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Cell Receptor-Xenobiotic Complexes as Exposure Biomarkers

Literature Summary and Recommendations

CELL RECEPTOR-XENOBIOTIC COMPLEXES AS EXPOSURE BIOMARKERS
LITERATURE SUMMARY and RECOMMENDATIONS

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ABSTRACT

This report presents a brief review of the literature relevant to the interaction between cell receptor macromolecules and chemicals in their immediate environment. An overview of the physiological role of cell receptors in relation to endogenous chemical regulators such as hormones is presented together with the essentials of receptor kinetics.

While a specific receptor type usually binds only one kind of endogenous chemical, any number of man-made molecules having the required structural characteristics (therapeutic drugs for example) may also be bound. Literature is cited demonstrating that certain toxic environmental chemicals, in addition to drugs, can also bind to receptor active sites. Further, their toxic action is dependent upon the binding.

The evidence at hand suggests that receptor-bound xenobiotic, rather than total tissue concentration, may provide a more accurate measure of exposure to environmental chemicals. However, since most tissues are not readily accessible for routine monitoring, a surrogate biomarker of exposure may be developed using receptor-bound xenobiotics present in the formed elements of the blood.

Recommendations are made for research needed to demonstrate the feasibility of blood cell receptor biomarkers of exposure.

CONTENTS

Page

Notice ii

Abstract iii

List of Figures and Tables v

Glossary vi

Introduction 1

Cell Receptors-Background 4

Xenobiotic Modulation of Receptor Numbers/Affinity 10

Xenobiotic-Immune System Interaction 23

Research Needs for Development of Receptor Biomarkers 24

Specific Recommendations 31

Literature Cited 35

LIST OF FIGURES

Number Page

1 6

2 9

LIST OF TABLES

I 11

II 25

III 26

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GLOSSARY

BIOMARKER

A change in composition or function of a biological system or sample which signals the occurrence of an event of concern.

CYTOSOL

The non-membranous cytoplasm bounded by the cell membrane.

DEXTRAN

A polymer of glucose having both 1-4 and 1-6 linkages with branches about every five units, giving it considerable resistance to enzymic hydrolysis. Molecular weight ranges from 30,000-25,000.

DIOXIN

A family of planar polychlorinated organic compounds consisting of two benzene rings connected by two oxygen bridges. The congeners differ in their structure and toxicity according to the number and positioning of chlorine atoms on the molecule.

ENDOCYTOSIS

A mechanism whereby cells can internalize molecules aggregated on its outer surface. An infolding of the membrane results in vesicle formation with envelopment of the aggregated material. The vesicle then separates from the boundary membrane, opens, and releases its contents to the cytoplasm.

GLUCOCORTICOIDS

Steroid hormones, produced by the adrenal cortex, which regulate metabolic interconversions between carbohydrates, fats and proteins and also exert anti-inflammatory effects.

HALOGENATED

The chemical designation of a molecule containing one or more halogen (e.g., chlorine bromine, etc.) atoms.

IMMUNOGLOBULIN

Any of five structurally and antigenically distinct antibodies produced by lymphoid tissue and present in the serum. Specifically, IgA, IgD, IgE, IgG, and IgM

INVAGINATE

To fold inward, creating a pocket or vesicle.

LIGAND

An organic molecule attached to a specific site on a macromolecule or membrane surface.

MICROSOMAL MONOOXEGENASE

Any of a group of enzymes, located in microsomes, that catalyze the oxidation of organic compounds.

MONOCLONAL ANTIBODIES

Antibodies derived from one B-lymphocyte cell line.

MUSCARINIC RECEPTOR

Receptors for acetylcholine located on smooth and cardiac muscle cells in contrast to those located on skeletal muscle cells, termed nicotinic receptors.

NEUROTRANSMITTER

Amine or peptide chemicals released by neurons which result in transmission of nerve impulses across synaptic junctions.

OUABAIN

A glycoside prepared from *Strophanthus gratis*. Acts similar to digitalis on the heart, resulting in increased strength and regularity of beat.

PHARMACOKINETICS

The mathematical description of the fate of drugs in the body including absorption, distribution, metabolism, and excretion.

POLYCLONAL ANTIBODIES

A mixture of antibodies produced by more than one type of B-lymphocyte.

STEREOSPECIFIC

Three-dimensional organic structures whose molecules have an ordered (crystalline) orientation rather than the random (amorphous) form.

XENOBIOTIC

Pertaining to organic substances foreign to the body.

CELL RECEPTOR BIOMARKERS

INTRODUCTION

Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and other environmental protection laws, the U.S. Environmental Protection Agency (EPA) is responsible for pesticide regulation and to ensure that unreasonable risks to human health or the environment do not result from exposure to pesticides. Quantitative health risk assessment requires that both toxicity and exposure assessments be made (1). Thus, accurate exposure assessments become critical to the evaluation of environmental and human health risks of pesticides and to the subsequent development of control regulations that may be needed for their protection.

Assessments may be made of external exposure, i.e., the sum of the xenobiotic material presented to the absorptive surfaces of an organism, or of internal dose, i.e., the amount of xenobiotic actually absorbed (2). Biological markers of internal dose may obviate the need for estimating the amount of xenobiotic absorbed, which must be done when exposure assessments are derived from environmental monitoring data. Indexes of internal dose range from

measurements of the tissue/body fluid concentration of a xenobiotic and/or its metabolites, to the measurement of a biochemical or physiological reaction at the cell, organ, or organism level.

At the molecular level, a chemical pollutant or its reactive metabolite binds to a receptor protein which may in turn produce a disturbance in intermediary metabolism at the cellular level. When the disturbance is of sufficient degree, a perceptible and measurable biochemical, physiological, or behavioral dysfunction occurs. The degree of dysfunction, as determined by controlled laboratory studies, provides a measure of the toxicity of the chemical. The close association of xenobiotic-receptor concentration with the metabolic processes leading to toxicity suggests that exposure assessments based on the amount of xenobiotic bound to receptor protein may support more accurate risk assessments than those based on the total amount of xenobiotic present in the organism or one of its tissue compartments.

In order to prevent unacceptable health risks, the measurement methods used must be sensitive enough to detect concentrations of receptor-bound xenobiotic below the threshold of physiological dysfunction. Immunoassays have the required sensitivity (3), and the antigenicity of receptor protein to which the xenobiotic is bound will facilitate production of the antibodies needed for the assay procedure. Biomarkers that measure exposure (i.e., receptor-bound pesticide) to the most sensitive (target) tissues will have the greatest relevance to risk assessment. However, sampling of that tissue often requires the use of invasive procedures and are thus unsuited for routine exposure monitoring of human populations. Accordingly, this report will evaluate, through a review of the literature, the occurrence of and potential for using

measures of xenobiotic compounds bound to receptor macromolecules in the formed elements of the blood as surrogate biomarkers of human exposure. The ease of access of blood samples compared to other tissues makes them a realistic choice. Receptors associated with cells or cell fragments found in other body fluids will also be addressed briefly when appropriate.

With certain exceptions (e.g., steroid hormones), receptors for hormones, neurotransmitters, and other chemical signals are believed to be located on the cell membrane surface (4). Cell membrane receptors are an integral part of the plasma membrane structure and serve to discriminate, with high specificity and efficacy, the ligand(s) present in the immediate environment surrounding the cell (5). The high affinity of receptor macromolecules assures binding of ligand at low concentrations.

The early conceptual framework for the study of receptor-ligand interaction evolved from pharmacologic investigations of drug action during the second half of the 19th century. Since most drugs bind to receptors designed for interaction with endogenous hormones and neurotransmitters, these studies also demonstrated receptor mediation of physiologic hormonal regulation. Xenobiotic-receptor interaction was also suggested in this period with the finding that lead has a greater affinity for certain tissues, notably those of the central nervous system (CNS), than for others (6). Experimental evidence for the binding of xenobiotics to cellular receptors has developed relatively rapidly in the past decade. For example, the Ah receptor, which is responsible for the induction of microsomal monooxygenases by polycyclic aromatic hydrocarbons, has also been found to mediate the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

(TCDD) (7). Also, three major classes of chlorinated hydrocarbon insecticides have been found to be potent, competitive, and stereospecific inhibitors of the γ-aminobutyric acid (GABA) receptor in brain tissue (8).

The foregoing emphasizes the three cornerstones of the rationale for developing membrane receptor biomarkers. First, the amount of xenobiotic bound to cell-receptor macromolecules at a specific time may afford a physiologically meaningful biomarker of exposure. This view is supported by the observation that the polybrominated biphenyl (PBB) concentration of the white blood cell fraction correlates better with severity of lymphocytic dysfunction than does the PBB concentration in the plasma fraction (9). Second, at very low concentrations xenobiotic-receptor complexes are measurable using immunoassay techniques. And third, xenobiotic-receptor binding also mediates a pollutant-induced adverse effect when the internal dose becomes sufficiently high. Therefore, information gained from investigations of xenobiotic binding to receptor macromolecules may advance the development of exposure- as well as effects-biomarkers.

CELL RECEPTORS - BACKGROUND

Receptors have been identified and have been shown to mediate physiologic as well as pathologic reactions for hormones, neurotransmitters, viruses, bacteria, immunoglobulins, and chemoattractants (10,11,12,13). Receptor-ligand interactions are reversible reactions involving

all the components of the membrane, viz., protein, carbohydrate, and lipid. Binding sites may be competitively inhibited by a molecule that resembles the ligand. They may also be inhibited non-competitively by molecules that react with sites other than the ligand binding site, causing a structural change that interferes with the signal transduction function of the receptor. The membrane-receptor macromolecule, composed of an assembly of proteins or glycoproteins, is embedded in the lipid bilayer. Receptor active sites are small portions of the molecule generally making up less than 10% of the total amino acid residues. They are often clefts in the surface of the macromolecule, composed mostly of hydrophobic residues but also containing polar groups that participate in binding (14). In glycoprotein receptors, the carbohydrate units are usually clustered on the periphery of the molecule so that this hydrophilic area is exposed to the external side of the cell membrane.

Some of the glycoproteins may extend their contact to the cytoplasmic side of the membrane, providing a means of internalizing an external chemical stimulus such as a hormone. The carbohydrate units of glycoproteins are covalently bonded to the polypeptide backbone. Carbohydrate prosthetic groups can, of course, be part of the ligand molecule as well as of the receptor macromolecule. In general, carbohydrate moieties of glycoproteins contribute to the stabilization of protein conformation and the regulation of metabolic half-life in the circulation of the intact animal, uptake by cells, message transmission, etc. (5). Some of the receptor macromolecule features described are represented in Fig. 1.

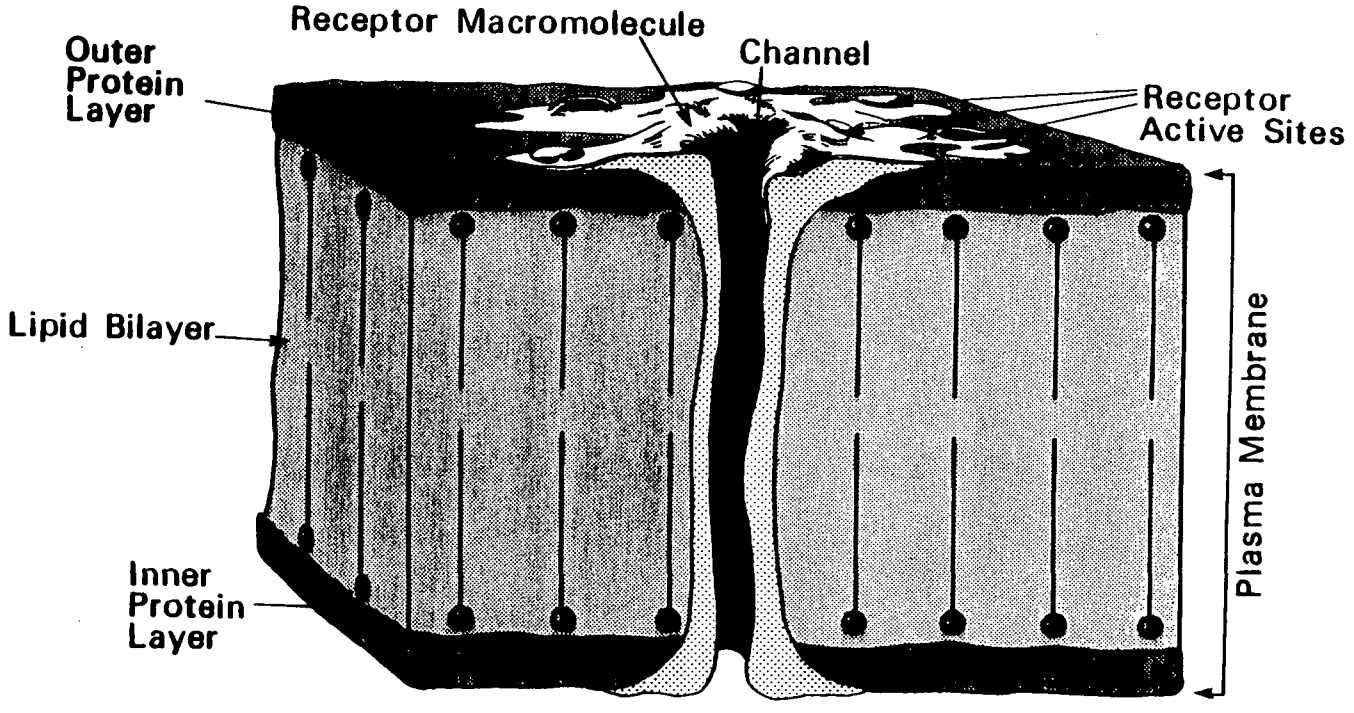

Figure 1. Schematic illustration of a cross-section of cell membrane and generalized receptor macromolecule. Note multiple receptor active sites and channel for the passage of ions and/or molecules

In most cells the number of receptors is under some regulation at the level of gene expression. Also, many receptor macromolecules contain one or more binding sites for specific regulatory molecules in addition to the ligand-specific binding site. Interaction between the subunits are of two types: either an alteration in the activity of the regulatory-molecule binding-site when ligand binds to the active site, or an alteration in the activity of the ligand-binding-site when the regulator site is occupied. The number of cell surface binding sites is also regulated by cycles of endocytosis, reutilization, and degradation (14).

From this brief overview, it can be seen that the magnitude of an effect resulting from a given concentration of hormone, drug, or xenobiotic may vary according to the available number of cell membrane receptors in existence at the time. Data from a number of systems suggest that all the necessary information for activation of the effector is contained in the receptor, i.e., some receptors phosphorylate, others aggregate, while still others exist in multiple-binding states. Receptor macromolecules can consist of subunits and can undergo reversible interactions in the lateral plane of the plasma membrane. Experimental techniques allow measuring the physical characteristics of the receptor while still a component of the membrane or whole cell as well as measuring its characteristics in purified form following membrane solubilization with suitable detergents (12).

A receptor is defined or identified, in the first place, in terms of its pharmacokinetics. Substances that effectively mimic or block the effects of the principal ligand should compete for

binding sites at low concentration. Others should compete only at very high concentrations, if at all.

To distinguish receptor-binding (specific) from "other" binding (non-specific) there must be a finite number of binding sites of rather high affinity, i.e., having dissociation constants in the nanomolar range or lower. This can be demonstrated by adding more and more radiolabeled compound and noting that binding increases until saturation occurs, i.e., all sites are occupied. In a binding assay, the membrane component (receptor) is selectively exposed to a radioactively labeled ligand to determine the rate at which the ligand attaches--k_1 (association), and is removed--k_2 (dissociation), i.e.,

k_1 = the rate constant for $[L]+[R] \Rightarrow [L.R]$, and

k_2 = the rate constant for $[L.R] \Rightarrow [L]+[R]$.

The apparent equilibrium dissociation constant of the receptor, K_d, can then be calculated (= k_2/k_1) and is usually on the order of 10^{-9} - 10^{-12} M. The K_d represents the concentration of ligand that results in one-half the total number of receptor sites being occupied at equilibrium. An increase in K_d correlates with a decrease in affinity. In addition, the maximum number of binding sites can be estimated by measuring the quantity of ligand necessary to saturate all the available binding sites (B_{max}).

This approach is complicated by the fact that radioligands will bind to a variety of membrane constituents, only one of which represents the receptor of interest. However, this

non-specific binding can be corrected by measuring the total radioligand bound following its addition alone, then determining its binding in the presence of excess (usually 100 times the K_d concentration) unlabeled ligand (blank). The difference (total - blank) represents receptor-specific binding (14). Binding properties of receptors are quantitated by Scatchard plots in which the ratio of bound-ligand to free-ligand is plotted on the ordinate against bound ligand on the abscissa (15). When a single ligand is interacting with a single population of receptors possessing a single affinity for the ligand,

$-1/K_d$ = the slope of the straight line obtained,

B_{max}/K_d = the y-intercept, and

B_{max} = the x-intercept (Fig. 2).

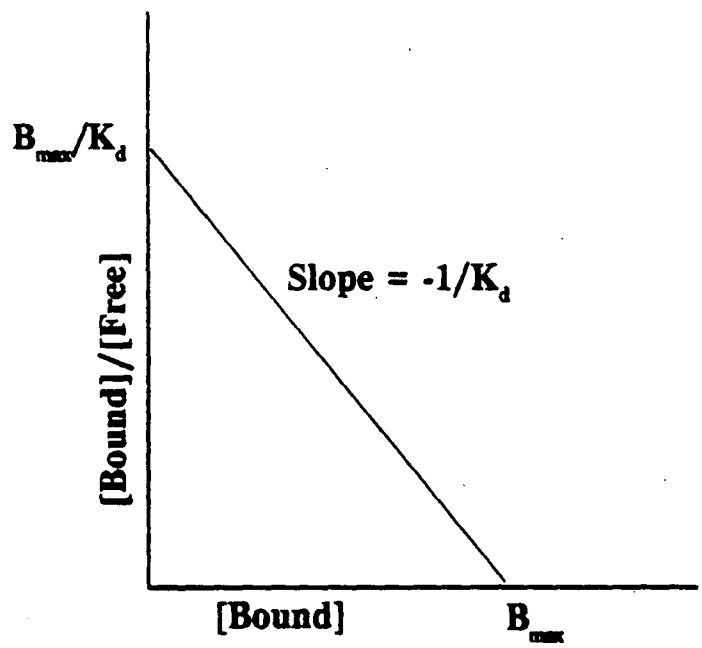

Fig. 2. Illustration of a Scatchard plot showing derivation of K_d and B_{max}.

However, as pointed out by Rosenthal (16), B_{max} must be interpreted as representing the total number of binding sites rather than the number of receptor macromolecules, since each macromolecule may have more than one binding site. Until the receptor is purified and its structure known, B_{max} is determined in units of fmol/mg membrane protein.

A number of mammalian cell membrane receptor systems which bind endogenous regulatory chemicals have been characterized functionally as well as structurally to varying degrees (Table I). Purification of some receptors, e.g., insulin receptors, may be hampered by a low occurrence or physical similarity to surrounding membrane protein, or both. On the other hand, the muscarinic cholinergic receptor that binds acetylcholine is relatively more abundant and easily separated and has been well characterized.

XENOBIOTIC MODULATION OF RECEPTOR NUMBERS/AFFINITY

Membrane Receptors

Ah Receptor The induction receptor for the polycyclic aromatic hydrocarbon-inducible microsomal monooxygenases, cytochrome(s) P_1-450, (sometimes designated the "TCDD" or "dioxin" receptor) was identified by Poland *et al.*(17). Later studies revealed that this protein regulates the expression of a gene battery which includes the structural genes for cytochrome(s) P_1-450 and in certain tissues, in concert with at least one other regulatory locus, alters patterns of cell proliferation and differentiation. For a review of these developments, see Poland and Knutson (18).

TABLE I. Verified hormone/transmitter receptor systems

Designation	Ligand(s)[1]	Cell Location[2]	Occurrence[3]	Ref.
β-adrenergic	E, NE	PM	wbc;s;sk;c;hp;ns	(11,13)
α_1-, α_2-adrenergic	E, NE	PM	hp;s;sk;c;wbc;ns	(11,13)
Acetyl Choline	Ach	PM	wbc;ns;s;sk;c	(10,13)
Insulin	Insulin	PM	wbc;wd	(11)
Glucocorticoid	Cortisol	C;N	wbc;wd	(13)
Estrogen	Estradiol	C;N	ut, wd	(20)
Thyroxine	T_3	PM,C,M,N	wbc;wd	(10,13)
Growth Hormone	Somatotrophin	PM	wbc;wd	(12,13)
Vasopressin/ Oxytocin	Vasopressin/ Oxytocin	PM	wbc;ns;sm	(13,21)
Substance P	Substance P	PM	wbc	(13)
Endorphins	β-Endorphins	PM	wbc	(13)
Histamine	Histamine	PM	wbc	(13)
GABA	γ-Aminobutyric acid	PM	ns	(12)
Dopamine	Dopamine	PM	ns	(13)

[1] E = epinephrine; NE = norepinephrine; Ach = Acetylcholine; T_3 = triidothyronine.
[2] PM = plasma membrane; N = nucleus; C = cytosol
[3] s = smooth muscle; sk = skeletal muscle; c = cardiac muscle; hp = liver; ut = uterus; pl = platelet; rbc = erythrocyte; wbc = leukocyte; ns = nervous system; pnc = pancreas; wd = widely distributed.

The administration of 3-methylcholanthrene (MC) and structurally related polycyclic aromatic hydrocarbons (PAH) results in the induction of cytochrome P_1-450 and associated monooxygenase activities, including aryl hydrocarbon hydroxylase (AHH) in some but not all strains of mice (19). PAH responsiveness in some strains of mice is inherited as a simple autosomal dominant trait. The gene locus controlling this trait was designated the Ah locus. Available data indicate that the receptor protein for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), MC, and other inducers is the product of the Ah locus (17). Studies to determine the subcellular distribution of the Ah receptor in mouse hepatic tissue suggest a cytosolic as well as a nuclear compartment. Comparisons have been made between species of the relative concentrations of Ah receptor in hepatic and extrahepatic tissues. Interspecies differences exist with respect to the tissue distribution of the Ah receptor as well as the relative concentrations of this protein in those tissues possessing the protein (19).

The direct demonstration of the occurrence of a receptor protein for TCDD in human lymphocytes (22) and human squamous cell carcinoma lines (23) has been reported. Binding studies using [^{3}H]TCDD demonstrated the presence of the Ah receptor in the cytosolic fraction from cultures of human squamous cell carcinoma of epidermal origin (17). The relative amount of receptor measured in different cell lines correlated with the maximally induced activity of the monooxygenase 7-ethoxycoumarin O-deethylase (ECOD)(23). Studies using cell cultures of cortical thymocytes (T-lymphocyte precursors) and medullary thymic epithelial cells treated with TCDD have suggested impaired differentiation of intrathymic precursor cells for

the observed TCDD-induced thymic atrophy seen in the intact animal (24). The potential for a direct effect of TCDD on cultured human lymphocytes has not been as extensively explored.

An excellent correlation was demonstrated between the binding affinities of 23 halogenated dibenzo-*p*-dioxin and dibenzofuran congeners *in vitro*, and a measure of their potencies *in vivo* (25). The charcoal-dextran binding assays were performed using rat hepatic cytosol receptor protein. At a given concentration, the amount of receptor-chemical complex formed is proportional to the affinity constant of the ligand. Potencies (ED_{50}) to induce AAH activity were determined in the intact rat.

Thus, the amount of receptor-chemical complex may provide a more physiologically meaningful measure of exposure to TCDD and related compounds than would total tissue concentration. Further, the ease of acquiring a blood sample for measuring complexed lymphocyte Ah receptor would facilitate routine monitoring of human exposures to these chemicals.

Gamma aminobutyric acid Receptor Neurotransmitters (neurohormones) are chemical signals used by nerve cells to communicate with each other and with muscle or gland cells. They are amino acids or small polypeptides released by one cell and received by another via stereospecific receptor sites. Binding of the neurotransmitter to its receptor causes a change in receptor conformation and initiates a change in membrane potential.

Neurons in the brain utilize a number of neurotransmitters in addition to the familiar acetylcholine and norepinephrine found in peripheral nerves. One of these, γ-aminobutyric acid (GABA), and its receptor complex have received considerable attention in recent years. The receptor model which has emerged is that of a protein having at least three interacting components: one that binds GABA, another that binds benzodiazepine (BZ) tranquilizers, and another which binds the convulsants picrotoxinin (PTX) and bicyclophosphates (26). These sites interact allosterically and affect each other's binding through conformational changes (27, 28). Activation of most GABA receptors (GABAR) in mammals leads to an influx of Cl^- causing membrane hyperpolarization (29).

The ligand of choice for studying agonists and antagonists of the PTX binding site within the GABAR complex is t-Butylbicyclophosphorothionate (TBPS) (30). It was selected because of its specificity (no more than 33% non-specific binding), high affinity (K_d = Ca 17 x 10^{-9} M), and because t-butylbicyclophosphite is easily labeled with ^{35}S in a single step to form [^{35}S]TBPS. The relatively high specific activity (about 30 Ci/mmole at the start), and the relatively short half-life of ^{35}S (about 90 days), permit a fresh batch of [^{35}S]TBPS to be used for about 6 months. Its binding and displacement properties remained constant over this period.

Most insecticides are neurotoxins that inhibit acetylcholinesterase at synapses or disturb sodium channels on nerve or muscle cell membrane. Some, however, exert their primary toxic action on the GABAR found in the vertebrate and invertebrate central nervous systems as well as in invertebrate neuromuscular junctions. Included are certain cyclodiene (e.g., dieldrin) and

hexachlorocyclohexane (e.g., lindane) insecticides (29), and three of the components of toxaphene, which probably account for most of the toxicity of the commercial product which has at least 188 components (31). These insecticides inhibit GABA-induced chloride permeability and may account for their stimulant and convulsant effects. On the other hand, competitive binding studies with some of the potent α-cyano-3-phenoxybenzyl pyrethroid insecticides suggest that they bind to a site closely associated with, but possibly distinct from, that of TBPS and PTX (32).

The density of specific [^{35}S]TBPS binding sites varies in different regions of the brain, being highest in cerebral cortex (Ca. 65 pmoles/g) and lowest in the pons-medulla (Ca. 14 pmoles/g) (30). Relatively few studies report a systematic search for the presence of GABAR in non-nerve tissues. Squires (30) found specific TBPS binding in rat liver and lung tissue to be about 7% and 2%, respectively, of that seen in cerebral cortex, while no specific binding was demonstrated in kidney tissue. [^{3}H]-muscimol, a potent GABA agonist used to label GABAR sites in vertebrate brain, was found to bind specifically to sites in a crude membrane fraction prepared from bovine cerebral blood vessels (33). GABA receptors have been reported in guinea pig ileum and vas deferens (34).

The BZ receptor in brain tissue occurs in a macromolecular complex that includes a binding site for the inhibitory neurotransmitter GABA. While most BZ binding sites are associated with GABA receptors, a few studies have demonstrated the existence of one without the other in some rat brain preparations (35). The GABA-BZ receptor complex also includes

recognition sites for barbiturates and possibly ethanol. In initial studies of ^{3}H-diazepam binding, peripheral tissues were examined as controls (36). Surprisingly, high densities of diazepam binding sites were observed in a variety of peripheral tissues with similar nanomolar affinity for diazepam as the central receptors. High densities of these receptors have been found in rat adrenal cortex (but not medulla), salivary glands, testes, and ovaries. Pancreas and pituitary tissue have relatively low densities of receptors. The physiological significance of these peripheral BZ receptors is unclear.

Nevertheless, the association of BZ and GABAR sites in central nervous system tissues and the occurrence of diazepam receptor sites in peripheral tissues give reason to investigate the possible occurrence of receptors for pesticides (e.g., dieldrin, lindane, toxaphene, and cypermethrin) in blood cell membranes.

Acetylcholine Receptor The acetylcholine receptor (AchR) is a well-characterized component of synaptic and neuromuscular transduction systems. This receptor translates the binding of the neurotransmitter acetylcholine (Ach) into a rapid increase, then decrease, in the permeability of the membrane to the passage of cations (Na^{+}, K^{+}). AchR sites are sub-units of a macromolecular receptor complex containing other agonist and/or antagonist binding sites. The enzyme acetylcholinesterase (AchE) and the AchR either occupy neighboring sites on the same macromolecule or sites on separate molecules that are coupled in such a way as to permit coordination of function (37). AchE is an extrinsic protein (i.e., it is readily dissociated from

membrane preparations of vertebrate muscle endplate), while AchR is an intrinsic protein embedded within the membrane structure (38).

The presence of AchE on erythrocyte membranes is well documented, and the inhibition of this enzyme's activity is used to assess exposure to organophosphate insecticides (39). The AchR has been reported in rat lymphocyte membranes (13). Whether the close association of AchR and AchE seen in neural and muscular tissue also holds for RBC and/or WBC membranes has not been reported.

There is evidence for interaction of organophosphate insecticides with RBC membrane protein other than AchE. Erythrocytes from humans exposed to organophosphate insecticides were found to have altered membrane stability (40). Human subjects with serum or blood AchE inhibition between 40-60% were considered mildly intoxicated, and those with more than 60% inhibition as severely intoxicated. Erythrocyte membranes from mildly intoxicated subjects were more resistant, while those from severely intoxicated subjects were less resistant to hypotonic hemolysis. Also, a significant increase in membrane phosphatidylcholine was seen in the severely intoxicated. This suggests a binding of organophosphate to RBC membrane macromolecules. Whether this binding involves the AchR receptor and/or can be utilized as a biomarker of exposure is unknown.

Dopamine Receptor The dopamine-sensitive site in the brain and pituitary to which neuroleptics bind with high affinity has been studied intensively in the search for new

therapeutic agents. Its functional molecular size is known, though it has not been characterized structurally. There appear to be multiple receptors for dopamine, some linked to the adenylate cyclase and others not (13).

The ligand spiroperidol binds specifically to dopamine receptors and is commonly used to study dopamine receptor agonists and antagonists. The specific binding of this ligand in membrane preparations from brain cortical tissue was found to be inhibited in the presence of lead and mercury (41). Tri-n-butyl lead was a more potent inhibitor than lead acetate, and mercurous chloride was more potent than methylmercuric chloride. Although the specificity of the heavy metal binding to receptor protein was not reported, clearly the affinity of the receptor site for dopamine was altered. The blocking action was not indiscriminate, since the heavy metals were relatively ineffective in blocking glycine, GABA, or diazepam receptors. However, they did inhibit the binding of ^{3}H-quinuclidinyl benzilate (^{3}H-QNB), a specific ligand for the muscarinic AchR.

Adrenergic Receptor Adrenergic receptors (adrenoceptors) have high specificity for the catecholamine neurotransmitters epinephrine and norepinephrine. While they are abundant in nerve and smooth muscle tissues, they can be found in most human tissues including erythrocytes and lymphocytes (42). β-receptor concentration increases from late fetal life to adulthood in most tissues and decreases with age thereafter. As rat reticulocytes mature to erythrocytes, the β-adrenergic receptors decrease by ca. 66%.

Four main receptor sub-types are recognized. The β_1- and β_2-adrenergic receptors stimulate the membrane-bound enzyme, adenylyl cyclase, thus raising intracellular cyclic AMP levels. The α_2-adrenergic receptors inhibit this enzyme, whereas the α_1-adrenergic receptor leads to the hydrolysis of polyphosphoinositides which, in turn, generates two secondary messengers, inositoltriphosphate and diacylglycerol (43).

Methyl mercury exposure to mice *in utero* produced a dose-dependent increase in the adrenergic receptor binding in liver and kidney tissue which persisted throughout the first 5 weeks of postnatal life. The animals' physiological reactivity to adrenergic stimulation was altered in a manner consistent with the increased receptor binding capacity (44). The authors suggest that methyl-mercury exposure *in utero* alters adrenergic responses through targeted effects on post-synaptic receptor populations in specific tissues. Modulation of β-adrenergic receptor activity by cardiac glycosides has also been reported. A single neonatal treatment with either ouabain or digoxin resulted in increased responsiveness to adrenergic excitation of two-month-old adult rats (45).

Additional work would be required to determine first, whether the receptor population increase would persist with continued exposure, and second, whether the phenomenon has potential as a biomarker of *in utero* and/or postnatal exposure. However, the results reported (44, 45) verify the link between receptor population and potential for response and lends further support for evaluating receptor-bound xenobiotic concentration as the physiologically meaningful measure of exposure.

Cytosolic/Nuclear Receptors

Receptors for some steroid hormones have been identified in target tissue cytosolic- and/or nuclear-fractions as well as in cell membrane-fractions. Receptors which are membrane-bound interact primarily with neighboring macromolecules, while the soluble and more mobile cytosolic receptors can interact with a greater variety of systems within the cytoplasm, including nuclear DNA. A review of the experimental data suggests the membrane-mediated effect of the hormone is probably different from the cytosolic/nuclear receptor-mediated effect (46). For example, the cytosolic/nuclear receptor for a steroid hormone may mediate a protein anabolic response, while the membrane-bound version of the same receptor may mediate an increase in intracellular cyclic AMP.

Receptors for most non-steroidal hormones/neurotransmitters are found only in the plasma membrane. One exception is the receptor for thyroxine, (T_3), which occurs as both membrane-bound and cytosolic (10), a finding which is consistent with the many intracellular effects induced by T_3.

Estrogen Receptor The receptor macromolecule for estrogen is found in a number of tissues including pituitary glands, gonads, smooth muscle of the reproductive tract, brain, etc., reflecting the rather widespread actions of estrogenic hormones.

An anti-estrogenic effect on rat pituitary cell cultures was reported for the pesticide chlordecone (Kepone) (47). Pretreatment of pituitary gonadotrophs with estradiol-17β (E_2) alone enhanced their response to gonadotrophin-releasing hormone (GnRH) in tissue culture. This response could be inhibited by pretreatment with E_2 plus Kepone. The approximate two-fold increase in secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) caused by E_2 pretreatment was depressed by Kepone in a dose-dependent manner. A related pesticide, Mirex, did not suppress the GnRH response at any concentration. It was noted that Kepone had little or no effect on basal FSH and LH secretion.

An estrogenic action has been reported for several pesticides which appear to bind to estrogen receptor macromolecules. Among these are o,p'-DDT and/or o,p'-DDE, Kepone, and the mono- and bis-phenol metabolites of Methoxychlor (48). The apparent disagreement concerning the effect of Kepone (47 vs. 48) may result from its ability to competitively inhibit E_2 in the *in vitro* test system, while expressing an inherent weak estrogenic activity in an *in vivo* test system lacking other sources of estrogen.

A rather extensive literature confirms the estrogenic activity exhibited by a number of pesticidal compounds or their metabolites, as evidenced by their ability to stimulate reproductive tract tissues *in vitro* and *in vivo*. On the other hand, demonstration of estrogenic effects on the CNS and/or behavior have been less consistent. For example, administration of these compounds to neonatal animals fails to influence sex differentiation. However, a recent report clearly shows a CNS/behavioral effect of methoxy-chlor when administered to

ovariectomized female rats and hamsters (49). These animals exhibited increased acyclic running wheel activity as well as persistent vaginal estrus, and displayed reproductive behaviors following progesterone administration.

While recent studies have demonstrated that TCDD significantly reduces uterine and liver estrogen receptor levels in rats (50), earlier studies had shown that several other inducers of the mixed function oxidase system decreased estrogen binding in rat liver preparations (51). Whether these compounds compete for the estrogen receptor or in some way alter its affinity was not determined.

Glucocorticoid Receptor Glucocorticoid receptors (GRc) are found in most tissues, an observation consistent with the regulatory action of the glucocorticoid hormones on fat, carbohydrate, and protein metabolism. GRc are also present in lymphocytes (Table I).

The maximal binding capacity (B_{max}) of skeletal muscle cytosolic GRc was reduced in rats treated with TCDD (52). A similar reduction of hepatic cytosolic GRc binding capacity was effected in pregnant mice treated with either 2,3,4,7,8-pentachlorodibenzofuran or 1,2,3,4,7,8-hexachlorodibenzofuran (53). Interestingly, these polychlorinated dibenzofurans also decreased the binding capacity of hepatic plasma membrane epidermal growth factor (EGF) receptors. Since both the GRc and EGF receptor systems have roles in controlling normal growth and differentiation, they are receiving considerable attention with respect to their possible involvement in xenobiotic-induced teratogenesis.

XENOBIOTIC-IMMUNE SYSTEM INTERACTION

Depressed antibody (B-lymphocyte) and/or cell-mediated (T-lymphocyte) immunity, indicative of impaired immunocompetence, occur after exposure to small but not essentially toxic amounts of certain environmental pollutant chemicals (9). Included are representatives from various chemical types. For example:

Halogenated aromatic compounds--2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polybromo-biphenyls, polychloro-biphenyls, and hexachlorobenzene,

Heavy metals--cadmium, cobalt, chromium, nickel, and lead,

Organometallics--methylmercuric chloride, and

Pesticides--Carbaryl, Carbofuran, DDT, Dieldrin, and Methyl Parathion.

The examples of immune system suppression presented in Table II were excerpted from a recent review of the subject (9) and are representative of the kinds of responses which have been observed. Additionally, the route and timing of the xenobiotic exposure appear to influence the immune response. For example, a single dose of Cd augmented the antibody response to an antigen if injected intraperitonealy and diminished the response when administered orally. Also, injection of Cd 14 days before the administration of antigen increased antibody synthesis, while injection 7 days before decreased it.

Despite the considerable attention given to immunosuppressive effects of environmental chemicals in recent years, relatively little is known concerning their mechanisms of action. While a discussion of the relevant research is beyond the scope of the present report, it is noteworthy that immunotoxicity by TCDD and PCBs is associated with stereospecific binding to the Ah receptor present in lymphoid tissue and cells (54).

RESEARCH NEEDS FOR DEVELOPMENT OF RECEPTOR BIOMARKERS.

A number of receptor systems that have been identified in various tissue types also are found in the formed elements of the blood. Much of the investigative effort concerning hormone receptors in blood cells originally focused on leukocytes and concerned interactions between the neuroendocrine and immune systems. Examples of these interactions include stress-induced immunosuppression mediated by epinephrine and glucocorticoids, and modulation of the neuroendocrine system by neuroactive peptides that are released by leukocytes responding to foreign stimuli (13).

In recent years, studies have extended to other formed elements in the blood. A serious, though not exhaustive, search of the literature revealed a respectable number of receptor systems that have been identified in blood cells (Table III). Clearly, the information base exists to warrant a systematic search for blood cell receptor systems that bind pesticidal and other toxic chemicals that have high Agency priority. Complexes thus identified can then be further evaluated for their potential use as biomarkers of exposure.

TABLE II. Xenobiotic Alteration Of Immunocompetence

Chemical[1]	Response
Pb	Incr. susceptibility of mice to *Salmonella typhimurium.*
Pb	Incr. susceptibility of mice to *Encephalomyocarditis virus.*
Pb	Incr. susceptibility of mice to *Langat virus.*
Cd	Incr. susceptibility of rats to inoculation with bacterial endotoxin.
Cd	Enhanced mortality of mice inoculated with *Encephalomyocarditis virus.*
Cd	Enhanced mortality of mice naturally infected with *Hexamitis muris.*
$Hg_{i/o}$	Enhanced mortality of mice inoculated with *Encephalomyocarditis virus.*
Pb,Cd,$Hg_{i/o}$	Lowered antibody response in mice to infectious agents.
Pb, Cd	Reduced I_gG antibody synthesis in rodents.
Hg_o	Suppressed both primary I_gM and secondary I_gG responses.
Heavy metals	Evidence of direct effect on B-lymphocytes to suppress humoral response.
PCB, HCB	Lowered rodent antibody synthesis to sheep RBC (antigen).
HCB	Suppressed cell-mediated and enhanced humoral immunity in rodents.
PBB	Decreased population of T-lymphocytes.
PBB	Better correlation of Lymphocyte dysfunction with PBB concentration in WBC fraction than in plasma fraction.
Dioxin	Progeny of mice exposed perinatally showed thymic atrophy.

1. Pb = lead ;Cd = cadmium; $Hg_{i/o}$ = inorganic/organic mercury; HCB = hexachlorobenzene; PCB = polychlorinated biphenyls; PBB = polybrominated biphenyls.

An early step in the evaluation of a candidate complex requires *in vitro* as well as *in vivo* studies which will: (1) compare the affinity constants, for a given xenobiotic, between receptors recovered from blood cells and those recovered from target tissue cells; (2) verify that the amount of receptor-bound xenobiotic in a blood sample is potentially detectable; and (3) determine the pharmacokinetics of the complex under different exposure scenarios *in vivo*, and the stability of the complex *in vitro* following withdrawal of the blood sample.

TABLE III
Receptor Systems Found In the Formed Elements of Blood
As Well As in Other Tissues

Receptor	Location[1]	Ref.
ACTH	(m)Leukocyte	(13)
Acetylcholine	(m)Leukocyte	(13)
α-Adrenergic	(m)Leukocyte, Platelet	(13, 11)
β-Adrenergic	(m)Leukocyte, platelet	(13, 55)
Ah	(c)Leukocyte	(22)
Endorphin	(m)Leukocyte	(13)
Enkephalin	(m)Leukocyte	(13)
Dopamine	(m)Leukocyte, Pituitary Cells	(56, 57)
Glucocorticoid	(m)Leukocyte	(13)
Growth Hormone	(m)Leukocyte	(13)
Histamine	(m)Leukocyte	(13)
Insulin	(m)Leukocyte, erythrocyte	(13, 58)
Interferon	(m)Leukocyte	(12)
Parathyroid	(c)Leukocyte	(13)
Prolactin	(m)Leukocyte	(13)
Prostaglandin $E_{1\&2}$	(m)Leukocyte, Erythrocyte	(59, 60)
Serotonin	(m)Leukocyte, Platelets	(13, 61)
Somatomedin	(m)Leukocyte, Erythrocyte	(12)
Somatostatin	(m)Leukocyte	(13)
Substance P	(m)Leukocyte	(13)
Thyroxine	(c)Leukocyte	(13)
Vasopressin	(m)Leukocyte	(13)

1. (c) = receptor occurs in cytosolic and/or nuclear fraction
(m) = receptor occurs in membrane fraction.

Regarding the comparison of blood-borne and target tissue receptors, the literature suggests that the same receptor system obtained from different tissues possess similar *in vitro* binding properties. Total binding capacity, reflective of receptor numbers rather than receptor binding kinetics, are the most frequently reported differences. Examples include: the AcR

(62); estrogen receptor (63); the Ah receptor (64); benzodiazepine receptors (36); adrenergic receptors (42); and insulin receptors (65). Insulin receptors from different human tissues appear to possess structural as well as functional similarity (66).

It is worth noting that interspecies similarities in functional and/or structural characteristics of GABA (35, 67), Ah (19), GnRH (68), and corticosteroid receptor systems (69) have also been reported.

Potential for detectability of the complex cannot be addressed directly because data on numbers of xenobiotic binding sites per blood cell are lacking. However, a calculation based on reported numbers of insulin receptors per erythrocyte suggests the feasibility of an immunoassay on a one milliliter blood sample. The abundance of insulin receptors per human erythrocyte has been estimated from 6.3-9.9 per RBC (70), up to >400 binding sites per erythrocyte (71). For purposes of illustration, taking the lower mean value of 8 sites per erythrocyte and $4x10^6$ RBC/mm^3 of blood, a one ml sample of blood contains $4x10^9$ erythrocytes and $3.2x10^{10}$ receptor sites. Dividing by 6×10^{23}(approx. Avogadro's number) gives about $5x10^{-14}$moles of binding potential, assuming one molecule of ligand per receptor site. Using the higher value for RBC insulin receptors, provides about $2x10^{-12}$ moles of ligand binding potential per ml of blood. Enzyme-linked immunosorbent assays using monoclonal antibodies are capable of detecting femtomole (10^{-15} moles) quantities of benzo[a]pyrene-DNA adduct (72). The numbers of hormone receptor sites reported for leukocytes are generally greater than those for erythrocyte insulin receptors, as can be seen from the following

examples: 2,000-7,000 β-adrenergic sites/cell, 200α-adrenergic sites/cell, 300 T_3 sites/cell, 4,000 growth hormone sites/cell, 7,000 substance P sites/cell, and 3,000 ACTH sites/cell (13), and 240 prostaglandin E_2 sites/cell (60). A similar calculation using 3,000 receptors per leukocyte and 7,000 WBC per mm^3 would provide for $3x10^{-12}$ moles of ligand binding. Thus, the potential for immunoassays of receptor-bound xenobiotic in the blood does appear to exist. Further, should a receptor type occur on both leukocytes and erythrocytes and have similar binding kinetics, an immunoassay screen could be performed on the total cell population of the sample.

Finally, the kinetics of ligand-receptor interaction which includes the association (k_1) and dissociation (k_2) rate constants, and the apparent equilibrium dissociation constant, K_d, (k_2/k_1) of the receptor can be readily determined (5). For exposure biomarker use, the K_d of the complex must be indicative of specific binding. Also important is the rate of dissociation of the ligand-receptor complex. The temperature of separation of bound from free ligand determines the loss of complex in the interval from removing the complex from its equilibrium melieu to its separation from the mixture (73). Thus, complexes that dissociate rapidly require separation techniques that minimize dissociation (assumes that separation began while binding reaction was at steady state. Since free ligand is infinitely diluted in the procedure prior to separation for all practical purposes, reassociation does not occur.

The loss of binding due to dissociation has been shown to increase approximately linearly through the first half-life ($t_{1/2}$) of dissociation of ligand-receptor complexes that follow first-order kinetics (73). When percent binding loss is plotted on the y-axis against separation

time (in dissociation half-life units) on the x-axis, it is seen that a separation procedure completed in less than approximately 0.15 $t_{1/2}$ avoids losing more than 10% of bound ligand.

Since

$$K_d = [L][R]/[LR] = k_2/k_1 = 0.693/t_{1/2}/k_1,$$

an association rate constant, (k_1), of 10^6 M^{-1} sec^{-1} (a value frequently obtained for neurotransmitter receptors) can be used to show that a ligand-receptor complex having a K_d = 10^{-8} M permits a separation time of about 10 sec., i.e.,

$$10^{-8}\ M = 0.693/t_{1/2}/10^6\ M^{-1}\ sec^{-1}$$

and

$$t_{1/2} = 69.3 \text{ sec. and } 0.15t_{1/2} = 10 \text{ sec. (66).}$$

Continuing the calculations, Yamamura (73) shows: for K_d = 10^{-7}M, separation must be complete within 0.10 sec; for K_d = 10^{-9}, 1.7 min; for K_d = 10^{-10}, 17 min; and for K_d = 10^{-11}, 2.9 hr. For the lower affinity constants, and depending on the separation time, it may be necessary to accommodate and correct for higher percentage losses.

Assessment of an exposure rate from the concentration of receptor-bound xenobiotic in the blood requires an understanding of the pharmacokinetics of receptor macromolecules *in*

vivo. This information is available for many receptor-bound therapeutic drugs, neurotransmitters, and hormones, but not for receptor-bound environmental chemicals.

Important pharmacokinetic parameters of receptor-bound chemical following acute exposure, assuming first order kinetics, include the rise-time and biological half-life of the receptor-xenobiotic complex *in vivo* for each exposure route of concern. These values must be estimated either from laboratory animal experiments, and then extrapolated to humans, or determined from successive measurements made on humans following a single accidental exposure.

Interpretation of receptor-bound xenobiotic levels resulting from chronic exposure requires, in addition, information on the total number of receptors present. The phenomenon of internalization of extracellular material has been described in which occupied receptor macromolecules migrate laterally, aggregate, invaginate, and enter the cytoplasm within vesicles. Endocytosis of receptors may be followed by return of the receptors to the surface unchanged or by receptor degradation and replacement (74). It is hypothesized that receptor numbers could remain unchanged, be decreased if replacement lagged internalization, or be increased if replacement was overcompensated. Thus, the total number of receptors in exposed individuals, compared to those found in non-exposed individuals, may help to establish a chronic exposure scenario.

Finally, the existence of body fluids, other than blood, containing cells and/or cell fragments should be acknowledged. The presence of renal tubular epithelial cells in urine and spermatozoa in semen may provide a source of receptor macromolecules either not found in blood, or found in greater numbers. Some of the evidence in support of exposure biomarker development using these fluids was presented in an earlier report (75).

SPECIFIC RECOMMENDATIONS

A study to evaluate the potential use of pesticide-receptor complex levels in human blood samples as biomarkers of exposure is timely for several reasons. Foremost is the Agency's recognition of the need for rapid, cost-effective screening tests as expressed in the research strategy for the 1990s (76). Further, the separate technologies needed to conduct a feasibility study, viz. receptor macromolecule-complex recovery, determination of receptor binding kinetics, and immunoassays have developed sufficiently to be well documented in the literature. Relatively minor modifications would be required for their application to a specific biomarker. Finally, immunoassays can be incorporated into biosensor devices which can measure the amount of analyte in the blood in real time, i.e., at the time of sample acquisition. Should feasibility be demonstrated, method standardization, protocol development, method comparison studies, QA/QC procedures, etc., would follow.

Ten specific tasks are recommended in order to evaluate receptor-xenobiotic complex concentration in the blood as a biomarker of human exposure. The approximate order of optimal undertaking is reflected in the following sequence:

1. Verify the occurrence of AhR and GABAR in experimental animal and human blood cells using radiolabeled ligands with high specific binding, e.g., [^{3}H]TCDD for AhR and [^{35}S]TBPS for GABAR.
2. For each receptor, determine the total binding capacity (B_{max}), association and dissociation rate constants (k_1 and k_2), apparent equilibrium dissociation constant (K_d), and stability of the receptor-xenobiotic complex within the sample *in vitro*. A considerable information base is already in existence for TCDD, making it the logical choice for the AhR. Of the insecticidal compounds bound by the GABAR, endrin is preferred because of its high affinity constant.
3. If blood cell GABA receptor concentrations are extremely low, the development of an immunoassay for one of the known pesticide ligands may not be practical. Accordingly, an alternative approach should be investigated concurrently with steps 2-5. Specifically, brain GABAR should be used to bind the pesticide of interest in a plasma sample followed by isolation of the complex and quantitation using an immunoassay procedure.
4. Obtain polyclonal antibodies for each receptor complex. Develop and evaluate an immunoassay for each complex. The specificity and/or sensitivity may be improved with the use of monoclonal antibodies if necessary.

5. Using the immunoassay procedure developed, identify the useful dose-response range by determining the concentration of complex formed at different xenobiotic concentrations *in vitro*.

6. Plan and conduct a controlled study using experimental animals to verify the methods and determine the relationship between exposure and receptor-bound xenobiotic concentration.

7. Initiate the development of a biosensor system which will transduce a quantitative chemical/physico-chemical reaction in the immunoassay system developed above into an electrical or optico-electrical signal which can be quantitatively processed.

8. When feasible, identify a human population with known exposure to one or both of the above chemicals and for which an epidemiological study is ongoing. Compare the concentration of receptor-bound pesticide in the blood with other measures of exposure such as total blood concentration, urinary metabolite concentration, etc.

9. A concurrent study should be carried on in conjunction with number one above. This would involve selecting four or five additional high priority pesticides to determine whether these chemicals show specific binding to blood cell membrane preparations *in vitro*. Small groups of xenobiotics, examined periodically as time permits, provide a reservoir of potential biomarkers to be developed as funds and need permit.

10. Three-dimensional molecular modeling software should be used to study the potential for binding of pesticides to specific receptors prior to initiating laboratory animal experiments. These programs permit quantitative information to be gained about

goodness of fit between a particular pesticide structure and the receptor based on comparisons with ligands with known structures and affinities.

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