convulsions) than with high phenytoin dose. Phenytoin does not induce neuropathological lesions in experimental animals. A comparison of seizure resistant versus seizure sensitive Mongolian gerbils has demonstrated that neuropathological lesions in the cerebellum were found only in the sensitive strain; the lesions were assumed to be caused by basic biochemical derangements associated with epileptic hyperactivity (Dam, 1983).

Phenytoin and to a lesser extent other extent AED can cause dyskinesias, an impairment of the power of voluntary movement resulting in fragmentary or incomplete movements. Coexisting organic cerebral damage may facilitate the development of drug-induced dyskinesias. Increases in dose of one drug or addition of another may trigger this impairment. The mechanism by which phenytoin causes dyskinesias may be related to its ability to alter brain neurotransmitter systems (Dravet et al, 1983).

Mutagenic and teratogenic effects. Valproic acid and phenytoin have been found to be teratogenic in an in vitro culture assay in a dose-dependent manner. The abnormalities produced in organ culture of embryonic mice included open neural tubes, abnormal body curvature and craniofacial deformities. Growth and development were also retarded in these embryos (Bruckner et al, 1983). Valproic acid is teratogenic in rats and results in .pa resorptions and a number of skeletal and visceral malformations (Ong et al, 1983).

Valproic acid appears to be readily transported across the placenta and achieves higher serum concentrations (1.4x) in the infant than the mother with a biological half-life three times longer

(Dickinson et al, 1979). Epidemiological studies have shown that valproic acid may cause neonatal transformations such as spina bifida aperta in humans (Robert et al, 1983). Various studies have shown that mothers on AED have a 6% rate of malformed children as compared to 2.5% in control mothers.

Chromosomal damage in leukocytes has been found in patients on long-term carbamazepine or phenytoin monotherapy. Both chromosomal and chromatid exchange-type aberrations were seen (Herna and Obe, 1977).

Overview of the Immune System

The immune system consists of an interconnecting network of lymphoid organs and tissues. In mammals, the central tissues are the thymus and bone marrow and peripheral tissues are the lymph nodes, spleen, tonsils, and Peyer's patches. Lymphocytes are produced in these tissues and move among these tissues in blood and lymph where they interact with antigens and with each other while in the system (Kimball, 1983).

An immune response is defined as a "altered reactivity to a specific configuration that develops following contact with it" (Kimball, 1983, pp.3). The response must meet the criteria of specificity and memory. An antigen is a substance that when introduced into an animal with a functioning immune system, can elicit a specific immune response. Two main effector mechanisms mediate immune responses: humoral immunity (antibody mediated) and cell-mediated immunity.

In the cell-mediated branch of the immune system, T-lymphocytes (T-cells) of different types and specificities are the main effector

cells. Antigenic stimulation of T-cells results in cell proliferation (division) and differentiation of T-cell populations which recognize specific antigenic determinants on the antigen. Clones (identical progeny) of these lymphoblast cells are responsible for such actions as delayed-type hypersensitivity (T_{DTH}), cytotoxic activity (T_C) directed against graft cells or infected cells, helper activity (T_H) which aids in T_C killing and antibody production, and suppressor activity (T_S) which prevents overaction of the immune system such as found in auto-immune disorders.

In the humoral branch of the immune system, antigenic stimulation of B-lymphocytes (B-cells) results in proliferation and differentiation of those cells which recognize antigenic determinants on the antigen. These lymphoblasts differentiate into plasma cells which produce and release antibody of the same specificity as the stimulated B-cells; i.e., they are specific for the eliciting antigen. Antibodies then act as intermediaries in a series of reactions, including the activation of the complement cascade for lysis, to help rid the body of the foreign antigen.

Natural killer (NK) cells are a subpopulation of lymphocytes responsible for the recognition and killing of tumor cells which arise spontaneously or via chemical or physical agents in the body. These cells are not truly immune cells because they require no prior exposure (sensitization) for effective killing and have no memory function as do other lymphocytes. They are thought to be important for surveillance and destruction of neoplastic cells in vivo. Lymphocytes responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) kill cells

coated with antibody specific to these target cells. These lymphocytes are possibly identical to the NK cells.

Blood monocytes and their tissue counterparts, the macrophages, are accessory cells of the immune system. They are phagocytic and responsible for the ingestion and breakdown of foreign particulate matter including live cells. Macrophages assist T- and B-lymphocytes in their tasks by the processing and presentation of antigen to T_H cells.

The various cells of the immune system are derived from bone marrow precursor cells. T-cells mature while in temporary residence within the thymus. Normal immune responses require recognition and cooperation of different cell types and coordination with a variety of factors. It is through this complex interplay that the body is protected from foreign agents and self destruction.

Effects of Antiepileptic Drugs on
The Immune System

Several of the widely used AED have been shown to have immunomodulating effects in some patients. Many patients on long-term phenytoin therapy have developed low secretory and serum IgA levels (Chiu et al, 1982; Gilhus and Aarli, 1981a; Martinez-Cairo et al, 1980; Fossan and Aarli, 1979; Aarli, 1976a, 1976b). Decreases in secretory IgA can result in an increase in frequency of respiratory infections (Gilhus and Aarli, 1981a; Aarli and Fontana, 1980) and there is an increased tendency for phenytoin-treated patients to develop recurrent respiratory infections (Aarli and Gilhus, 1983). Decreased IgA levels have also found in duodenal fluid of patients receiving phenytoin (Martinez-Cairo et al, 1980). Predisposition to develop IgA deficiency appears to be genetically determined (Aarli and Gilhus, 1983). Anderson and Moseklide (1977) have shown decreases in serum IgA in some patients but increases in others. Serum IgA deficiencies can be reversed by withdrawal of phenytoin therapy (Aarli, 1976a). Serum IgA deficiency may be associated with increased serum IgM and IgD levels (Pereira et al, 1983).

The levels of other serum immunoglobulins have also been altered following phenytoin therapy. Suppression of IgG (Czlonkowska et al, 1981; Anderson and Moseklide, 1977; Aarli, 1976a), imbalance of IgG subclasses (Fontana et al, 1978c), elevation of IgM (Anderson et al, 1981; Anderson and Moseklide, 1977), depression of IgM (Aarli, 1976b) and increases in IgE and IgD (Blanco et al, 1977) have been reported. Many of these changes were slight such that the overall effects on the patients were likely to be insignificant. An alteration of the

composition or quality of the cerebrospinal fluid IgG has also been found in phenytoin-treated patients (Fossan and Aarli, 1979) and reduction of cerebrospinal fluid IgG concentrations has been observed (Fossan, 1976). Deposits of immunoglobulins in the skin have been found (Meistrup-Larsen et al, 1979). A severe panhypogammaglobulinemia has been found in a few patients (Pereira et al, 1983). It has been suggested that B-cell differentiation is altered by phenytoin (Seager et al, 1975). In particular, there may be several blocks of the normal progression in B-cell differentiation in a manner similar to that found with adult-onset hypogammaglobulinemia. These blocks could lead to the increase in IgD found in some of the IgA-deficient patients (Pereira et al, 1983).

A decrease in numbers of T-cells has been found in epileptic and non-epileptic patients receiving phenytoin (Martinez-Cairo et al, 1980). The mean percentages of T-cells and monocytes were decreased in patients on phenytoin therapy (Chiu et al, 1982). Evidence of T-cell dysfunction has also been observed (Shakir et al, 1978) and decrease in total lymphocyte numbers have been noted (Gilhus et al, 1982b and 1982c).

Suppression of in vitro lymphocyte blastogenesis with phytohemagglutinin and pokeweed mitogen by serum factors has been found in patients with brain tumors treated with phenytoin (Neuwelt et al, 1983). In epileptic patients receiving phenytoin, phytohemagglutinin blastogenesis was slightly depressed (Gilhus et al, 1982b; Czlonkowska et al, 1981; Yabuki and Nakaya, 1976). Phenytoin added directly to lymphocyte cultures from patients depresses lymphocyte response but a comparison of these patients after initiation of drug therapy versus

pre-drug responses showed a trend to increased responsiveness to pokeweed mitogen, conconavalin A and suboptimal levels of phytohemagglutinin in those with low serum IgA (Gabourne1 et al, 1982).

A number of patients on long-term hydantoin treatment, including phenytoin, have developed lymphoma (malignant or Hodgkin's). There are possible correlations between the immunosuppressive action of hydantoin derivatives and tumor induction (Jungi et al, 1975).

The percentages of lymphocytes forming rosettes with sheep red blood cells was decreased in epileptic patients receiving no drug therapy (Czlonkowska and Korlak, 1980) and with patients on AED therapy (Fontana et al, 1978c); however elevated responses (Czlonkowska et al, 1981) and normal responses have also been observed (Gilhus et al, 1982c).

Patients on phenytoin therapy have been found to have reduced concentrations of complement components C1-inhibitor and C4 (Aarli and Gilhus, 1983).

Several changes in normal immune function have been reported following carbamazepine therapy. An increase in serum IgA and IgG were found in one study where the increase was positively correlated to increasing carbamazepine concentraions and inversely correlated with age (Strandjord et al, 1980). In another study, a trend for increased serum IgG and IgM levels was noted as well as a significant fall in phytohemagglutinin-induced blastogenesis (Czlonkowska et al, 1981). A marked decrease in serum IgA and IgM levels ocured in some patients during the first month of carbamazepine therapy (Gilhus et al, 1982a). However other studies have failed to confirm immunomodulating effects of carbamazepine: Gilhus et al (1980) found no significant difference

in salivary IgA although a trend of elevated salivary IgA, IgG, and IgM levels was found. Gilhus et al, (1982d) found no significant differences in nasal and serum IgA, IgG and IgM levels or in the number of days with respiratory tract disease symptoms in epileptic patients receiving carbamazepine therapy.

One patient developed lupus erythematosus while receiving carbamazepine therapy. Salivary, gastric juice, and serum IgA levels were low or absent. No IgA-producing plasma cells were found on the rectal mucosa and bone marrow. Numbers of surface IgA+ B-lymphocytes were below normal, and there was a decreased capacity to develop both circulating antibodies and delayed hypersensitivity against test antigens (Takigawa et al, 1976).

There is little indication that other AED have effects on the immune system. Cells from patients with brain tumors treated with phenobarbital have a decreased in vitro lymphocyte blastogenic response to mitogens via a suppressive factor in serum (Neuwelt al, 1983). Blanco et al (1977) found serum IgA deficiency in one out of 20 children being treated with phenobarbital.

Patients on multiple drug therapy have shown decreases in production of specific antibody to such antigens as parainfluenza virus III (Czlonkowska and Iwinska, 1977).

The varying degrees of immunomodulation seen in patients on AED indicates that there are likely other factors besides the AED that have a role in suppression or stimulation of various components of the immune system. For example patients on phenytoin may have no change in IgA levels (Modeer et al, 1981), mild depression or severe depression (Pereira et al, 1983). In one study, IgA suppression was found to be

more related to the history of febrile convulsions than to the type of epilepsy or drug therapy (Gilhus and Aarli, 1981b). Epileptic patients not receiving medication have shown no antibody changes in some studies (Aarli, 1976b; Yabuki and Nakaya, 1976) whereas follow up studies on patients on phenytoin therapy show a decrease in serum IgA only after start of treatment (Aarli, 1976b; Fontana et al, 1976). Relatives of epileptics have at times shown antibody imbalances (Fontana et al, 1978b; Haldorsen and Aarli, 1977). While some studies have shown that phenytoin may depress in vitro blastogenetic response to phytohemagglutinin, other studies have shown that a greater than expected number of patients have a low responsiveness to mitogens prior to treatment (Gabourel et al, 1982), and in fact serum IgA-deficient epileptic patients receiving phenytoin have shown increased lymphocytic responses to mitogens.

Various authors have stated the importance of acquired or genetic factors in determining whether a patient will suffer either suppression or stimulation of immune responses while receiving AED (Chiu et al, 1982; Anderson et al, 1981; Blanco et al, 1977). HL-A2 antigen is associated with decreased IgG titers seen with phenytoin and carbamazepine therapy and with immunoglobulin subclass imbalance (Fontana et al, 1978c). HL-A7 antigen has a significantly increased frequency among epileptic patients while HL-A12 antigen has a low frequency. HLA-B7 is associated with Lennox-Gastaut epilepsy (Smeraldi et al, 1975). Tartara et al (1981) found the common feature of immunological disorders more related to early onset of seizures and AED treatment rather than the specific therapy or the clinical type of epilepsy.

Both epilepsy itself and AED have been associated with autoimmune

phenomena. It is possible that epilepsy itself may have an immunological basis in some cases. Divalent and monovalent antibodies to GM 1 ganglioside injected into the sensori-motor cortex of the rat induces recurrent epileptiform activity (Karpiak et al, 1982). It has been proposed that epileptic discharges could be the result of an autoimmune response to either an antigen released during tissue destruction or an infective agent; antibodies may block transmitter receptor sites at synapses and reduce synaptic transmission (Ettlinger and Lowrie, 1976).

The types of auto-antibodies found in some epileptic patients include antinuclear (Chiu et al, 1982; Anderson and Moseklide, 1977), anti-RNA (Ooi et al, 1977) anti-mitochondrial (Anderson and Moseklide, 1977), and anti-muscle and anti-brain nicotinic acetylcholine receptor (Fontana et al, 1978a). Popova et al (1975) found a 31% incidence of brain autoantigens and an 8% incidence of anti-brain antibodies in epileptic patients receiving AED. Lymphocytotoxins with activities against T- and B-lymphocytes were found in 30% of patients on phenytoin (Ooi et al, 1977). It has been postulated that AED can activate a latent autoimmune response and modify its natural course for the worst (Alarcon-Segovia et al, 1972).

Ethosuximide (Tor et al, 1979), carbamazepine (Takigawa et al, 1976), and other AED are considered lupus erythematosus-activating drugs (Alarcon-Segovia and Fishbein, 1975). In several patients an unquestionable correlation was found between the appearance of seizures and the action of certain allergens. However, allergy is only rarely considered to be the cause of epileptic seizures (Dziulek, 1975).

Pechadre et al (1977) have reported that out of 10 children with severe epilepsy that were treated with repeated large doses of gamma

globulin, 7 showed marked improvement in behavior and 8 showed a disappearance in seizures with EEG improvement. Epilepsy may then be triggered by a combination of an inadequate immune system and an infectious agent.

Despite what is known concerning immune deficiencies associated with epilepsy and AED, a unified concept of their relationships cannot as yet be formulated (Cereghino, 1983).

A number of disorders, ranging from mild to severe may affect the same individual receiving AED (Christe et al, 1983). A number of factors appear to predispose a patient to chronic toxicities: young children may be more susceptible to the toxic effects of these drugs. Long-term therapy increases the risk of chronic toxicities as does multiple drug therapy. There is evidence that multiple drug therapy has little advantage over single drug therapy in controlling seizures and may greatly increase toxicity risk. Institutionalization, poor diet, pregnancy, and the presence of concurrent illnesses and disabilities increase toxicity risk. Brain damage and mental retardation increase toxicity risk. Repeated acute toxicity can increase risk by damaging peripheral nerves. Reynolds (1983b) has outlined the factors predisposing individuals to chronic toxicities and has recommended that both physicians and patients be aware of the side effects of AED and their signs. The importance of correct diagnosis and therapy is stressed. Furthermore, early and effective treatment is a safeguard to patients. Lastly, therapy should be withdrawn when possible.

CHAPTER III
REDUCED NATURAL KILLER CELL ACTIVITY, NUMBERS OF
LEU11+ CELLS, AND OKT4+/OKT8+ RATIO
IN EPILEPTIC PATIENTS.

Introduction

The drugs effective in controlling seizures are variously associated with a number of chronic toxicities including toxicity to the immune system (Oxley et al, 1983). Included among these toxicities are depression of serum and secretory IgA titers seen in patients on long-term phenytoin treatment (Yabuki and Nakaya, 1976; Martinez-Cairo et al, 1980) and decreases in specific antibody titer found in patients on multiple antiepileptic drug (AED) therapy (Sorrel et al, 1971; Czlonkowska and Iwinska, 1977).

A limited number of studies have been conducted on lymphocytic function of patients on long-term AED therapy. A decrease in mitogenic response to phytohemagglutinin (PHA) and pokeweed mitogen (PWM) has been found in association with phenytoin and phenobarbital treatment of patients with brain tumors (Neuwelt et al, 1983). Further, Shakir et al. (1978) found evidence of suppressed T-cell function in patients receiving phenytoin. However, it has been suggested that immune alterations found in epileptic patients are not necessarily a result of AED since abnormalities have been found in patients not receiving medication (Gabournel et al, 1982). Limited data are available on the relative roles that AED, disease processes of epilepsy, and genetic factors play in immune abnormalities seen in epileptic patients.

The present study assessed natural killer and killer cell

activities of peripheral blood mononuclear cells (PBMC) isolated from children and adult epileptic patients. Healthy siblings were also studied as a means to assess a genetic role in immune function. PBMC responses to the lymphocyte mitogens concanavalin A (con A), PHA and PWM were also investigated. Enumeration of the lymphocyte subpopulations and subsets which expressed the markers OKT4, OKT8, 7.2(Ia), and Leu 11 were made. Immunologic studies of epileptic children were of particular interest since few data are available from young patients.

Materials and Methods

Experimental subjects. The human subjects used in this study included both epileptic patients and age- and sex-matched healthy volunteers. The epileptic children were affiliated with the Developmental Center for Handicapped Persons at Utah State University. The patients were receiving a variety of medications and differed in their treatment regimens and medical histories. The ages of the children ranged from 4-16 years (patients) and from 3-18 years (controls). The siblings included 5 children aged 6-14 years and 1 adult (age 25). Four adult patients, ranging in age from 24-35 years, and up to 46 healthy adults aged 20-45 were also studied.

Separation of PBMC. PBMC were separated by centrifuging freshly drawn blood on a Ficoll-Hypaque density gradient, rinsed, and suspended in RPMI-1640 medium (Gibco, Grand Island, New York) containing penicillin (100 .pa units/ml), streptomycin (100 mcg/ml), and 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah).

Natural killer cell assay. The details of the ⁵¹Cr technique have been described (Warren et al, 1976). PBMC in concentrations of 5, 2.5, 1.2, and 0.6 x 10⁶ cells/ml were incubated with ⁵¹Cr-labelled K562 target cells suspended at 1 x 10⁵ cells/ml. PBMC and target cells at 0.1 ml each were incubated together in triplicate in round bottom tissue culture plates for 4 h at 37C in 5% CO₂. An aliquot of supernatant was removed and counted in a Beckman gamma counter. Results were expressed as a percent ⁵¹Cr-release relative to spontaneous (target cells in medium alone) and maximal release (target cells in 0.25 % saponin):

$$\text{Percent Release} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100$$

Lymphocyte enumeration. PBMC were depleted of macrophages by allowing adherent cells to attach to plastic culture dishes for 1 hr. The non-adherent PBMC were enumerated with the rosette assay for total T-cells and the complement-mediated cytotoxicity assay for lymphocyte and lymphocyte subpopulation proportions. For the latter assay, the cells were incubated in Microtest II plates with various monoclonal antibodies: OKT4 for helper/inducer T-lymphocytes, OKT8 for suppressor/cytotoxic T-lymphocytes, (Ortho Diagnostic Systems, Raritan, New Jersey), 7.2 for the Ia antigenic determinant on B-lymphocytes (New England Nuclear, Boston, Massachusetts), and on a few of the subjects, with Leu 11 for an NK cell subpopulation (Becton Dickinson Monoclonal Antibody Center, Mountain View, California). Complement was then added followed by another incubation and the percent viable cells was determined by trypan blue exclusion. Percent lysis was calculated as lysed cells/total number of lymphocytes counted.

Lymphocyte blastogenesis. The lymphocyte blastogenesis assay was carried out as previously described (Mickelson et al, 1981). Briefly, lymphocytic responses to the mitogens PHA, and PWM (Gibco) and con A (Flow Laboratories, McLean, Virginia) were assayed by culturing 1×10^5 PBMC with various concentrations of mitogens in flat bottom 96 well tissue culture plates. After the cultures were incubated for 68 h in 5% CO₂, tritiated-thymidine was added for an additional 4 h incubation. The cells were harvested and tritiated thymidine incorporation counted with a scintillation counter.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay. This assay was carried out in a fashion similar to that of the NK assay except the targets were YAC-1 murine lymphoma cells. PBMC (0.05 ml) in concentrations of 10, 5, 2.5, and 1.2×10^6 cells/ml and in 40% heat-inactivated fetal bovine serum were incubated in round bottom wells with 0.1 ml ⁵¹Cr-labelled target cells suspended at 2×10^5 cells/ml and 0.5 ml of rabbit anti-mouse thymocyte serum (M.A. Bioproducts, Walkersville, Maryland) with a final dilution of 1/1000. ⁵¹Cr -release was quantitated as in the NK assay.

Statistical analysis. Data were analyzed with t-test for different means, with analysis of variance, or simple linear regression and the 95% confidence interval for a normal distribution. Blastogenesis data were analyzed after log transformations of the counts using geometric means and variances.

Results

NK cell assay. PBMC from epileptic patients, healthy siblings, and

unrelated healthy subjects were tested for natural killer cell activity (Figure III-1). The mean percent ⁵¹Cr releases effected by PBMC of the epileptic patients and their healthy siblings were significantly lower at each effector to target cell ratio than that of the healthy control subjects. Although the siblings of the epileptic patients had induced somewhat lower mean percent releases than the patients, these lower releases were not significant. Mean releases effected by cells from epileptic children were similiar to those of the patients shown in Figure III-1 and were significantly decreased from those of the healthy children ($p < .01$, separate data not shown). Also, 3 of 4 adult patients had lower activities than healthy adults.

Lymphocyte subset analysis. The monoclonal antibody anti-Leu 11 was used to estimate the proportion of NK cells bearing the Leu 11 determinant in 13 of the patients and healthy subjects. A significant correlation was found between the proportion of Leu 11+ cells and NK cell activity (Figure III-2, $r = 0.68$ for the 50:1 effector to target cell ratio). In addition, 6 epileptic patients included in this portion of the study had a significantly lower proportion of Leu 11+ cells (mean of $9.5 \% \pm 5.0$) than 6 unrelated healthy subjects ($14.4 \% \pm 3.9$).

The epileptic patients had a significantly lower OKT4+/OKT8+ cell ratio as compared to that of healthy subjects (Table III-1). Healthy siblings also had a lower ratio although not significantly. In addition to data shown in Table III-1, epileptic children had a mean ratio of 1.54 ± 0.5 which was significantly lower than that of healthy children who had a mean ratio of 2.3 ± 0.9 . Adult patients had a lower

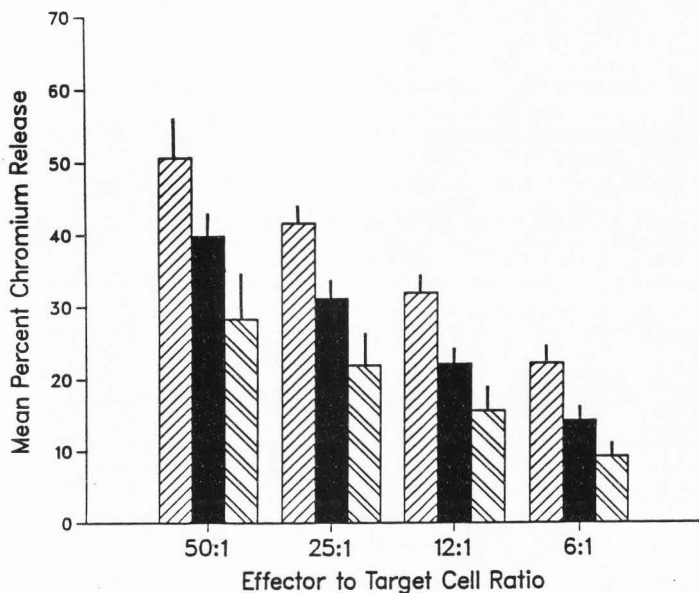


FIGURE III-1. Natural killer cell activity expressed as mean percent ⁵¹Cr release and standard error of the mean at various ratios of

effector to target cells. Peripheral blood mononuclear cells (PBMC) were isolated from 46 healthy unrelated subjects including 10 children and 36 adults (hatched), 19 epileptic patients including 15 children and 4 adults (solid black), and 6 healthy siblings including 5 children and 1 adult (diagonal lines). Mean releases effected by PBMC from patients and their siblings were significantly lower at each effector to target cell ratio ($p < 0.001$).

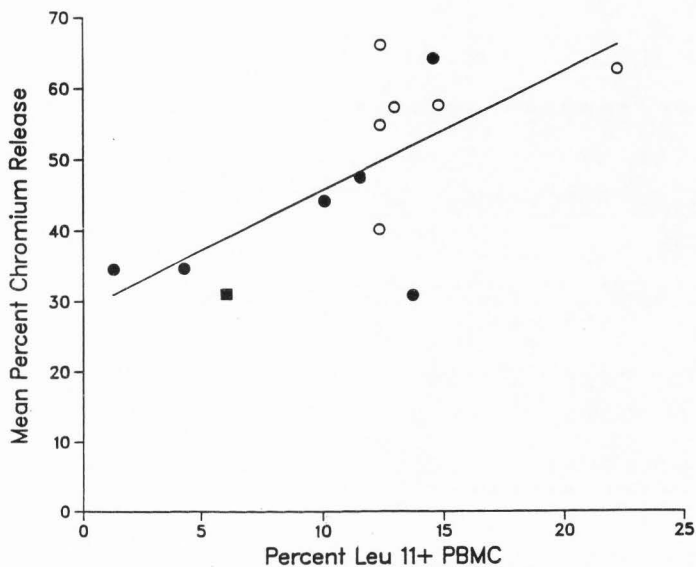


FIGURE III-2. A significant correlation ($r = 0.68$, $p < 0.05$) was found between percent Leu11+ peripheral blood mononuclear cells and natural killer cell activity of various subjects: 6 healthy ○, 6 patients ●, and 1 sibling ■. Cytotoxicity shown is from the effector to target cell ratio of 50:1.

TABLE III-1.

LYMPHOCYTE ENUMERATION OF EXPERIMENTAL SUBJECTS

| Group | ^a N | ^b OKT4+/OKT8 | ^c 7.2+ | ^d RFC% | ^e WBC |
|---------------------|-------------------|----------------------------|----------------------|----------------------|---------------------|
| Healthy Subjects | 15 | 2.14 _f | 13.4 | 77.4 | 7235 |
| | | 0.75 _f | 5.5 | 7.8 | 1911 |
| Patients | 17 | 1.54 _g | 15.9 | 77.9 | 7235 |
| | | 0.42 | 7.5 | 8.3 | 2261 |
| Siblings | 6 | 1.68 | 14.6 | 79.4 | 6290 |
| | | 0.37 | 4.7 | 2.7 | 0831 |

a. Numbers of subjects studied.

b. Mean ratio of the proportion of peripheral blood mononuclear cells which were OKT4+ to OKT8 +.

c. Mean percent of monocyte-depleted peripheral blood mononuclear cells positive for the monoclonal antibody determinant 7.2.

d. Mean percent of rosette forming cells from peripheral blood mononuclear cells depleted of macrophages.

e. Total white blood cells as determined from aliquots of whole blood.

Values expressed as mean $\times 10^3$ per mm³.

f. Standard deviation of the mean.

g. Significantly reduced ($p < 0.05$).

ratio of 1.7 ± 0.4 compared with healthy adults with a ratio of 1.9 ± 0.4 , however this difference was not significant.

The monoclonal antibody anti-7.2 was used to estimate the numbers of B-cells in PBMC depleted of monocytes (Table III-1). No differences were found in the percent of 7.2+ cells from epileptic patients as compared with that found in cells from healthy subjects. A T-cell rosetting assay with sheep red blood cells was done as an indication of total T-cells and the percent rosette-forming cells (RFC) are listed in Table III-1. Cells from neither children nor adult patients showed deviations from the normal range of 70-80 percent. Also presented is the total white blood cell (WBC) counts for the various groups of subjects. These counts were quite variable and there were no significant differences seen between the counts obtained from patients and healthy subjects.

Lymphocyte blastogenesis assays. Responses to several concentrations of the T-cell mitogens PHA and Con A and the B-cell mitogen PWM are shown in Figures III-3, III-4, and III-5, respectively. Considerable variation was observed in response to these mitogens and no significant differences among PBMC from the various groups were found.

Mitogenic responses of PBMC from 5 epileptic children who displayed symptoms of autism were analyzed separately and were found to be significantly decreased for PHA, Con A, and PWM. Details of these studies will be presented and discussed elsewhere (Warren et al, 1985).

ADCC assay. In contrast to findings with the NK cell assay, ADCC activity by cells of epileptic patients was not significantly

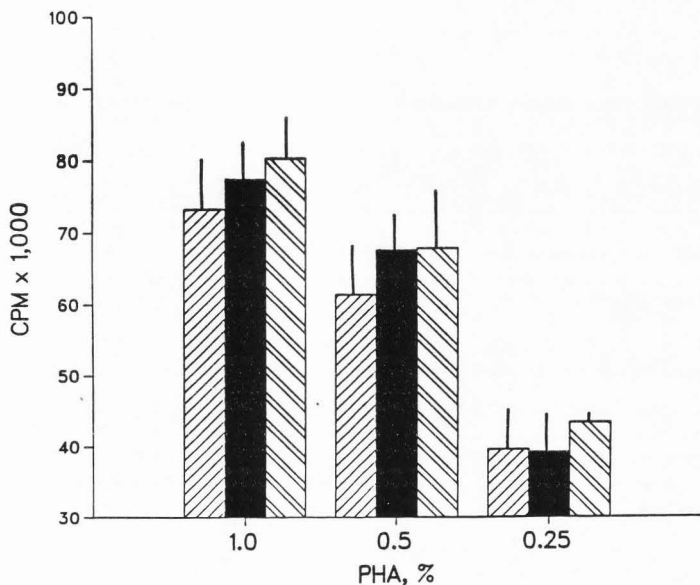


FIGURE III-3. Mean incorporation of counts per minute of tritiated thymidine and standard error of the mean by peripheral blood mononuclear cells from 17 unrelated healthy subjects including 9 children and 8 adults (hatched), 13 epileptic patients including 9 children and 4 adults (solid black), and 5 healthy siblings including 4 children and 1 adult (diagonal lines), following incubation with various concentrations of phytohemagglutinin. No significant differences were found between results obtained using cells from epileptic patients and healthy subjects.

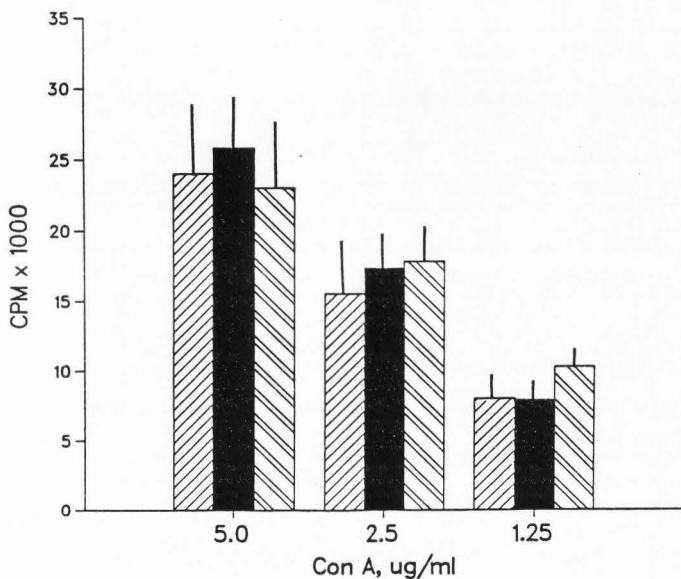





FIGURE III-4. Mean incorporation of counts per minute of tritiated thymidine and standard error of the mean by peripheral blood mononuclear cells from 17 unrelated healthy subjects including 9 children and 8 adults , 13 epileptic patients including 9 children and 4 adults , and 5 healthy siblings including 4 children and 1 adult , following incubation with various concentrations of concanavalin A. No significant differences were found between results obtained using cells from epileptic patients and healthy subjects.

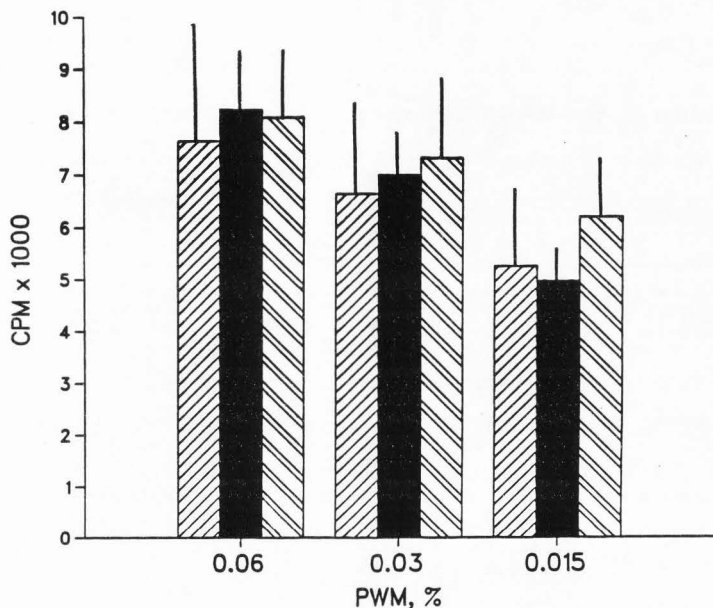





FIGURE III-5. Mean incorporation of counts per minute of tritiated thymidine and standard error of the mean by peripheral blood mononuclear cells from 16 unrelated healthy subjects including 7 children and 9 adults , 13 epileptic patients including 9 children and 4 adults , and 5 healthy siblings including 4 children and 1 adult , following incubation with various concentrations of pokeweed mitogen. No significant differences were found between results obtained using cells from epileptic patients and healthy subjects.

different from that induced by PBMC of the siblings of the patients or the other healthy subjects (Figure III-6).

Discussion

The current study presents evidence for altered immune function and lymphocyte subsets of epileptic patients and their siblings. Specifically, NK cell activity was found to be significantly depressed in epileptic patients and their siblings. In addition, the proportion of Leu 11+ NK cells was significantly lower in epileptic patients than in healthy subjects. A correlation was found between NK cell activity and percent Leu 11+ cells, suggesting that the low NK cell activity observed in some of the patients may be due to a low proportion of cells in the blood bearing this determinant. However, other mechanisms, such as impaired lytic activity, could be operating.

Evidence indicates that NK cells play a vital role in host defense against virally infected and transformed (tumor) cells (Herberman, 1984). The reduced NK cell activity found in the patients and their siblings may not be of a level considered to be clinically meaningful, and in fact none of the experimental subjects in this study were considered to be immunologically compromised. However, a depressed NK cell activity could be responsible for the slightly increased susceptibility to respiratory infections seen in epileptic patients treated with phenytoin (Gilhus and Aarli, 1981a). Since only 6 siblings were available for study, the association of a reduced NK cell activity in these subjects must be regarded as tentative.

NK cells produce and are activated by various lymphokines and are involved in immune regulation and activation (Herberman, 1984; Scala et

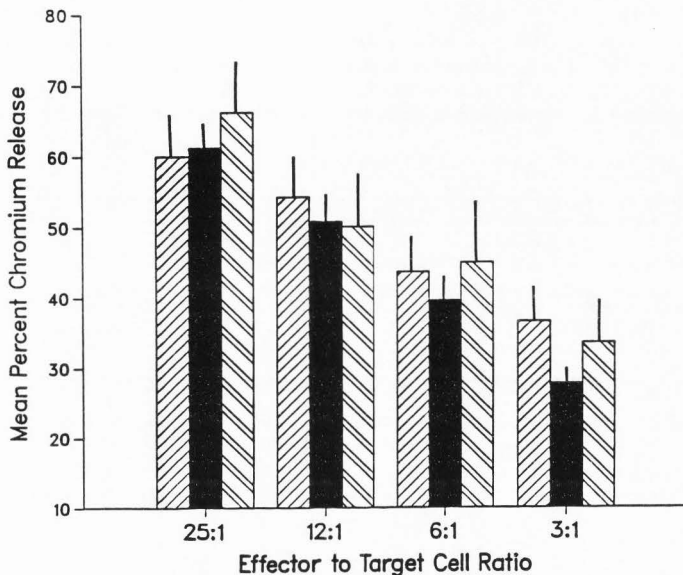





FIGURE III-6. Antibody-dependent cell-mediated cytotoxicity expressed as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells. Peripheral blood mononuclear cells were isolated from 9 unrelated healthy subjects including 1 child and 8 adults , 13 epileptic patients including 9 children and 4 adults , and 5 healthy siblings including 4 children and 1 adult . No significant differences were found between results obtained using cells from epileptic patients and healthy subjects.

al, 1984). Thus, low numbers or reduced activity of NK cells could influence immune functions other than NK cell activity or may be a reflection of other immune abnormalities.

A significantly lower ratio of OKT4+/OKT8+ cells was found in epileptic patients. Altered ratios sometimes are associated with immune deficiency if the ratio is low, such as that seen in acquired immunodeficiency syndrome (Fauci et al, 1984) or autoimmune mediated disease such as multiple sclerosis (Bach et al, 1980; Reinherz et al, 1980) if the ratio is high. Epileptic patients on multi-drug therapy have shown lower specific antibody titers for parainfluenza virus III (Czlonkowska and Iwinska, 1977) and some patients on phenytoin therapy lack delayed-type hypersensitivity to common test antigens (mumps, Candida, and Streptococcal antigen) and fail to produce antibody to Salmonella typhi antigen (Sorrel et al, 1971).

In contrast to observed differences in NK cell killing, no deviations in ADCC activity were found from cells isolated from patients versus healthy subjects. Evidence is accumulating that ADCC is likely mediated by a different mechanism than NK cell killing (Koren and Williams, 1978; Suthanthiran et al, 1984). Blastogenic response to T-cell and B-cell mitogens were found to be variable and no significant differences were seen except in epileptic patients who also expressed the symptoms of autism (discussed separately in Warren et al, 1985). The percent of monocyte-depleted PBMC forming rosettes with SRBC (an indication of total T-cells in this population) from the patients were within the normal range. This finding contrasts with previous studies demonstrating decreased RFC in epileptic patients not receiving AED (Czlonkowska and Korlak, 1980) and patients receiving AED (Fontana et

al, 1978c) but agrees with the report of Gilhus et al. (1982b) who found normal proportions of RFC in epileptic patients treated with AED.

The varying degrees of immune alteration seen in epileptic patients on AED therapy indicate that factors other than those associated with AED likely play a role in modulating various components of the immune system in these patients. Chief among these factors is probably a genetic mechanism. It has been reported that IgA deficiency in association with phenytoin therapy appears to be genetically determined and linked to the HLA-A2 antigen (Gilhus et al, 1982c; Aarli and Gilhus, 1983). Also, various HLA antigens are associated with immune alterations in epileptic patients and with epilepsy itself: HLA-A2 antigen has been linked to decreased IgG titers and altered immunoglobulin subclasses (Fontana et al, 1978b) while HLA-B7 antigen has an increased frequency among epileptic patients (Smeraldi et al, 1978). The current study has shown a depressed NK activity and a low OKT4+/OKT8+ ratio in both epileptic children and their siblings. Thus, it would appear that these immune system abnormalities also have a genetic basis.

The question of which factors cause immune alterations in epileptic patients remains a complex one. There is evidence for drug effects and disease effects: that is immune alterations may result from disease processes or conversely, epilepsy in some cases may be a result of an immune abnormality such as autoimmunity. It is interesting that various types of autoantibodies have been found in epileptic patients: anti-nuclear (Chiu et al, 1982), anti-mitochondrial (Anderson and Moskilde, 1977), anti-muscle and anti-brain nicotinic acetylcholine receptor (Fontana et al, 1978a), and brain autoantigens (Popova et al,

1975). It has been suggested that AED may activate a latent autoimmune response (Alarcon-Segovia et al, 1972) and various AED are considered to be lupus erythematosis-activating drugs (Alarcon-Segovia and Fishbein, 1975).

This study has demonstrated immune alterations not previously found in epileptic patients. Further, evidence indicated that the depressed NK function and low OKT4+/OKT8+ ratios in epileptic patients is likely not caused by either AED therapy or disease processes of epilepsy but rather may be genetically determined. These results indicate the importance of appropriate control subjects in immune studies of epileptic patients: both family members, such as siblings, and unrelated age- and sex-matched control subjects are necessary in order to properly interpret observations. Such studies would shed further light on the basis of immune abnormalities seen in epileptic patients.

CHAPTER IV
EFFECTS OF PHENYTOIN AND CARBAMAZEPINE ON
NATURAL KILLER CELL ACTIVITY
IN VITRO

Introduction

Natural killer (NK) cells are a subpopulation of lymphoid cells with the innate ability to recognize certain target cells as abnormal and mount a cytotoxic attack against these target cells resulting in their lysis. Killer (K) cells are also contained within the lymphoid population and are responsible for antibody-dependent, non-complement mediated killing. The activities of both cells reside predominantly in the large granular lymphocyte fraction. Their activities are thought to be important in host defense and immunosurveillance based on evidence in both human subjects and experimental animals.

Phenytoin and carbamazepine, widely used antiepileptic drugs (AED), have been associated with immune function alterations in man. In particular, phenytoin has been found to depress IgA levels (Yabuki and Nakaya, 1976; Martinez-Cairo et al, 1980), specific antibody titers and cell mediated reactions (Sorrel et al, 1971). More severe reactions to this drug include complex immunodeficiencies (Masi et al, 1976), agranulocytosis (Tsan et al, 1976) possibly autoimmune in nature (Taetle et al, 1979), lymphadenopathies (Bellido et al, 1977), lymphoma (Lukes and Tindle, 1975), and a multitude of hypersensitivity reactions (Booker, 1975; Haruda, 1979). Carbamazepine has also been linked with agranulocytosis (Luchins, 1984), neutropenia, and leukopenia (Cereghino et al, 1974; Hart and Easton, 1981).

There appears to be a different individual susceptibility to the immunosuppressive effects of AED (Massimo et al, 1976). Evidence is emerging that these drugs do have many effects on the immune system, particularly phenytoin, as shown in clinical studies. This investigation studied the effects of two AED on killer cell activities.

Materials and Methods

Effector cells. Freshly drawn blood was obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were obtained by centrifuging whole blood on a Ficoll-Hypaque density gradient. PBMC were then rinsed and suspended in RPMI-1640 medium (GIBCO, Grand Island, New York) containing penicillin (100 units/ml), streptomycin (100 mcg/ml), and 10 % fetal bovine serum (Hyclone Laboratories, Logan, UT).

Chemicals. Phenytoin (powder) was a gift from Parke-Davis Warner-Lambert Co. (Ann Arbor, MI). Phenytoin was dissolved in 0.9% NaCl at pH 11 with 4N NaOH and diluted with RPMI-1640 medium to the appropriate concentrations. Carbamazepine (powder) was obtained from Sigma Chemical Co. (St. Louis, MO), dissolved in propylene glycol (Sargent Welch Scientific Co., Skokie IL), and diluted with RPMI-1640 medium.

Experimental protocol. Three concentrations of phenytoin and carbamazepine chosen for this study were representative of levels used clinically. Plasma concentrations of 10-20 ug/ml phenytoin and 8 ug/ml carbamazepine were considered therapeutic levels. Levels of phenytoin and carbamazepine of 40 and 10-16 ug/ml, respectively, were in the

range considered acutely toxic: central nervous system effects such as nystagmus and ataxia become apparent in patients with plasma levels near or above these concentrations (Rall and Schleifer, 1980).

PBMC were pre-incubated for 20 hr in either supplemented medium alone or medium with drug. The cells were rinsed and resuspended in medium with or without drug present followed by assessment of natural killer cell activity.

Natural killer cell assay. This assay was carried out as described in the methods section of Chapter III.

Interferon pre-treatment. PBMC were pre-incubated with either 500 or 250 units/ml human alpha-interferon (Sigma) 18 hr prior to the NK cell assay. The PBMC were then rinsed and resuspended in medium followed by assessment of NK cell activity.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay. This assay was carried out as described in the methods section of chapter III.

Statistical analysis. Data were analyzed with ANOVA and the least significant difference test, with a 95% confidence interval for a normal distribution.

Results

Phenytoin and NK cell activity. Phenytoin in concentrations of 40, 20, and 10 ug/ml suppressed NK cell activity in a dose-dependent manner in vitro (Figure IV-1, $p < 0.001$). Phenytoin did not appear to alter lymphocyte viability since cells incubated for 24 hr in concentrations

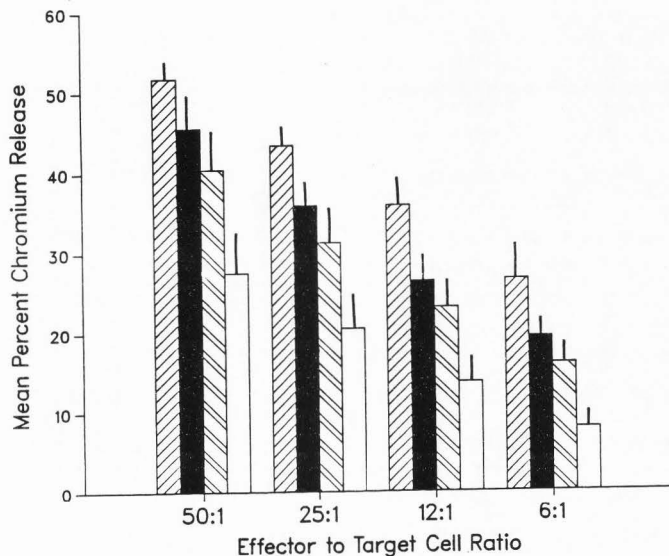






FIGURE IV-1. Peripheral blood mononuclear cells were pre-incubated with phenytoin and assessed for natural killer cell activity in the presence of 0 , 10 , 20 , and 40  ug/ml of phenytoin. Natural killer cell activity is expressed as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells: 50:1, 25:1, 12:1 and 6:1. Phenytoin at each concentration significantly depressed ^{51}Cr release ($p < 0.05$).

of phenytoin up to 40 ug/ml had the same viability as diluent-treated cells as shown by trypan blue exclusion (98.0%, data not shown).

PBMC pre-incubated for 20 hr with concentrations of phenytoin up to 40 ug/ml, rinsed several times, and suspended in drug-free medium effected NK cell cytotoxicity at levels not significantly different from that of diluent-treated PBMC (Table IV-1).

Carbamazepine and NK cell activity. The drug carbamazepine had a variable effect on NK cell activity (Figure IV-2). PBMC treated with 8 ug/ml carbamazepine had a slightly depressed activity as compared with diluent-treated cells though this decrease was not significant. Treatment of cells with 10 ug/ml resulted in a significant increase in activity as compared to cells treated with 8 or 16 ug/ml ($p < 0.05$). Carbamazepine may have a slight but variable effect on NK cell cytotoxicity depending upon its concentration.

Propylene glycol and NK cell activity. A comparison of the NK cell activity from PBMC treated with the standard medium diluent control for phenytoin which contains saline and RPMI-1640 with that for carbamazepine, which has a 1.4% final concentration of propylene glycol in RPMI-1640, indicated a significant depression of activity caused by the propylene glycol (Figure IV-3, $p < 0.001$). In another series of experiments it was found that propylene glycol concentrations as low as 0.1% v/v depressed NK cell activity of PBMC (data not shown).

Phenytoin and interferon. Because of the pronounced effect of phenytoin on NK cell activity, it was of interest to investigate the effect of this drug on the ability of alpha-interferon to augment NK

TABLE IV-1
PHENYTOIN AND NATURAL KILLER CELL ACTIVITY^a

| Treatment | Release at effector to target cell ratio | |
|---------------------------|--|------|
| | 12:1 | 6:1 |
| Diluent | 28.2 | 20.3 |
| | 9.7 | 8.0 |
| 10 ug/ml +/- ^b | 24.1 | 16.2 |
| | 5.8 | 6.8 |
| 20 ug/ml +/- | 21.1 | 12.4 |
| | 6.7 | 5.0 |
| 40 ug/ml.+/+ | 9.5 | 2.6 |
| | 4.1 | 2.9 |
| 10 ug/ml +/- ^c | 27.3 | 19.6 |
| | 9.7 | 8.7 |
| 20 ug/ml +/- | 30.0 | 20.6 |
| | 8.4 | 10.1 |
| 40 ug/ml +/- | 29.5 | 21.6 |
| | 12.6 | 10.8 |

a. Human peripheral blood mononuclear cells were assessed for natural killer cell activity following incubation with phenytoin. Activity is expressed as mean percent chromium release and standard deviation at the effector to target cell ratios indicated: N=4 for all means.

b. Phenytoin, included in both the pre-incubation and in assay of peripheral blood mononuclear cells, significantly depressed natural killer cell activity ($p < 0.001$).

c. Phenytoin, included in the pre-incubation of peripheral blood mononuclear cells but not in the assay, did not depress natural killer cell activity compared to cells treated with diluent only.

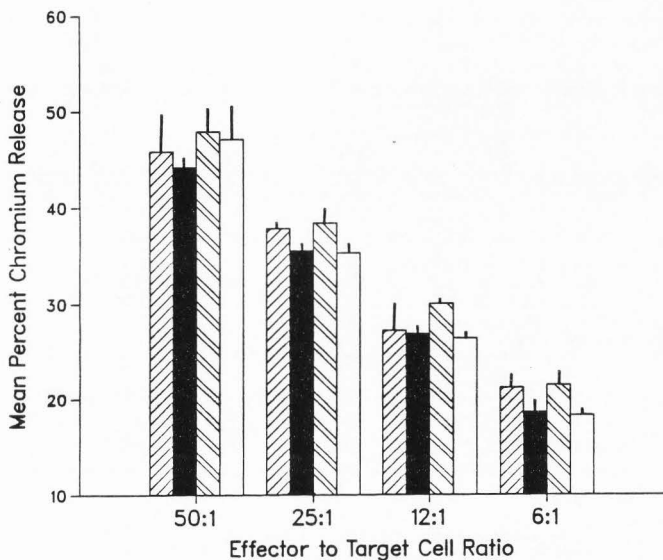






FIGURE IV-2. Peripheral blood mononuclear cells were pre-incubated with carbamazepine and assessed for natural killer cell activity in the presence of 0 , 8 , 10 , and 16  ug/ml of carbamazepine. Natural killer cell activity is expressed as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells: 50:1, 25:1, 12:1, and 6:1. Carbamazepine had no significant effect on mean percent ^{51}Cr release.

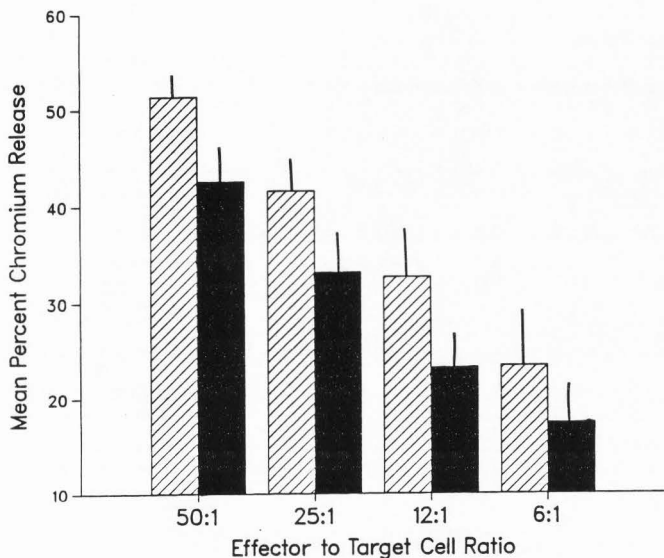




FIGURE IV-3. Peripheral blood mononuclear cells were pre-incubated with propylene glycol and assessed for natural killer cell activity in the presence of medium alone  or 1.4% v/v concentration of propylene glycol . Natural killer cell activity is expressed as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells: 50:1, 25:1, 12:1 and 6:1. Propylene glycol significantly depressed ^{51}Cr release ($p < 0.001$).

cell cytotoxicity. PBMC were incubated with 250 units alpha-interferon/ml and phenytoin, rinsed, and assessed for NK cell activity in the presence of drug. Phenytoin did not block the ability of PBMC to respond to interferon at any of the phenytoin concentrations but did depress NK activity in a dose-dependent manner (Figure IV-4, $p < 0.001$). The pattern of phenytoin-induced depression of interferon-treated cells was similar to that seen with PBMC not treated with interferon. Similar results were found when PBMC were incubated in 500 units of interferon/ml.

Phenytoin and ADCC. Phenytoin also inhibited ADCC in a dose-dependent fashion (Figure IV-5, $p < 0.001$). However, the depression of this activity occurred only at phenytoin concentrations of 20 and 40 ug/ml and not at 10 ug/ml as seen with NK cell killing.

Discussion

In the current study, phenytoin depressed basal and interferon-augmented NK cell cytotoxicity in a dose-dependent manner in vitro. This depression appeared reversible since PBMC incubated with phenytoin and resuspended in medium without drug showed normal activities. The pattern of inhibition of NK cell activity of PBMC treated with interferon and those not treated with interferon appeared the same, indicating that inhibition results from the ability of phenytoin to interfere with the basal cytotoxic mechanisms and not mechanisms of interferon augmentation. In fact, a comparison of the depression caused by phenytoin with or without interferon pre-treatment suggests that interferon may act in an antagonistic fashion to phenytoin. For example, at the effector to target cell ratio of 6:1, interferon

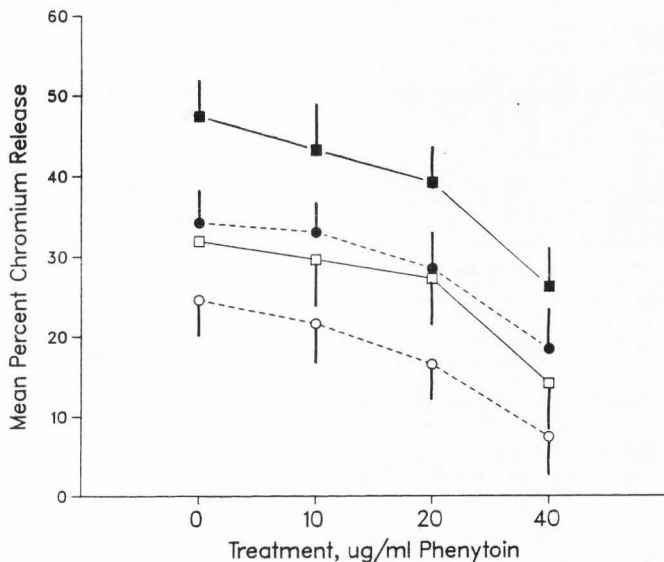


FIGURE IV-4. Peripheral blood mononuclear cells were pre-incubated with phenytoin and 250 Units alpha interferon/ml and assessed for natural killer cell activity in the presence of 0, 10, 20, and 40 ug/ml of phenytoin. Natural killer cell activity is expressed as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells and pre-incubation with and without interferon: 12:1 + interferon ■, 12:1 - interferon □, 6:1 + interferon ●, and 6:1 - interferon ○. Phenytoin at each concentration significantly depressed ^{51}Cr release of interferon treated and untreated cells ($p < 0.05$).

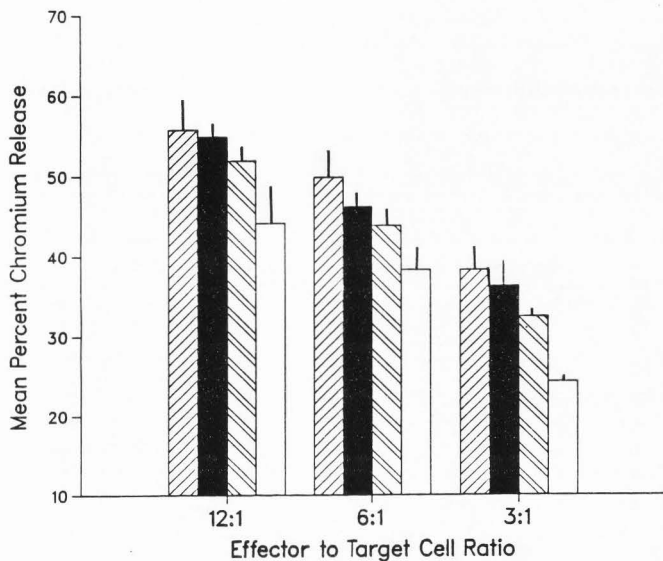






FIGURE IV-5. Peripheral blood mononuclear cells were pre-incubated with phenytoin and assessed for antibody-dependent cell-mediated cytotoxicity in the presence of 0 , 10 , 20 , and 40  ug/ml of phenytoin. Antibody-dependent cell-mediated cytotoxicity is expressed as mean percent ^{51}Cr release and standard error of the mean at various ratios of effector to target cells: 25:1, 12:1 and 6:1. Phenytoin at 20 and 40 ug/ml concentration significantly depressed ^{51}Cr release ($p < 0.05$).

stimulated control and phenytoin-treated, 40 ug/ml, cells by 139% and 248%, respectively. However, additional work would be needed to confirm this effect.

The present study shows a pronounced phenytoin-induced depression of NK cell activity by PBMC isolated from healthy donors and so is a short-term assessment of phenytoin's effect on this activity. This depression was found at concentrations of phenytoin representing plasma levels considered to be therapeutic to neurotoxic in range. The long-term effect of phenytoin on NK cells is unknown and is presently being investigated using an in vivo animal model.

NK cells are thought to play a vital role in host defense and to be a first line of defense against developing neoplasms. Evidence to support these roles have been found in both man and animals (Herberman, 1984). This effect of phenytoin on NK cell mediated cytotoxicity is interesting in light of the association of hydantoin medications, including phenytoin, with lymphoproliferative disorders and lymphoma. There are numerous reports of patients on long-term hydantoin treatment who have developed these disorders (Lukes and Tindle, 1975) and a 10-fold increase in the expected incidence of lymphoma in epileptic patients on hydantoin therapy has been reported (Anthony, 1970).

The mechanism by which phenytoin depresses NK cell activity is presently unknown. Phenytoin may alter the binding of the effector cell to its target and/or inhibit the ability of the effector cell to lyse its target once a conjugate is formed. To distinguish between these requires a single cell assay which is currently under investigation.

One theory on the mechanism by which phenytoin suppresses seizure activity is by alteration of Ca^{++} fluxes and Ca^{++} dependent processes (Greenberg et al, 1984; Sugaya et al, 1984 and 1985; Caldwell and Harris, 1985) and by inhibition of Ca^{++} release from stores (Sugaya et al, 1985). Ca^{++} and the calcium-binding protein calmodulin have been shown to be important in NK cell cytotoxicity (Rochette-Egly and Tovey, 1984) and phenytoin may depress NK cell activity by altering Ca^{++} fluxes across cell membranes or Ca^{++} binding proteins. The ability of interferon to augment NK cell activity is also dependent upon movements of Ca^{++} and the presence of calmodulin. Phenytoin depressed basal and interferon-induced NK cell lysis, thus phenytoin may depress NK cell activity via its effect on Ca^{++} and its effector proteins. DeLorenzo (1982) has indicated that phenytoin inhibits the Ca^{++} -calmodulin kinase system but not calmodulin itself. Thus the effects of phenytoin on Ca^{++} and Ca^{++} -binding proteins are unclear. It is conceivable that phenytoin alters NK cell activity by altering Ca^{++} channelling and Ca^{++} mediated events either directly such as by inhibition of calmodulin or indirectly such as through decreased phosphorylation of specific proteins after the Ca^{++} signal, as has been shown in synaptic vesicles (DeLorenzo, 1980).

Phenytoin also depressed ADCC but only at the 2 higher concentrations studied. Large granular lymphocytes contained within the PBMC population have been shown to be capable of both NK cell cytotoxicity and ADCC (Bradley and Bonavida, 1982) although by a different mechanism (Koren and Williams, 1978). Killing by either cell type is a multi-step though ill-defined process, involving lytic granules (Podack, 1985), reactive oxygen intermediates (Suthanthiran et al, 1984), and

outward K⁺ currents during the lethal hit stage (Chandy et al, 1985). It is possible that phenytoin depresses both NK cell killing and ADCC by the same mechanism: that is other pharmacologic agents have been shown to depress both activities (Henney and Gillis, 1984). This indicates that some steps may be shared in the mechanism of these two types of killing.

It is reasonable to assume that depression of NK cell activity by phenytoin can result in lymphoma and lymphoproliferative states. Diethylstilbestrol, a synthetic non-steroidal agent used therapeutically in man, has been found to depress NK cell activity in vivo (Kalland and Haukaas, 1981) and the lytic phase of this killing in vitro (Kalland and Campbell, 1984). There is a clear association between in utero exposure to diethylstilbestrol and the development of vaginal and uterine cancer (McLachlan and Dixon, 1976). Ultraviolet radiation has also been found to suppress the post-binding lethal stage of NK cell cytotoxicity and ADCC (Weitzen and Bonavida, 1984).

Various studies suggest that phenytoin causes chromosomal aberrations (Herna and Obe, 1977) and sister-chromatid exchanges (Kulkarni et al, 1984) in humans exposed to this drug long-term. Furthermore, De Oca-Luna et al (1984) found a significant increase in micronucleated polychromatic erythrocytes in mice exposed to multiple injections of phenytoin, an indication of chromosomal aberrations as shown by nuclear fragments. Phenytoin may then act two ways in the development of neoplasms: by a direct or genotoxic mechanism and by immunosuppression, an epigenetic mechanism.

Phenytoin has also been found to depress mixed lymphocyte reaction in vitro (Bluming et al, 1976) and alter lymphocyte blastogenesis to

mitogens (Neuwelt et al, 1983). Thus, there may be a variety of mechanisms by which phenytoin causes immune abnormalities, possibly leading to immunodeficiencies on the one hand and lymphoma and lymphoproliferative states on the other. NK cells, as well as being important for immunosurveillance, are thought to be important in combating viral infections (Herberman, 1982) and in immunoregulation, such as through the production of lymphokines (Scala et al, 1984). As a result, depression of NK cell activity by phenytoin could lead to a variety of immune system alterations.

Carbamazepine did not significantly depress NK cell activity from diluent-treated cells and slightly increased activity at 10 ug/ml concentration. This effect was not dramatic however, and may be biologically insignificant. Carbamazepine has been found to depress Ca⁺⁺-dependent phosphorylation of synaptosomal proteins similar to phenytoin but at much greater concentrations than found therapeutically (DeLorenzo, 1980). It is interesting that phenytoin and carbamazepine are useful in controlling similiar seizure types and have considerable overlap in their 3 dimensional structures (Rall and Schleifer, 1980), yet are dissimilar in their effects on NK cell activity.

Propylene glycol depressed NK cell activity in a dose-dependent manner. This effect should be kept in mind with its use as a diluent for drugs in immunological assays. Ethylene glycol has also been found to depress NK cell activity in vitro, presumably due to its ability to act as a hydroxyl radical scavenger (Suthanthiran et al, 1984). Due to its structural similarities to ethylene glycol, propylene glycol probably depresses NK cell activity by this same mechanism.

CHAPTER V
EFFECT OF PHENYTOIN ON ANTIBODY
PRODUCTION: USE OF A
MURINE MODEL

Introduction

Phenytoin, a widely used antiepileptic drug (AED), has been found to alter both humoral and cellular immunity in man. These alterations include reduced antibody response and delayed-type sensitivity *in vivo* (Sorrel et al, 1971), and decreased blastogenic response (Neuwelt et al, 1983), lymphocyte protein synthesis, and numbers of T rosette-forming cells (Fontana et al, 1978c) from patients treated with phenytoin.

A complex interplay of drug effects, disease processes of epilepsy, and genetic factors are all thought to be associated with immune abnormalities seen in epileptic patients treated with AED. For example, long-term phenytoin treatment is associated with IgA deficiency in up to 21% of patients (Sorrel et al, 1971): evidence indicates that susceptibility to this deficiency is genetically determined and linked to the HLA-A2 antigen (Gilhus et al, 1982c; Aarli and Gilhus, 1983). Furthermore, Gilhus and Aarli (1981b) found IgA suppression to be more related to a history of febrile convulsions than to the type of epilepsy or drug therapy for some patients.

An animal model to study effects of AED on immune function would allow a clear investigation into the effects of AED on the immune system. Animals have been used successfully in studies of epilepsy (Chung and Johnson, 1984; McNamara, 1984; Morishita et al, 1984) and

AED: for example in investigations of valproate-induced hepatotoxicity (Turnbull et al, 1983; Becker and Harris, 1983; Granneman et al, 1984; Kesterson et al, 1984) and phenytoin-associated teratogenesis (Hicks et al, 1983; Watkinson and Millicovsky, 1983). However few animal studies have been used to investigate effects of AED on immune function.

The present report describes a murine model to study effects of phenytoin on specific antibody production. Results, and the model's usefulness to assess other immune function disorders observed in clinical studies, are discussed.

Materials and Methods

Drug and dosage levels. Phenytoin (powder), a gift from Parke-Davis Warner-Lambert Co. (Ann Arbor, MI), was dissolved in 0.9% NaCl at pH 11 with 4N NaOH. Three dosage levels for the mice were chosen from a pilot study. A low dose of 10 mg/kg (10 mg/ml) was near the dose found to be effective in controlling electroshock seizures in other strains of mice (Swinyard and Woodhead, 1982; Loscher and Meldrum, 1984; Zelger et al, 1983) and was considered to be a therapeutic dose. A high dose of 40 mg/kg (40 mg/ml) approached the neurotoxic dose 50% in this strain at 2 hr post injection as indicated by neurological defect. An animal was considered to be suffering from acute toxicity if it was unable to maintain balance on a rotating rod (6rpm) for 1 min in each of 3 trials at the time of peak drug effect (2 hr) (Swinyard and Woodhead, 1982). This dose was taken to represent frequent periods of acute toxicity. An intermediate dose of 20 mg/kg was also chosen.

Experimental animals. Mice of the NFS strain were bred at our facility and given food and water ad libitum. Animals 5 weeks of age

were divided into groups using both sexes per treatment, and dosed with phenytoin or diluent by intraperitoneal injection daily for 28 days.

Immunization. Mice were injected subcutaneously with 2 mg (0.1 ml) bovine serum albumin (BSA) on day 3 of diluent or phenytoin treatment and challenged with 1 mg on day 23 (Koller et al, 1983). BSA was dissolved in water at 40 mg/ml and emulsified 1:1 with Freund's incomplete adjuvant (Sigma Chemical Company, St. Louis, MO). On day 29 the animals were lightly anaesthetized with ether and bled to determine hematocrits, white blood cell (WBC) counts, and titers of antibody to BSA.

Antibody measurements. Antibodies to BSA were titered using an indirect enzyme-linked immunosorbent assay (ELISA). BSA at a concentration of 1 mg/ml was aliquoted into a 96-well Falcon Flexible plate 3912 (Beckton and Dickinson and Co., Oxnard, CA) in a volume of 0.1 ml/well followed by a 30 min incubation at room temperature. Liquid was then decanted out of the wells and 0.2 ml of 1% human serum albumin (Sigma) added to the wells as a blocking agent. The contents within the wells of the plate were incubated for 30 min followed by decanting of the liquid. Mouse serum was then added in a volume of 0.05 ml at dilutions ranging from 1/10 to 1/1,000 and allowed to incubate for 1 hr. Liquid was then decanted out and the wells rinsed 3 times with phosphate buffered saline (PBS). A 0.05 ml aliquot of peroxidase-conjugated goat anti-mouse IgG (Tago, Inc., Burlingame, CA) diluted 1/1000 was added to the wells followed by 90 min of incubation. Liquid was then decanted out, the wells rinsed 4 times with PBS, and 0.05 ml of the substrate ABTS (2,2'-azinodi-(3-ethylbenzthiazoline

sulfonic acid diammonium salt, Sigma) at a concentration of 0.91mM was added. Color changes from negative control wells were read 30 min after this with a MicroElisa minireader (Dynatech) using a 410 nm filter. Normal mouse serum was used as a negative control.

Statistical analysis. Data were analyzed with analysis of variance and least significant difference test with the 95% confidence interval for a normal distribution.

Results

Immunization and antibody titer. Phenytoin induced a dose-dependent decrease in IgG production against BSA in mice (Figure V-1). This alteration in antibody production was significant for mice receiving both the 20 and 40 mg/kg dose at each of 2 dilutions of serum ($p < 0.05$). No differences in antibody production between male and female mice were observed.

Body and specific organ weights. In general, the animals gained or maintained their weights during the course of the experiment with the exception of the female mice given 40 mg/kg phenytoin. These female mice lost weight during the first 2 weeks of treatment and during the third week the mouse that had lost the most weight died. By day 22 these mice had weights not significantly different from female mice given diluent alone (Table V-1). Specific organ weights on the basis of g/g body weight for thymus, spleen, and liver were variable and no significant differences were seen between phenytoin-treated and diluent-treated mice (Table V-2). Grossly, no changes in the appearance of these or other organs were seen in phenytoin-treated mice.

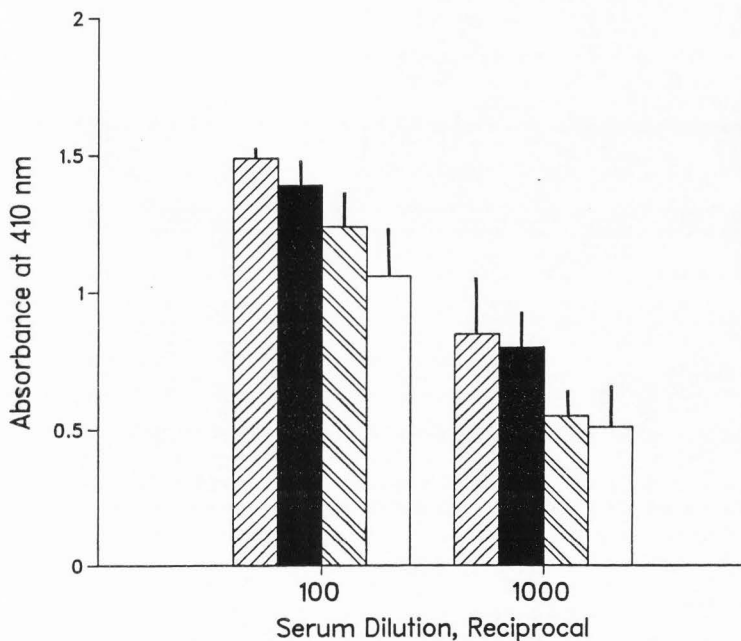






FIGURE V-1. Mice received diluent alone , or 10 , 20 , or 40  mg/kg phenytoin for 28 days during which time they were sensitized and challenged with bovine serum albumin (BSA). Serum collected on day 29 was diluted and assayed for IgG production specific for BSA. IgG production is expressed as a mean absorbance and standard error of the mean for serum dilutions of 1/100 and 1/1000. A significant decrease in IgG specific for BSA was found in mice given 20 and 40 mg/kg phenytoin ($p < 0.05$).

TABLE V-1
ANIMAL WEIGHTS AND PHENYTOIN EXPOSURE

| Treatment ^a | Female mice | | | | Male mice | | | |
|------------------------|--------------------------|-------------|-------------|--------------------------|-------------|-------------|-------------|-------------|
| | Diluent | 10 | 20 | 40 | Diluent | 10 | 20 | 40 |
| Day 0 ^b | 18.6 1.2 ^c | 17.6 0.7 | 17.8 0.8 | 18.4 1.7 | 20.3 1.4 | 21.3 1.6 | 20.5 1.5 | 21.0 1.1 |
| Day 8 | 19.5 1.5 | 19.2 0.8 | 18.9 0.4 | 17.7 1.8 | 21.1 1.6 | 23.0 1.9 | 21.8 2.2 | 21.5 1.0 |
| Day 15 | 20.6 1.5 | 19.7 1.3 | 19.5 0.0 | 17.5 ^d 2.0 | 23.1 1.1 | 24.4 2.5 | 22.2 2.3 | 21.5 0.7 |
| Day 22 | 20.9 1.1 | 20.7 0.8 | 20.3 0.4 | 19.9 2.0 | 24.6 1.1 | 26.1 2.7 | 22.6 2.8 | 22.6 1.1 |
| Day 29 | 22.0 0.0 | 21.4 0.8 | 20.8 0.8 | 20.7 1.6 | 25.3 1.3 | 26.3 2.1 | 22.2 1.1 | 23.7 1.2 |

a. Animals were injected daily with diluent or phenytoin at doses of 10, 20, or 40 mg/kg for 28 days during which time they were sensitized and challenged with bovine serum albumin.

b. Mean weights at the beginning of each week and at the time of sacrifice. Weights were listed separately for female and male mice due to a difference in weight between the sexes. N=5 for days 0, 8, and 15. N varies from 2-4 on days 22 and 29.

c. Standard deviation of the mean.

d. Significantly decreased ($p < 0.05$).

TABLE V-2
 SPECIFIC ORGAN WEIGHTS and BLOOD VALUES
 AFTER PHENYTOIN EXPOSURE

| Treatment : ^a | Diluent | 10 | 20 | 40 |
|----------------------------|---------------------------|--------------|--------------|--------------|
| Mice per Treatment | 5 | 7 | 6 | 8 |
| Thymus Weight ^b | 2.44 0.45 ^c | 3.38 1.08 | 3.27 0.80 | 2.55 0.65 |
| Spleen Weight ^d | 5.85 1.04 | 5.48 0.81 | 6.05 0.76 | 5.04 0.89 |
| Liver Weight ^e | 7.40 0.63 | 6.65 0.59 | 6.71 0.61 | 7.95 1.03 |
| Hematocrit ^f | 46.4 2.1 | 46.7 2.2 | 47.0 1.8 | 43.4 4.2 |
| WBC Count ^g | 10.2 6.3 | 16.5 10.4 | 20.0 10.0 | 13.8 13.5 |

a. Mice were injected daily with diluent alone or phenytoin at doses of 10, 20, or 40 mg/kg for 28 days during which time they were sensitized and challenged with bovine serum albumin. Parameters studied were at time of sacrifice. No significant differences were found between mice treated with phenytoin or diluent only in any of the specific organ weights, hematocrits, or white blood cell counts.

b. Mean thymus weights in units of g/g body weight $\times 10^{-3}$.

c. Standard deviation of the mean.

d. Mean spleen weights in units of g/g body weight $\times 10^{-3}$.

e. Mean liver weights in units of g/g body weight $\times 10^{-2}$.

f. Mean hematocrit.

g. Mean white blood cell count in units of leukocytes/mm blood $\times 10^3$ at time of sacrifice.

Blood values. Female mice given 40 mg/kg had slightly lower hematocrits than diluent-treated mice, although this difference was not significant. White blood cell counts were variable and no significant differences were found between phenytoin- and diluent-treated animals (Table V-2).

Discussion

The experimental protocol was tolerated relatively well by the animals. Body weights were maintained or increased throughout the injection period except for the first 2 weeks for the female mice given 40 mg/kg phenytoin. Specific organ weights for thymus and spleen, which are central and peripheral lymphoid tissues, respectively, and for liver, were variable and no significant differences were found between mice given phenytoin or diluent. The organs were normal in appearance at sacrifice. White blood cell counts and hematocrits were similar in mice given phenytoin or diluent. These results suggest that phenytoin has no overt toxicity to these particular organs and blood cells.

The use of this animal model appears to be suitable for the study of effects of AED on immune function even at high doses of drug. Mice have been used to study the effects of immunomodulators (Nacy and Meltzer, 1984) and immunotoxins such as polycyclic aromatic hydrocarbons (Wojdani et al, 1984), 2,4-dichlorophenol (Exon et al, 1984), and heavy metals such as lead (Neilan et al, 1983).

The depression by phenytoin of IgG production in mice against the T-cell dependent antigen BSA, though not dramatic, appears to be dose-dependent with high doses having a more pronounced effect than the therapeutic dose. Phenytoin is considered the AED most associated with

immune system abnormalities in man (Aarli and Gilhus, 1983) having been found to alter immunoglobulin levels. In particular, serum IgA levels are depressed in some patients which may lower their resistance to infections (Gilhus and Aarli, 1981a). However, a predisposition to develop IgA deficiency while being treated with phenytoin on a long-term basis appears to be genetically determined (Gilhus et al, 1982a, Aarli and Gilhus, 1983).

Phenytoin has also been found to moderately alter IgG and IgM levels (Anderson and Moseklide, 1977; Czlonkowska et al, 1981). Fontana et al (1976) found IgG4 deficiencies in 13/21 patients receiving phenytoin therapy. Cerebrospinal fluid IgG levels have been found to be decreased in epileptic patients given phenytoin (Fossan and Aarli, 1979). Thus, the present finding of a moderate reduction in IgG production in mice is not surprising. Decreased IgG production may result in a depression of immune functions requiring IgG, for example antibody-dependent cell-mediated cytotoxicity by large granular lymphocytes and macrophage-mediated phagocytosis and killing.

It is interesting that offspring of pregnant mice given phenytoin displayed humoral immune dysfunction as a result of prenatal exposure to this drug (Chapman and Roberts, 1984). Mice showed a dose-dependent decrease in antibody production to pneumococcal polysaccharide, a T-independent antigen, but no changes in cell-mediated immunities measured by delayed-type hypersensitivity response to the contact allergen oxazolone. These results suggest that phenytoin given prenatally can adversely affect the normal development and expression of humoral immune function. Levo (1982) noted a decrease in antigenic challenge to sheep red blood cells in mice exposed to phenytoin.

However, this finding must be viewed with caution since neither data nor the protocol used were presented in this report. The above results and those presented in this report indicate the suitability of mice in studies of phenytoin on immune function: mice display alterations in immune function to phenytoin in ways similar to man without the complications and limitations associated with human studies.

The use of a murine model to study effects of various AED on immune function would be useful in investigating situations where it is thought that an interplay of drug and constitutional factors results in abnormalities. These would include altered lymphocyte numbers (Blanco et al, 1977) and populations (Chiu et al, 1982), immunoglobulin levels (Fontana et al, 1976; Tartara et al, 1981), and antibody production (Anderson et al, 1981) all of which have been seen in patients receiving phenytoin. Phenobarbital has also been shown to be potentially immunosuppressive (Park and Brody, 1971) and carbamazepine has been associated with hematopoietic and immunologic effects including neutropenia and leukopenia (Cereghino et al, 1974; Hart and Eastman, 1981). Both drug dose effects and mechanisms could be investigated using an animal model.

CHAPTER VI
EFFECT OF PHENYTOIN ON MURINE HEPATITIS
VIRUS INFECTION AND NATURAL
KILLER CELL ACTIVITY

Introduction

Phenytoin is a widely used drug for the treatment of epilepsy, psychosis, and cardiac arrhythmias. A number of immune system abnormalities are associated with use of this drug including deficiencies resulting in increased frequency of respiratory infections in patients receiving phenytoin on a long-term basis. Recurrent or chronic respiratory infections are especially predominant in patients with an IgA deficiency caused by phenytoin (Aarli and Gilhus, 1983). Phenytoin has also been associated with liver damage, however the incidence is low and appears to be a result of a host idiosyncratic reaction.

Few data are available in studies of the effects of phenytoin on other types of infections. This investigation examined effects of long-term phenytoin treatment on liver function and hepatitis infection in mice. Since hepatitis virus infects hepatocytes causing jaundice and degeneration of the liver, liver function tests were used as indicators of infection. Natural killer (NK) cell activity was also ascertained in cells from mice given phenytoin since these cells are thought to be important in host defense against viral infection (Herberman, 1984).

Materials and Methods

Phenytoin preparation and dosage levels. Phenytoin was obtained and prepared as described in the materials and methods section of Chapter V. Dosage levels of phenytoin chosen for this study, 10, 20, and 40 mg/kg, were considered to range from therapeutic to neurotoxic as described in Chapter V.

Virus. Murine hepatitis virus (MHV) preparations, a gift from Dr. Robert Sidwell (Utah State University), were prepared from liver homogenates of Swiss Webster SPF mice infected with MHV (titer of $10^{5.6}$ cell culture infectious dose 50 percent/ml). To determine a suitable dose for infection into NFS mice used for this study, serial dilutions of the virus were made and a single (0.1 ml) intraperitoneal dose given to groups of male and female NFS mice. The animals were observed daily and their deaths recorded. A dilution of the virus was chosen for the experiments which would give a low incidence of deaths in infected NFS mice.

Experimental protocol. Male and female NFS mice were bred at Utah State University and randomly assigned to 3 experimental groups each with 3 mice of each sex per treatment. Groups 1 and 3 received diluent or phenytoin daily for 23 and 18 days, respectively, except on day 15 when mice were injected with 0.1 ml of virus at a dilution of 10^{-5} . In group 1, dates of the animals deaths were recorded and animals still alive on day 28 were sacrificed. Group 3 animals were sacrificed on day 19, and the livers excised, photographed, and scored for jaundice. Serum was collected, frozen, and later analyzed for bilirubin content and activities of the enzymes aspartate aminotransferase (ASAT) and

alanine aminotransferase (ALAT). Group 2 animals received diluent or phenytoin for 18 days and were sacrificed on day 19. Serum was collected, frozen, and later analyzed for serum bilirubin, ASAT and ALAT levels. Spleens were removed and a cell suspension prepared by forcing the spleens through a screen. Red blood cells were removed by hypotonic shock and the cells rinsed and resuspended in RPMI-1640 medium (Gibco, Grand Island, New York).

Serum bilirubin and aminotransferase assays. Serum bilirubin levels were determined using Sigma diagnostic kit # 605-D (St. Louis, MO). Briefly, bilirubin in an aliquot of serum is coupled with diazotized sulfanilic acid (p-diazobenzenesulfonic acid) to form azobilirubin. The aliquots are then made alkaline and read on a spectrophotometer at 600 nm. Bilirubin standards are used to convert absorbances into mg/dl total bilirubin.

Serum ASAT and ALAT enzyme levels were determined using Sigma diagnostic kit #505-0P. Briefly, an aliquot of serum was added to the substrates aspartic acid and alpha-ketoglutaric acid, or alanine and alpha-ketoglutaric acid for ASAT and ALAT analysis, respectively. The oxalacetic or pyruvic acid formed by ASAT and ALAT respectively, are then reacted with 2,4-dinitrophenylhydrazine to form the highly colored phenylhydrazine product. The reaction mixture is then read spectrophotometrically at 490 nm. Aminotransferase standards are used to convert absorbances into Sigma/Frankel enzyme units/ml.

Natural killer cell assay. This assay was carried out as described in the materials and methods section of chapter III except that effector splenic cells (0.1 ml) in concentrations of 100, 50, 25, and

12.5 x 10⁶ cells/ml were incubated with 0.1 ml Cr-labelled Yac-1 murine lymphoma cells.

Statistical analysis. Data were analyzed with ANOVA and the least significant difference test with the 95% confidence interval for a normal distribution.

Results

Virus. A virus dilution of 10⁻⁵ gave a low incidence of mortality in diluent-treated mice and was selected for use in the phenytoin experiments. No difference in mortality between male and female mice was observed. Deaths occurred on days 4-8 post virus infection. Mice given 40 mg/kg phenytoin (group 1) had a significantly higher mortality rate as compared with diluent-treated mice ($p < 0.05$; Table VI-1).

Liver appearance. All livers from Group 3 mice were normal in appearance with the exception of a male mouse given 10 mg/kg phenytoin whose liver was jaundiced.

Bilirubin levels. Serum collected from mice in groups 2 and 3 were analyzed for assessment of liver function. In mice of group 2, no differences in total bilirubin levels were found in animals receiving any of the phenytoin doses (Table VI-2). However, in group 3 (virus infected mice), animals treated with 20 and 40 mg/kg phenytoin had significantly higher bilirubin levels than diluent-treated mice. Significantly higher bilirubin levels were found after virus infection in mice receiving 20 and 40 mg/kg of phenytoin.

Serum ALAT levels. Phenytoin treatment alone did not significantly

TABLE VI-1

PHENYTOIN AND MURINE HEPATITIS VIRUS INFECTION: LETHALITY

| Group 1 Experimental Mice | | |
|---------------------------|------------------------------|-----------------|
| Treatment ^a | Number deaths/number animals | Death incidence |
| Diluent | 1/9 | 11.1% |
| 10 mg/kg | 0/9 | 0.0% |
| 20 mg/kg | 0/9 | 0.0% |
| 40 mg/kg | 4/10 | 40.0% |

a. Male and female NFS mice were injected with diluent or 10, 20, or 40 mg/kg phenytoin for 23 days. On day 15, all mice were infected with murine hepatitis virus. Deaths were recorded and death incidence was calculated. Mice treated with 40 mg/kg of phenytoin had a significantly increased mortality as compared with mice given diluent alone ($p < 0.05$).

TABLE VI-2

PHENYTOIN AND MURINE HEPATITIS VIRUS INFECTION:
SERUM BILIRUBIN LEVELS

| Treatment ^a | Group 2 - MHV | Group 3 + MHV |
|------------------------|--|------------------|
| Diluent | 2.31 ^b 0.46 ^c | 2.12 1.90 |
| 10 mg/kg | 1.95 0.23 | 2.19 0.67 |
| 20 mg/kg | 2.03 0.54 | 2.35 0.55 |
| 40 mg/kg | 2.41 1.95 | 4.19 0.30 |

a. Male and female NFS mice were injected with diluent or 10, 20, or 40 mg/kg phenytoin for 18 days. On day 15, all mice in group 3 were infected with murine hepatitis virus (MHV). All animals were sacrificed on day 19. Total serum bilirubin levels were determined spectrophotometrically and expressed as mg/dl. Phenytoin alone did not alter serum bilirubin levels (Group 2 mice). Virus infection significantly increased bilirubin levels in mice given 20 and 40 mg/kg phenytoin ($p < 0.05$). Significant increases in bilirubin levels were also found in MHV-infected mice given 20 and 40 mg/kg phenytoin as compared with infected mice injected with diluent only ($p < 0.05$, Group 3 mice).

b. Mean, N=6.

c. Standard deviation.

alter serum ALAT levels (Table VI-3, group 2), however a comparison of animals in group 3 showed a significant increase in ALAT levels in mice receiving the diluent, 10, and 20 mg/kg phenytoin after virus infection. In these virus-infected mice of group 3, mice treated with 10 mg/kg phenytoin had significantly higher ALAT levels while those treated with 40 mg/kg phenytoin had significantly lower levels than diluent-treated animals.

Serum ASAT levels. Phenytoin treatment alone did not significantly alter serum ASAT levels in mice treated with virus or not. However, a trend toward increased levels were found except that mice treated with 40 mg/kg phenytoin showed lower ASAT levels than mice in other treatments: this difference was significant in mice not treated with virus (Table VI-4, $p < 0.05$).

Natural killer (NK) cell activity. NK cell activity of group 2 male mice was reduced in a dose-dependent manner in mice given phenytoin. This depression was significant in male mice given 40 mg/kg (Figure VI-1, $p < 0.05$). Female mice in all treatments had significantly lower activity than male mice and much variation was observed ($p < 0.01$). No significant differences in NK cell activity were found in female mice given phenytoin (Figure VI-2).

Discussion

The effects of phenytoin on liver function and virus infection were investigated with an animal model used to study the effects of immunomodulators on virus infection (Sidwell et al, 1977). Since phenytoin has not been associated with direct drug-induced hepato-

TABLE VI-3

PHENYTOIN AND MURINE HEPATITIS VIRUS INFECTION:
SERUM ALANINE AMINOTRANSFERASE LEVELS

| Treatment ^a | Group 2 - MHV | Group 3 + MHV |
|------------------------|---------------------------------------|------------------|
| Diluent | 43.1 ^b 3.0 ^c | 75.2 32.7 |
| 10 mg/kg | 45.3 6.6 | 114.1 78.9 |
| 20 mg/kg | 52.0 8.7 | 71.8 16.5 |
| 40 mg/kg | 48.2 8.6 | 47.9 25.3 |

a. Male and female NFS mice were injected with diluent or 10, 20, or 40 mg/kg phenytoin for 18 days. On day 15, all mice in group 3 were infected with murine hepatitis virus (MHV). All animals were sacrificed on day 19. Serum alanine aminotransferase (ALAT) levels were determined spectrophotometrically and expressed as Sigma/Frankel units/ml. Phenytoin alone did not alter ALAT levels (Group 2 mice). Virus infection significantly increased ALAT levels in mice given diluent, or 10 and 20 mg/kg phenytoin ($p < 0.05$). Significant increases in ALAT levels were found in infected mice (Group 2) given 10 mg/kg phenytoin as compared to mice treated with diluent only ($p < 0.05$). Mice given 40 mg/kg of phenytoin showed depressed levels.

b. Mean, N=6.

c. Standard deviation.

TABLE VI-4

PHENYTOIN AND MURINE HEPATITIS VIRUS INFECTION:
SERUM ASPARTIC AMINOTRANSFERASE LEVELS

| Treatment ^a | Group 2 - MHV | Group 3 + MHV |
|------------------------|---|------------------|
| Diluent | 130.9 ^b 25.3 ^c | 149.7 34.0 |
| 10 mg/kg | 149.8 30.2 | 164.3 72.8 |
| 20 mg/kg | 148.3 19.2 | 173.5 71.2 |
| 40 mg/kg | 85.7 18.6 | 120.2 25.2 |

a. Male and female NFS mice were injected with diluent or 10, 20, or 40 mg/kg phenytoin for 18 days. On day 15, all mice in group 3 were infected with murine hepatitis virus (MHV). All animals were sacrificed on day 19. Serum aspartic aminotransferase (ASAT) levels were determined spectrophotometrically and expressed as Sigma/Frankel units/ml (mean of 6 mice and standard deviation). Phenytoin alone did not alter ASAT levels (Group 2 mice). ASAT levels increased slightly in mice infected with virus. Mice given 40 mg/kg phenytoin had low ASAT levels which were significant in mice not treated with virus ($p < 0.05$).

b. Mean, N=6.

c. Standard deviation.

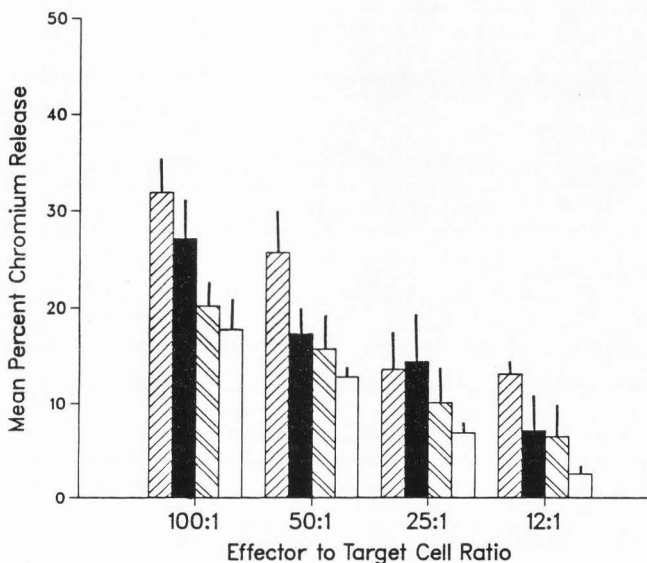






FIGURE VI-1 Splenic cells were isolated from male mice treated with diluent , or 10 , 20 , or 40  mg/kg phenytoin for 18 days and assessed for natural killer cell activity. Natural killer (NK) cell activity is expressed as a mean percent ⁵¹Cr release (N=3) and standard error of the mean at various ratios of effector to target cells. A significant decrease in mean releases were effected by mice treated with 40 mg/kg phenytoin ($p < .05$).

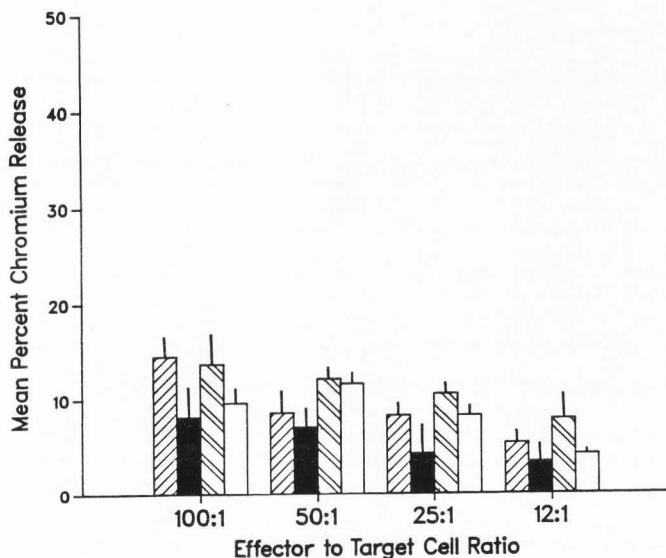






FIGURE VI-2 Splenic cells were isolated from female mice treated with diluent , or 10 , 20 , or 40  mg/kg phenytoin for 18 days and assessed for natural killer cell activity. Natural killer (NK) cell activity is expressed as a mean percent ⁵¹Cr release (N=3) and standard error of the mean at various ratios of effector to target cells. A significant decrease in mean releases were effected by female mice as compared with male mice in all 4 treatments ($p < 0.001$). Phenytoin treatment had no effect on NK cell activity in female mice.

toxicity, MHV was chosen as the infectious agent. Liver function was ascertained by measuring bilirubin and ALAT and ASAT levels in serum. Jaundice and degeneration of the liver caused by MHV infection results in an increase in total bilirubin levels in blood due to impairment of liver metabolism and excretion of conjugated products into bile. An increase in the liver enzymes ALAT and ASAT in blood after infection are due to leakage of these enzymes following hepatocyte degeneration and loss of membrane integrity. Animals in group 3 were sacrificed at an early stage of infection to prevent deaths in these mice.

A high dose of phenytoin (40 mg/kg) caused a significant increase in mortality from MHV infection. Phenytoin alone did not alter serum bilirubin, ALAT or ASAT levels: these levels generally increased in virus-infected animals. Increase in bilirubin levels following virus infection was most notable in mice given 40 mg/kg phenytoin. Serum ALAT levels were higher from infected mice given 10 and 20 mg/kg phenytoin, but lower in mice given 40 mg/kg. Serum ASAT levels in the latter mice were low in comparison to mice in other treatments with or without virus infection.

Since the mortality and bilirubin data indicate an increase in susceptibility to MHV infection caused by high doses of phenytoin, the low ALAT and ASAT levels in the serum of these mice is puzzling. However, high levels of phenytoin in the serum may be interfering with the aminotransferase assay, possibly by inhibiting these enzymes. If high phenytoin levels inhibit aminotransferase activities in vivo, intermediary metabolism by the liver may be altered. Phenytoin has been associated with height and weight abnormalities in children (Trimble and Corbett, 1983) and interference with transaminase activity

could potentially alter protein metabolism sufficiently to depress growth.

A dose-dependent depression of NK cell activity by phenytoin was found in male mice and was significant in mice treated with 40 mg/kg. Phenytoin has been found to depress, in a dose-dependent, reversible manner, both basal and interferon-augmented NK cell activity of human cells in vitro (Results, Chapter IV). The results presented here indicate a long-term effect of phenytoin on NK cell-mediated lysis. Female mice had variable and significantly lower NK cell activity than male mice and phenytoin treatment did not alter this activity. The low NK cell activities found in female mice may not be as sensitive to depression as higher activities in male mice. A larger number of .pa animals might be necessary to examine NK cell activity in female mice and its modulation by phenytoin. It is possible that female hormones affect NK cell activity, and the phasic nature of these hormones could increase variation in NK cell activity. However, there is no information available on this topic.

The depression of NK cell activity by high doses of phenytoin in mice may contribute to the increased deaths found after MHV infection. However, both male and female mice appear to have the same increase in mortality even though no evidence for NK cell depression by long-term phenytoin was found in female mice. Thus phenytoin may depress immune function in other ways besides NK cell activity. One possibility is that interferon augmented NK cell activity may be depressed by phenytoin in vivo as has been found in vitro (Results, Chapter IV). Interferons are lymphokines with antiviral properties. Effects of phenytoin on cytotoxic T-cell (T_C) mediated lysis are unknown: these

cells are also important in host defense against viral infection. If phenytoin alters T_C activity, this may also contribute to a decreased resistance to MCV or other viral infections.

CHAPTER VII

CONCLUSIONS

Clinical Studies

A number of immune abnormalities have been found in epileptic patients. Several but not all of these defects appear to be related to the toxic effects of antiseizure medications. In order to examine the basis of immune abnormalities in epilepsy, various populations and subsets of peripheral blood mononuclear cells (PBMC) from epileptic patients and their siblings were enumerated and their functions investigated. Significantly reduced natural killer cell (NK) activity was found in cells isolated from epileptic patients and their siblings. A decreased ratio of OKT4+ /OKT8+ cells was also found in epileptic patients. Enumeration of the PBMC showed a significantly lower proportion of Leu 11+ cells in the patients which may account for the low NK activity found. Antibody-dependent cell-mediated cytotoxicity (killer) activity was similar in patients and control subjects. Patient blastogenic responses to the T-cell mitogens concanavalin A and phytohemagglutinin and to the B-cell mitogen pokeweed mitogen were variable and no significant differences were found. Total rosette-forming cells of the patients were within the normal range. The results indicate a possible genetic basis for some of the immune alterations seen in epileptic patients and suggest that appropriate control subjects in immune studies of epileptic patients should include close relatives of the patients as well as unrelated control subjects for correct interpretation of experimental observations.

In Vitro Studies

Several of the widely used antiepileptic drugs (AED) have been found to alter immune function in man. Effects range from minor alterations in immunoglobulin levels and antibody production to more severe reactions including hypogammaglobulinemia and agranulocytosis. In addition, phenytoin has been associated with lymphoproliferative states and lymphoma. In the present paper effects of phenytoin on natural killer (NK) and killer (antibody-dependent cell-mediated cytotoxicity or ADCC) cell activity were investigated. Phenytoin depressed NK cell activity in a dose-dependent manner in vitro. This effect was observed following short-term exposure at levels of drug considered therapeutic and was reversible. Phenytoin also depressed interferon-augmented cytotoxicity in a dose-dependent manner and suppressed ADCC but at concentrations slightly higher than that required for suppression of NK cell activity. The AED carbamazepine had a slight and variable effect on NK cell activity, either stimulating or depressing activity depending upon the concentration of drug that was used. Propylene glycol, a commonly used diluent for drugs, also depressed NK cell activity at concentrations as low as 0.1% v/v.

NK and killer cells are thought to be vital in host defense and a first line of defense against developing neoplasms. Inhibition of their activity by phenytoin may be a mechanism by which patients develop lymphoma after long-term treatment by this drug.

In Vivo Antibody Production

Phenytoin, a widely used antiepileptic drug (AED), has been found to cause immune alterations in man. However, a complex interplay of drug effects, disease processes of epilepsy, and genetic factors are thought to be important in immune abnormalities seen in epileptic patients treated with AED. An animal model can be used to study effects of AED on immune function without the limitations and complications present in clinical investigations. In the present study, inbred NFS mice given phenytoin and immunized with bovine serum albumin (BSA) demonstrated a dose-dependent decrease in specific IgG production. The treatments were relatively well tolerated by the mice even at high doses of phenytoin as judged by body weights, specific organ weights for thymus, spleen, and liver, white blood cell counts, and hematocrits. Current findings in the mice indicate that phenytoin causes alterations in immune function in ways similar to those observed in man. This animal model should allow investigations into toxic dose levels and mechanisms by which phenytoin and other AED alter immune function.

Effects of Phenytoin on Murine

Hepatitis Virus Infection

Phenytoin, a widely used antiepileptic drug, has been found to alter immune functions in man. In patients receiving this drug on a long-term basis, an increase in frequency of respiratory infections has been seen, particularly in patients with phenytoin-induced IgA deficiency. Few information are available on the effects of phenytoin on other types of infections. The present study investigated the effects of long-term phenytoin treatment on liver function and hepatitis virus infection in mice. A high dose of phenytoin (40 mg/kg) significantly increased mortality after virus infection, but phenytoin alone did not alter serum bilirubin or alanine aminotransferase (ALAT) or aspartate aminotransferase (ASAT) levels. In contrast, virus-infected animals given either diluent or phenytoin generally showed increases in serum bilirubin, ALAT and ASAT levels. Virus-infected mice given 10 or 20 mg/kg of phenytoin generally showed higher bilirubin and ALAT and ASAT levels than diluent-treated mice, however mice given 40 mg/kg phenytoin showed high bilirubin levels but low ALAT and ASAT levels. Since both mortality and bilirubin levels increased in virus-treated animals given high-dose phenytoin, this decrease in aminotransferase activity may be due to interference by serum phenytoin in the assay for these enzymes, possibly by altering enzyme activity.

Natural killer (NK) cell activity was significantly lower in male mice given 40 mg/kg of phenytoin. NK cell activity of female mice was variable and not significantly lower in mice treated with phenytoin. Since NK cells are thought to be important in combating viral

infections, depression of NK cell activity in mice given a high dose of phenytoin may contribute to the decreased resistance to murine hepatitis virus infection observed.

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Professional Societies:

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Honors:

First place poster presentation. Sigma Xi Poster Contest, April 3, 1985. Utah State University. Depression of natural killer cell activity by antiepileptic drugs.

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Professional Publications:

Margaretten, N., R.P. Warren, and W. Thain. 1985. Reduced natural killer cell activity, numbers of Leu 11+ cells and OKT4+/OKT8+ ratio in epileptic patients. Submitted for publication.

Warren, R.P., A. Foster, N.C. Margaretten, and N.C. Pace. 1985 Immune abnormalities in patients with autism. *J. Autism Developmental Disorders*. In press.

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Abstracts:

Margaretten, N. and E. Hays. 1982. Adverse effects of long-term oral administration of cadmium chloride on hemopoiesis. *Blood*. Abstract 60(38a):78.

Presentations with Abstracts Published:

Margaretten, N. and R. Warren. 1984. Suppression of natural killer cell activity by antiseizure drugs. *Society of Toxicology March, 1985 Annual Conference*. San Diego, Ca.

Margaretten, N, Reed Warren and Wilbur Thain. 1984. A murine model to study effects of anticonvulsant drugs on the immune system. *American Association of Laboratory Animal Science. Scientific Meeting, Utah Branch*. Salt Lake City, Utah.

Margaretten, N. 1981. Effects of long-term cadmium exposure on the immune system in mice. *Sigma XI Student Research Symposium*. Ca. State Northridge Chapter.

Presentations:

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