12th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals: Susceptibility to Environmental Hazards

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The 12th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC) considered the topic of methodologies for determining human and ecosystem susceptibility to environmental hazards. The report prepared at the meeting describes measurement of susceptibility through the use of biological markers of exposure, biological markers of effect, and biomarkers directly indicative of susceptibility of humans or of ecosystems. The utility and validity of these biological markers for the study of susceptibility are evaluated, as are opportunities for developing newer approaches for the study of humans or of ecosystems. For the first time a SGOMSEC workshop also formally considered the issue of ethics in relation to methodology, an issue of particular concern for studies of susceptibility. — *Environ Health Perspect* 105(Suppl 4):699–737 (1997)

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Introduction

Biological Basis of Human and Ecosystem Susceptibility to Environmental Hazards

Individuals vary greatly in their likelihood of developing specific diseases and in their response to environmental hazards. A large number of interacting factors contribute to an individual's risk for disease; these factors include environmental exposures, genetic factors, diet, socioeconomic status, age, and gender. The intrinsic susceptibility of an individual is altered by inherited mutations in genes involved in predisposition to specific diseases, genes involved in the metabolic activation or detoxification of environmental toxins, and genes controlling the repair of DNA or cellular damage. Epigenetic changes in expression of these genes can also affect host susceptibility. These alterations may be due to inherited differences in genes that control the expression of other genes, to past environmental exposures, to the physiological state of an individual, to age-related differences, or to developmentally controlled processes. The interaction between genes and the environment, the cross talk between genes, and the interplay of environmental factors, which include diet and lifestyle, illustrate the complexity in understanding the susceptibility to environmental hazards.

Recent advances in the understanding of molecular biology, the human genome, toxicology, and disease mechanisms and ecosystem functions have led to significant advances in the study of susceptibility factors for environmental hazards. These methods can be used to identify environmental causes of human diseases and harm to ecosystems; to identify susceptible subpopulations; and to understand interindividual and interethnic differences in response to environmental hazards, which, we hope, will lead to disease prevention, an important translation of molecular medicine. The complex interplay between genes and environment represents a tremendous challenge to scientists but also an important opportunity to reduce the burden of disease and dysfunctions to humans and the ecosystem.

The number of biomarkers available to study responses of biological systems to environmental factors is growing rapidly. The term "biomarker" is a general term for specific measurements of an interaction of a biological system and an environmental agent (*1,2*). Biomarkers of exposure measure an exogenous substance or its metabolite and its interaction with a biological molecule. Because a number of factors determine whether a chemical exposure reaches its biological target for a toxic response, the most accurate measurement of "dose" is the biologically effective dose at the target tissue, which can be more reliably measured by biomarkers of exposure than estimated by measurements of administered or ambient chemical exposure.

Biomarkers of effect are measurable biochemical, physiological, behavior, or other alterations within an organism (*1*). Biomarkers of effect are primarily concerned

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Abbreviations used: AAS, atomic absorption spectrophotometry; AHH, aryl hydrocarbon hydroxylase; δ-ALA, δ-aminolevulinic acid; 1-HP, 1-hydroxypyrene; CA, chromosome aberration; CDGE, constant gradient gel electrophoresis; CYP, cytochrome P450; DGGE, denaturing gradient gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediamine tetraacetic acid; EROD, ethoxyresorufin *O*-deethylase; FISH, fluorescent *in situ* hybridization; GPA, glycophorin A; GSTM1, glutathione *S*-transferase M1; GSTT1, glutathione *S*-transferase T1; HPLC, high performance liquid chromatography; HPRT, hypoxanthine phosphoribosyl-transferase; HUGO, Human Genome Project; ICSU, International Council of Scientific Unions; MN, micronuclei; ILO, International Labor Organization; IPCS, International Programme on Chemical Safety; MFO, mixed-function oxidase; MXR, multixenobiotic resistance mechanism; OP, organophosphate; PDGF, platelet-derived growth factor; PCB, polychlorinated biphenyl; PCDF, polychlorinated dibenzofuran; PCDD, polychlorinated dibenzo-*p*-dioxin; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; PSA, prostate-specific antigen; QC, quality control; QA, quality assurance; RFLP, restriction fragment length polymorphism; RBP, retinal binding protein; RT, reverse transcription; SGOMSEC, Scientific Group on Methodologies for the Safety Evaluation of Chemicals; SCOPE, Scientific Committee on Problems of the Environment; SSCP, single-strand conformation polymorphism; SCEs, sister chromatid exchange; TCB, tetrachlorobiphenyl; TCDD, tetrachlorodibenzo-*p*-dioxin; TEF, toxic equivalent factor; WHO, World Health Organization; XRF, X-ray fluorescence; ZPP, zinc protoporphyrin.

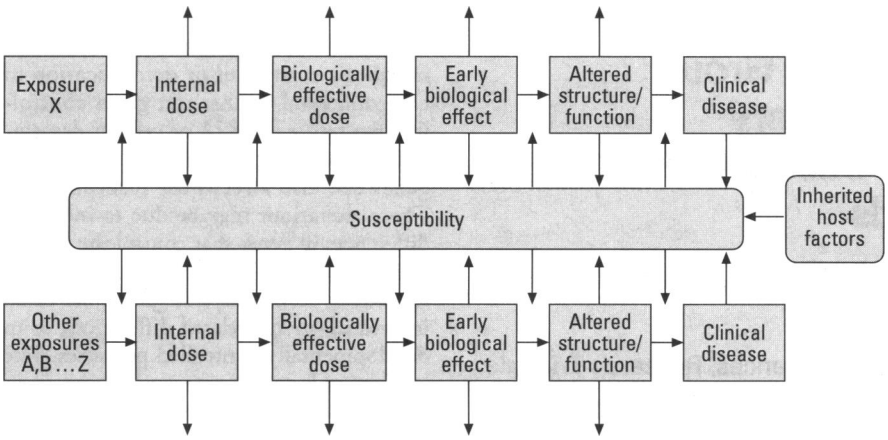

Figure 1. The role of susceptibility in the continuum between biological markers of exposure and of effect.

with adverse effects, although the level of evidence varies for the relationship between a given measured effect and specific, pathological responses, which occur often after a long period and after chronic exposures. There is an overlap between biomarkers of exposure and biomarkers of effect, because the same biomarker can be used for both measurements. Some of the same biomarkers are also used to measure interindividual differences in response and thus further serve as biomarkers of susceptibility.

There is a continuum between biological markers of exposure and those of effect (Figure 1). Exploring the relationship between biological markers on this continuum can be a useful means for detecting susceptibility. Biological markers of susceptibility can be defined as indicators of the mechanistic processes that cause variability among the compartments in the continuum between exposure and effect.

As a simplification, there are five different mechanistic avenues through which factors affecting susceptibility can influence the interaction between biological systems and environmental exposures. These avenues are body uptake, metabolism, target cell uptake, subcellular or molecular interaction, and the baseline status of the individual or ecosystem. The mechanistic avenues are bounded by compartments in which exposure or effect can be quantitated. These compartments are external exposure level, internal exposure level, extracellular level of toxic agent or metabolite, cellular level of toxic agent or metabolite, target cell toxicity, and adverse effect to the individual or ecosystem. In each case one or more of the mechanisms responsible for susceptibility produce a variation in the relation between compartments that is potentially detectable through use of biomarkers.

In the simplest formulation, susceptibility can be defined as a variation in the quantity of exposure/effect in a compartment among individuals or populations who have similar levels of exposure/effect in the preceding compartment. Of necessity, this can only be accounted for by mechanisms operative in the avenue between these two compartments. Examples of compartments and mechanistic avenues are the following:

- For a given level of external exposure, there is an interindividual variation in the uptake into the body (e.g., increased Fe uptake in hemochromatosis; variations in uptake of air pollutants related to respiratory rate).
- For a given level of a toxic agent, there is interindividual variation in the uptake into the target cell (e.g., increase in radioactive iodine uptake into the thyroid in iodine-deficient individuals; tumor resistance to chemotherapeutic agents).
- For a given level of uptake into the body, there is interindividual variation in the level of a metabolically produced active toxic agent (e.g., differing activities of metabolic enzymes).
- For a given level of uptake into the target cell there is a variation in the extent of effect (e.g., glucose 6-phosphate dehydrogenase deficiency or methemoglobin reductase deficiency rendering the red cell susceptible to oxidant stress; differences in DNA repair).
- For a given level of effect in the target cell, there is a variation in the impact on the individual (organ functional reserve capacity variations due to age; disease factors).
- For a given level of effect in an organism, there is a variation in the impact on communities and ecosystems (e.g., shifts in biomass, species diversity, energy cycling due to heavy metals or organochlorines).

Each mechanistic avenue can have multiple inputs in different directions, which influence the extent to which a quantity in one compartment affects the quantity in the next compartment. For example, even when the relation between the absorbed dose of a xenobiotic and the level of a toxic metabolite is the result of a single enzyme, the activity of this enzyme in any individual may be a function of a genetic polymorphism as well as the presence of other pollutants or of dietary factors that affect enzyme activity. The potential order of the compartments may differ depending upon the mechanistic pathways; e.g., metabolism may occur in a nontarget cell and precede uptake of the toxic metabolite into the target cell (reversal of the second and third sections in the above example). The relationship between compartments can be modeled mathematically so as to explore the extent of variability in the relationship between two compartments among a specified population group, i.e., the distribution of susceptibility due to this particular mechanistic avenue.

In essence, the true distribution of susceptibility in a population is the relation between the first compartment and the last one—between external exposure and disease outcome, with the intervening mechanistic avenues contributing to this distribution. For a given individual it is possible that a mechanism that increases susceptibility to a disease outcome in one avenue may be counterbalanced by a mechanism that decreases susceptibility in another avenue. It is thus important to recognize that analysis of only one mechanistic avenue in the pathway between exposure and disease does not necessarily provide definitive information on the susceptibility of any single individual in the population.

Biomarkers can be divided into those that measure compartments, e.g., blood levels, DNA adducts, or tissue damage; and those that measure mechanisms, e.g., enzyme activity levels or gene polymorphisms. Each has its advantages and its limitations. Measurement of the compartment tells little about the reasons for the level; measurement of the mechanism is not always a definitive indicator of what will be happening in the next compartment. For individuals or populations at risk, it is preferable to have measurements both from mechanistic avenues and from

related compartments to more effectively target prevention strategies.

Genetic Susceptibility

Individuals may vary in terms of their responses to environmental hazards due to differences in their genetic constitution. The human genome encodes for 50,000 to 100,000 genes, some of which are key regulators of biological processes. Mutations (heritable alterations in the primary coding sequences of genes or their controlling elements) in specific genes ("disease genes") greatly predispose an individual to a disease. For example, mutations that inactivate the retinoblastoma gene, a central regulator of cell growth and division, can result in a 105-fold increased probability of a rare eye cancer in children (retinoblastoma). Due to recent advances in gene mapping and isolation, a number of major disease genes have been discovered, including genes involved in cystic fibrosis; Huntington's disease; Alzheimer's disease; and breast, colon, lung, and many other cancers. Mutations in disease genes lead to a very high probability of disease development, often approaching a 100% incidence of disease in carriers of the mutations.

Mutations in disease genes are usually rare; depending on the gene, 1 in 200 to 1 in a million people are affected. However, multiple genes may regulate a specific disease, therefore, the percentage of disease cases for which there is a genetic component may be high. For example, in certain cancers (e.g., colon, breast, and prostate) it is estimated that up to 10% of the cancers are due to a genetic predisposition. These very strong susceptibility genes may not be influenced by environmental factors, but there is evidence for environmental influences in some diseases even with a strong genetic predisposition.

Many of the genes in the genome of humans and other species influence the impact of environmental agents on the organism. Genetic controls on the uptake, activation, detoxification, or repair of environmental insults are known. The exact number of genes involved in the organism's response to environmental hazards is unknown but could be very large. For example, the estimates of the number of the individual P450 genes in any mammalian species range from 60 to 200 (*3*).

All genes commonly have variations in their sequences that may or may not have functional consequences. Changes in DNA sequence that occur frequently (in >1% of the population) are called genetic polymorphisms. Polymorphisms often affect the function of a gene but some may change the level of expression of a gene or change the activity of the gene product, for example, an enzyme. Genetic polymorphisms that are functionally significant are quite important when the gene controls the response of an organism to environmental hazards. For example, polymorphisms in the genes that metabolize carcinogens can affect the response of individuals to that carcinogen. Given that a large number of genes are involved in responses to environmental hazards and that a large number of polymorphisms exist in these genes, genetic differences are important susceptibility factors in environmental responses. Many of these commonly occur in human populations.

Recent advances in the identification and cloning of specific genes and methods for the detection of mutations and polymorphisms in these genes have led to significant advances in our understanding of genetic susceptibility to environmental hazards. There is an important difference between individuals with genetic alterations that lead to disease susceptibility and individuals with genetic susceptibility to environmental factors. Individuals who inherit a mutation in a disease susceptibility gene have a high risk of developing that disease regardless of environmental exposures, although environmental factors may increase the incidence or rate of disease development. Individuals who have a mutation or polymorphism in genes involved in response to environmental hazards will only have an increased risk of disease development when they are exposed to specific environmental hazards. Therefore, risk to these individuals is influenced strongly by gene–environment interaction. Also, because multiple genes are involved in response to the same environmental hazards, two individuals with the same genetic susceptibility and environmental exposure may have different risks because of the interplay between genes involved in response to xenobiotics. For example, two individuals may both have a polymorphism in a gene that increases the rate of carcinogen activation but different polymorphisms in a gene that inactivates the same carcinogen. Because many environmental response genes remain to be identified and characterized and because many mixtures of environmental exposures are present in the environment, it is difficult to determine an individual's risk. Great advances have been made in the identification of susceptibility factors for subpopulations exposed to environmental hazards.

Susceptible Populations versus Susceptible Individuals

Environmental and occupational medicine, like medicine in general, is traditionally concerned with individuals and their health. It may also seek to identify individuals who are liable to develop a specific disease (predictive medicine). This approach implies the possibility of dividing the people into two groups: those who are disease prone, and the healthy remainder. This is, of course, a considerable oversimplification, because susceptibility is rarely confined to a distinct high-risk minority and because our ability to predict the disease outcome for these individuals is therefore weak.

Epidemiology, as a discipline of public health, includes the study of the distribution of diseases and risk factors in populations. Epidemiologic research focuses on populations in order to elucidate a broad range of risk factors for disease. A distinction exists between the populations that epidemiologists study and the individuals comprising those populations. Within a low risk population, there are individuals who develop disease, and within high-risk populations there are disease-free individuals. An individual's risk will be determined by a complex interplay of multiple genes, multiple exposures, diet, age, and chance. The application of biomarkers of exposure, effect, and susceptibility to the assessment of an individual's risk from environmental hazards is not conceptually different from the application of common clinical measures in medical practice. For example, blood pressure, blood lipids, relative weight, and smoking habits are applied to the assessment of an individual's risk for coronary heart disease in clinical medicine. Combining biomarkers with epidemiological study design has led to the development of molecular epidemiology, an approach that generates a great deal of interest and expectation. The use of biomarkers in human studies raises challenging ethical questions that must be addressed in advance of applying new technologies in this area.

Susceptible Populations and Ecosystems

Ecologists are concerned with understanding differences in susceptibility among individuals within communities of thousands of species. One important methodological approach is the development of

biomarkers of exposure and effect that are applicable across a broad range of species that range from invertebrates to birds and mammals. However, ecologists also are concerned with understanding and predicting—through the use of biological indicators—differences in susceptibility of communities and ecosystems. Here, vulnerability refers to changes in the structure and function of ecosystems. The challenge for ecologists is to develop a broad range of methodologies from molecular through ecosystem measures that can be used to assess susceptibility.

In many cases, cellular and molecular biomarkers that can be used to assess the susceptibility of a range of indicator species will be most useful for communities and ecosystems as well. Such biomarkers could be used to assess both the health of the ecosystem and the potential of the ecosystem to support human populations and provide ecological services.

Ethics

The study of susceptibility in human populations and in ecosystems poses a number of ethical challenges. Regarding humans, both the manner in which information is obtained and the uses to which it could be put raise urgent concerns. Regarding ecosystems, the ethical duty to work towards protecting our shared environment and its life support systems requires ongoing attention and the development of clear guidelines.

Specifically relevant to susceptibility studies in humans is the sensitivity of the information and the likelihood of labeling certain individuals or subgroups in ways that could cause unanticipated harm. Scientists and public health professionals investigating susceptibility or implementing related preventive screening programs must be aware of the potential for harmful social and psychological side effects arising from susceptibility studies. For example, denial of employment, denial of insurance, fear for the premature onset of illness or death, social marginalization, reconsideration of family planning and of both medium and longer term life goals, and the need for psychological counseling are possible untoward effects that may arise from the identification of susceptibility in individuals.

Scientists also must remain sensitive to cross-cultural differences in perception of personal risks associated with susceptibility studies and must strive for equity in their research and in its potential consequences. For example, there may be greater need to focus resources on populations subjected to more severe pollution than on those populations experiencing lower levels of exposure.

Scientists must understand ethical issues to be able to address the challenges presented by susceptibility studies and to learn how to resolve them. Preparation would include defining the key ethical issues, identifying the principles behind them, and then attempting to resolve the ethical dilemmas that emerge. This objective requires a certain amount of formal training in ethics as well as ongoing dialogue involving a broad range of stakeholders who try to anticipate ethical dilemmas. Stakeholders include, among others, other professionals as well as patient advocates.

Markers of susceptibility are of a particularly sensitive nature. Therefore, serious attention must be paid to quality assurance so that data integrity can be even more highly assured than in most other types of studies. In addition, existing guidelines that protect confidentiality and ensure the participants' "right to know" must be revisited and made more specific in light of the potential consequences of susceptibility studies.

Where legal protections are not clearly in place to ensure confidentiality of personal information gathered in the conduct of research into susceptibility, and to protect the various stakeholders against retroactive actions, the risks associated with such studies must be made clear to any research participants in advance of their commitment to participate. Such risks include birth with inherited negative traits, and illness induced or associated with environmental/occupational exposures only subsequently recognized as having been associated with susceptibility traits.

Finally, susceptible ecosystems exist, the destruction of which can have both direct and indirect negative consequences for human health and well-being. For example, the susceptibility of the earth's atmosphere has consequences for global warming associated with, among other things, increasing skin cancer rates, coastal flooding disasters, and crop failures. Scientists engaged in such work must bring to the attention of human populations their stewardship duty and responsibility to protect the ecosphere.

Conclusions and Recommendations

The following conclusions and recommendations have been formulated in recognition of human and ecosystem vulnerability to environmental hazards:

a) Determination of susceptibility to chemicals in the workplace and general environment is becoming increasingly feasible through rapid advances in biological sciences, particularly molecular biology. Parallel advances have occurred in epidemiology, ecology, toxicology, and related sciences which have greatly facilitated understanding and measurement of susceptibility.

b) Increased understanding of the pathways leading to susceptibility of individuals, of populations, and of ecosystems to chemical and physical agents is of value in protecting human health and the environment.

c) Identification of biological markers of exposure and of effect is a useful avenue for determining susceptibility. Markers of susceptibility in essence operate in the pathway between the various compartments of exposure and effect, reflecting mechanisms responsible for variations in response to the levels in the previous compartment.

d) There is an important distinction between studies of the susceptibility of populations at risk and studies of the susceptibility of individuals. It is important not to misinterpret from population studies the risk to an individual.

e) The ethical, legal, and social dimensions should be recognized in studies of individuals, populations, communities, and ecosystems at risk. Ethical dilemmas must be considered in advance of research activities or of any use of susceptibility markers in evaluating populations at risk. In some cases public debate and legislation will be required to clarify social norms and to provide legal protection for the participant. Ethical issues should be incorporated in the development of methodologies to study and evaluate communities and ecosystems and should be reflected in research proposals.

f) Studies of biomarkers of susceptibility should not be undertaken in circumstances in which confidentiality and privacy safeguards cannot be assured.

g) Training programs for professionals engaging in susceptibility studies of biomarkers need to integrate formal ethics education. Continuing education should include an ongoing discourse through ethics workshops, symposia, and discussion in the journals of the respective professions.

h) Numerous other methodological issues must be taken into account before accepting a marker of susceptibility as being potentially useful in protecting public health and the environment. These issues include technical feasibility, cost, quality assurance and control, and cultural and

logistic issues related to sample availability, collection, and storage.

i) The determination and use of susceptibility markers pose both technical and ethical issues in developing countries. These need to be taken into account when designing studies or applying the use of susceptibility markers validated in industrialized countries.

j) Additional research is needed to focus biological advances on increasing understanding of factors affecting the susceptibility of human and nonhuman components of ecosystems, and to determine how best to apply this understanding to protect public health and the environment. Good science should be tied to good ethics and vice versa.

Biological Markers of Exposure

Definition of Exposure Markers

In the narrowest sense, biomarkers of exposure refer to measurement of the specific chemical of interest or its specific metabolite in a body compartment or fluid. In the broadest sense, it can refer to any biomarker used to estimate current or past exposure for either medical, epidemiologic, or risk assessment purposes (*4*). This can even include the clinical detection of evidence of exposure, although this domain is usually excluded from discussion of biomarkers.

Many of the biomarkers of effect are used also to assess exposure, and some of these will be discussed in this section. Not all biomarkers can be used in all settings, particularly in developing nations (*5*).

Objectives for Using Biomarkers of Exposure

Exposure can be viewed as both a population and an individual phenomenon. Exposure markers can function in several ways. *a*) They may lead to early detection of exposure at a point where significant health effects have not occurred. This must focus attention on external means of reducing exposure, i.e., on primary prevention. *b*) They may provide validation of exposure for use in epidemiologic studies. *c*) They may facilitate comparison of exposure levels in different compartments (external, blood, cellular) to identify susceptibility differences.

Criteria for Screening, Using Biomarkers. The application of any biomarker approach must be conducted within the context of a viable screening and prevention program. The World Health Organization (WHO) has established a set of criteria to be met before instituting a screening program. These will be discussed in relation to exposure markers.

CRITERION 1. The screening must be conducted for a condition of public health significance. Overall the exposure, health status, or susceptibility of populations or subpopulations is clearly of public health significance. Thus, criterion 1 is usually met (but see the section on ethical considerations).

CRITERION 2. The natural history of the exposure marker must be well understood, and there must be a distinguishable subclinical phase. There is tremendous variability in our knowledge regarding the natural history of various exposure markers and their relation to subsequent effects.

CRITERION 3. There must be a scientifically defensible and socially and personally acceptable intervention. The usual intervention when an exposure is detected is to remove the hazard and reduce the exposure.

CRITERION 4. The tests used must have appropriate sensitivity and specificity. Usually the sensitivity limitation is imposed by the analytic method available. Increasingly sophisticated instrumentation has allowed the measurement of various analytes at infinitesimally small levels, although in most cases this sensitivity is not required. However, it is essential to choose the appropriate tissue or fluid and methodology to assure adequate test sensitivity. Sensitivity also depends on the proper timing of analysis with respect to exposure.

Measuring the agent of concern is usually highly specific. For example, a blood lead determination reflects lead exposure. However, it may not be specific to the source of lead being investigated since there are many sources of lead in our environment. Specificity in part depends on the question being asked. For example, one might be interested in recent exposure or historic exposure and would design a different testing approach for each. In some cases, to address specific questions, chemical speciation of the agent or analyte is necessary (e.g., chromium VI vs chromium III, methyl vs inorganic mercury, organic vs inorganic arsenic) and speciation, in turn, may reduce sensitivity.

The metabolites one measures may be specific or nonspecific. Thus phenol is commonly used as a marker of benzene exposure but is not specific (other compounds are metabolized to phenol and some consumer products, such as cough medicines, contain phenol). Nonetheless it may still be useful in quantifying exposure in individuals known to have relatively high levels of exposure to benzene. Other metabolites are more highly specific (e.g., muconic acid for benzene), and others are very specific (DDE for DDT). Some metabolites are also active agents.

CRITERION 5. The condition or exposure being sought must be sufficiently common that the tests have acceptable predictive value. Exposure is a phenomenon common to both a population and an individual. On the individual level, the predictive value of a screening test depends not only on its sensitivity and specificity, but on the underlying prevalence of the exposure. One can estimate population exposure by randomly sampling a subgroup of the population before extending screening for exposure biomarkers to a larger population.

CRITERION 6. The testing must be acceptable to the target population. Biomarkers of exposure are most often detected with noninvasive (breath sampling, urine testing) or minimally invasive (milk collection, blood testing) approaches. At the other end of the spectrum are fat biopsies which are limited to clinical diagnostic settings or to specialized research protocols.

CRITERION 7. The testing program must be cost effective (see below, on analysis and quality assurance).

Use of Biomarkers

Applications of Biomarkers of Exposure. Monitoring biomarkers of exposure is usually part of a preventive activity. It can contribute to identifying and reducing exposure or to identifying at-risk or susceptible populations that need to be protected in special ways. In occupational health, the major goal of detecting biomarkers is the prevention of health impairment by the recognition of excessive exposure and the elimination of hazards (*6*). Ideally, exposure is controlled and measured at the source so that no excessive exposure occurs (*7*), but often there is supplemental reliance on biological monitoring as part of a medical surveillance program, to assure that the primary preventive strategies have been effective.

A marker can be applied on an individual basis to estimate the amount of pollutant absorbed or retained, usually through the measurement of the agent or its specific metabolites (*8*). A marker can reflect individual differences in the rate of absorption and in toxicokinetics (metabolism, distribution, and excretion).

The traditional definition of an exposure biomarker involves measurement of a xenobiotic or its metabolite in a body tissue or fluid, whereas markers of effect include any measurable alteration attributable to a xenobiotic that can be recognized as a health impairment. Between the two is a continuum of subtle effects caused by chemicals that can be measured but which are not indicative of a disease. Some of these are early stages in a significant pathogenic process (e.g., DNA adducts of alkylating agents), others are early, often trivial, stages in a pathogenic process (zinc protoporphyrin [ZPP] elevation in lead exposure), and others may be the early signs of damage that are reversible and not of clinical significance (for example, depression of cholinesterase activity or elevation of some tubular proteins in persons exposed to heavy metals). In the latter case, it is recognized that kidney function may show no clinically detectable decrement until about 90% of the nephrons are damaged; hence substantial changes can occur with no immediate overt clinical significance except a reduction in reserve capacity.

Blood lead is a marker of exposure and a surrogate marker of effect. Elevations of ZPP or erythrocyte protoporphyrin and urinary δ-aminolevulinic acid (δ-ALA) or the activity of δ-ALA dehydratase are all effects caused by lead; the inhibition of enzymes in the heme synthesis pathway results in anemia, yet anemia is rarely the end point of concern in lead poisoning, especially in adults. Thus, they are useful as exposure as well as effect markers.

A major criterion for distinguishing markers of exposure from markers of effect is the purpose to which they are put. In many cases enzymatic or cellular damage markers are used for dose reconstruction or for the classification of exposure status for epidemiologic studies. Van Schooten et al (*9*), for example, treat DNA adducts in smokers as a means of estimating their polycyclic aromatic hydrocarbons (PAH) exposure.

For the purposes of this discussion we recognize the following as biomarkers of exposure: *a*) measurement of the xenobiotic itself (i.e., lead in blood, DDT in milk); *b*) measurement of specific metabolites; *c*) measurement of specific subclinical effects that are reversible. Many effect markers that are used to estimate exposure or tissue dose are described in more detail below.

Choice of Tissue or Fluid and Analyte. Factors that influence the choice of tissue or fluid and analyte include the toxicodynamics of the agent (where it is distributed, stored, excreted), speciation (its chemical form), and chronology (acute vs chronic exposures and temporal aspects of its kinetics in the body).

An exposure marker should be able to distinguish long-term from short-term exposures (*10*). Thus blood lead may reflect recent exposure, while bone lead reflects cumulative exposure.

An example of the importance of appropriate quality control (QC) is reflected in the experience with routine blood lead determinations by clinical laboratories. As recently as the early 1990s, when laboratory proficiency testing programs were concerned with accuracy in the presumed toxic range of 40 μg/dl, it was found that most laboratories were unable to provide reliable results below 15 μg/dl, even though the Centers for Disease Control had designated 10 μg/dl as a "level of concern." Two years later, when proficiency testing began to include unknowns in the 10 μg/dl range, most laboratories rapidly improved to the point where they provided acceptable results. It is essential that a quality assurance (QA) program be designed for the range of values that will be encountered in a population.

DNA and Protein Adducts. Many compounds form covalent adducts with nucleic acids and other macromolecules including hemoglobin and other proteins. In the case of DNA adducts, the changes in the DNA molecule may relate directly to mutational or repair events and the possibility of subsequently developing cancer. Elevated levels of DNA adducts are seen in a number of populations with known elevated cancer risks, including smokers and coke-oven workers.

DNA adducts can be measured by the ^{32}P-postlabeling technique or by immunoassays. For certain adducts, mass spectrometry, fluorescence spectrometry, and liquid chromatography/electrochemical detection have been used. For quantitative analysis a standard compound is necessary for the calculation of recovery as discussed by Hemminki (*11*). In the case of complex mixtures, identification of individual adducts may not be possible. In such cases recoveries cannot be calculated and quantification is not possible. Data on half-lives of adducts in humans are very limited. Biomonitoring of protein adducts applies to either hemoglobin or albumin. Adducts can be released hydrolytically from the protein and assayed by gas or liquid chromatography, or by mass spectrometry. N-terminal valine of hemoglobin can be specifically released in the Edman degradation process and assayed by mass spectrometry. Adducts are generally not thought to alter the half-lives of the proteins.

DNA–protein cross-links are another end point that has been used to estimate exposure to mutagens such as hexavalent chromium (*12*). A standardized DNA extraction procedure is used that fails to extract cross-linked DNA that can be quantified as a percentage of the total DNA.

Assessing Population Exposures. Examples of exposure markers used to document the effectiveness of regulatory interventions are the dramatic decline in blood leads in countries that have eliminated lead from gasoline (*13*), and the decline in the levels of polychlorinated dioxins and dibenzofurans in breast milk in Sweden (*14*), attributable partly to the ban of chlorinated phenol herbicides.

Overall Ethical Considerations. The estimation of exposure is performed to benefit the individual subject or population, usually by subsequently reducing their exposure to hazards. It is unethical to perform a testing program merely for its own sake, without having the goal of reducing harmful exposures that may be detected. Therefore, in a workplace, a biological monitoring program using markers of exposure should be performed as part of a comprehensive medical surveillance program linked to an industrial hygiene program capable of discovering and eliminating hazards.

Zielhuis (*10*) cautioned that the results of a screening examination must be examined in the light of many individual factors. In turn the physician must impart objective information to the participant. "In the perception of the examined subject, biological sampling for assessment of internal exposure, and henceforth of health risk, is not distinguished from assessment of their health" (*10*). Individuals should be informed of their health risks, along with the degree of uncertainty, regardless of the technologic and economic consequences.

Some question the ethics of biological monitoring for exposure, arguing that humans should not be the guinea pigs or the detectors of their own exposure. However, biological monitoring offers the advantage of taking into account absorption by all routes (whereas air monitoring would not detect potential exposure by other routes). We caution that biological monitoring can be an adjunct, but it cannot substitute for environmental monitoring and controls. [See below and Soskolne

Table 1. Examples of biological exposure markers.

Agent	Exposure marker
Aniline	Total *p*-aminophenol in urine; methemoglobin in blood
Benzene	Total phenol or muconic acid in urine; benzene in expired air
Cadmium	Cadmium in urine or blood
Carbon disulfide	2-Thiothiazolidine-4-carboxylic acid in urine; CO in end-expired air
Chlorobenzene	Total 4-chlorocatechol or *p*-chlorophenol in urine
Chromium VI	Total Cr in urine
N,N-Dimethylformamide	*N*-Methylformamide in urine
Ethylbenzene	Mandelic acid in urine or ethylbenzene in end-expired air
Fluorides	Urinary fluoride
Furfural	Total furoic acid in urine
n-Hexane	2,5-Hexanedione in urine or *n*-hexane in expired air
Lead	Lead in blood or urine, ZPP
Methanol	Methanol or formic acid in urine
Methemoglobin inducers	Methemoglobin
Methyl chloroform	Methyl chloroform in end-expired air or trichloroacetic acid in blood or urine
Methyl ethyl ketone	Urinary methyl ethyl ketone
Nitrobenzene	Total *p*-nitrophenol in urine or methemoglobin in blood
Organophosphates	Red cell or serum cholinesterase activity
Parathion	Total *p*-nitrophenol in urine or RBC cholinesterase
Pentachlorophenol (PCP)	Total PCP in urine or free PCP in plasma
Styrene	Mandelic acid in urine, styrene in blood; phenylglyoxylic acid in urine
Toluene	Hippuric acid in urine; toluene in blood or end-expired air
Trichloroethylene (TCE)	Trichloracetic acid in urine; TCA or trichlorocethanol in blood; TCE in end-expired air
Xylenes	Methylhippuric acids in urine

(*15*) elsewhere in this issue for a further discussion of ethical issues.]

Examples of Biological Monitoring for Selected Agents (Table 1)

Heavy Metals. Most heavy metals are of toxicologic concern, although some are essential trace elements in humans as well. Since these elements are not metabolized, they can be tracked in the body, and can be measured in various body compartments and fluids. A variety of standardized analytic procedures are described in readily available references (*16*). Most of these are analyzed with atomic absorption spectrophotometry (AAS), usually using a graphite furnace as the source. Flame photometry works with higher concentrations of metals. Mercury is usually measured with a cold vapor technique.

LEAD. A variety of tests of lead exposure and early effects have been used, including: δ-ALA in urine, δ-ALA dehydratase activity in red blood cells, free erythrocyte protophorporin or ZPP in blood (still widely used), urinary coproporphyrin (very limited use), and blood lead (the standard measure).

The measurement of blood lead is considered the best sampling approach for both adults and children (*17*). Analysis for populations with negligible lead exposure (no occupational exposure in countries without leaded gasoline) requires the sensitivity of graphite furnace AAS; this is currently the most widely used instrument for any population, although anodic stripping voltammetry is also used. In the absence of this capability, excessive lead exposure can be detected by measuring ZPP in a fingerstick blood sample with a portable fluorometer. Alternative methods still available in some laboratories include measuring the urinary excretion of δ-ALA.

These measurements reflect relatively recent and ongoing exposure. They do not provide information regarding the body burden or effects of long-term accumulation. The challenge test, which uses a dose of a chelating agent (usually EDTA) and monitors urinary lead excretion in the following 24 hr, provides an estimate of the amount of lead that can be mobilized and is used to judge whether chelation will be effective. The chelation test carries a level of risk, including the mobilization of large amounts of lead which can then reach the brain and kidneys.

The past decade has seen the development of *in vivo* X-ray fluorescence (XRF) of bone as a way of measuring the relative concentration of lead stored in the skeleton. Although increasingly available, the technique is still under development, and there is not adequate concordance among laboratories (*18*). Two types of machines are used, the K-wave and L-wave sources, which differ in their penetration and apparently in the precision of measurements. Almost all machines in use today employ K-wave XRF.

An example of using blood lead as a marker of exposure and susceptibility is a recent study in Mexico. Romieu et al. (*19*) found a significant correlation among the lead content of ceramics used to prepare food, the soil lead on children's hands, and the children's blood lead; yet all of the environmental variables explained only 19% of the variance in blood lead. Thus even assuming that there are uncertainties in measurement of the independent variables, this leaves tremendous room for individual variability in the uptake and distribution of inorganic lead in these children. Thus blood lead identifies variation in susceptibility as well as exposure among these children.

Analysis of lead in urine is of relatively little value for quantifying exposure, even in those with organolead exposure.

Many studies have shown that there is a low correlation between lead in blood and lead in air, due in part to the sampling duration; concurrent exposures through water, diet or other jobs; alternative routes of exposure (ingestion); variations in the use of protective equipment; variations in circumstances of exposure, including respiratory rate, exercise, and other microniche characteristics; and individual variation of either a genetic or epigenetic nature (*17*).

MERCURY. The uptake, toxicokinetics, and end points associated with organic mercurials (particularly methyl mercury) differ greatly from those associated with inorganic mercurials. Inorganic mercury is excreted mainly in urine, organic mercury mainly in feces. Both mercurials can be deposited in hair. Accordingly, inorganic exposure is usually monitored with urinary mercury, although blood mercury testing is also useful and helps distinguish exposure within the past week from that occurring in the past month. Blood mercury is used to assess organic mercury exposure. When dietary exposure (especially fish consumption) is the source of mercury, it is usually not necessary to speciate the mercury, since almost all of the mercury is methyl mercury. There is a strong correlation between either blood or urine mercury and air mercury (*20*).

Hair mercury is useful for screening populations. The digestion of hair, however, causes difficulties in some laboratories (*21*). Urinary mercury testing is mainly used for occupational exposure or for residential exposure to metallic (elemental) mercury.

CADMIUM. Cadmium is usually measured in blood or urine by graphite furnace AAS. Lauwerys et al. (*7*) documented the relationship between exposure and blood levels of cadmium in humans. Many

sources recommend the concomitant measurement of β_2-microglobulin, but that marker of renal tubular damage is insensitive and should not be relied on. By the time it becomes elevated, significant kidney damage has occurred. However, a variety of new tubular markers such as retinal binding protein (RBP) and *N*-acetyl-β-D-glucosaminidase (NAG) are more sensitive but not more specific (*22*).

The body burden of cadmium has been measured by neutron activation *in vivo* (*23*), but this is a highly specialized technique of only research application.

Pesticides. Pesticides include any substance or mixture that destroys or controls plant or animal pests or vectors of disease. "Pesticide" is a broad term including, in addition to pesticides, a variety of biocides (fungicides, herbicides, acaricides, molluscicides, rodenticides, etc.) Pesticides remain a global occupational health problem (*24*).

Some pesticides, such as the organophosphates, are short-lived both in the environment and in the body, and it is possible to detect only acute exposure (by measuring a metabolite) or recent exposure (past 3 months) by measuring cholinesterase levels. Chlorinated hydrocarbon pesticides are persistent both in the environment and in the body and can be measured for many months or years after exposure has terminated (*25,26*).

PESTICIDE EXPOSURE. Absorption resulting from dermal exposure is the most important route of uptake for pesticide-exposed workers, while ingestion is the most important nonoccupational route. Within the body, the pesticide may be eliminated or transferred to a target, unchanged or after metabolism. Organophosphates are typically broken down rapidly, while many organochlorine pesticides are stored in the fat. The actual pesticide exposure (uptake) can be measured by biological monitoring of human tissues and body fluids. Insecticides and their metabolites can be measured after occupational exposures (*26–29*).

WHERE TO MEASURE VARIOUS PESTICIDES. Different sampling approaches are required for different classes of pesticide. Examples include the measurement of dialkylphosphates in urine after exposure to organophosphorus insecticides (*27*), of *p*-nitrophenol after exposure to parathion and methylparathion (*28*), and of 1-naphthol after exposure to carbaryl (*29*). Altered liver enzyme activities have been reported among pesticide workers exposed to organophosphorus pesticides alone or in combination with organochlorine or other pesticides (*30*). Monitoring changes in vitamin K levels is useful for identifying exposure to anticoagulant rodenticides (*31*).

Adducts to hemoglobin have been detected with several pesticides (*32*). The advantages of such measurements include the possibility of assessing dose closer to the target, of assessing individual capacity to form electrophiles, and of extrapolating data on toxicity more easily across species. When the mechanism of action of a pesticide is understood, more specific markers can be used (*30*).

ORGANOCHLORINES. Certain organochlorine (OC) pesticides are highly persistent. Although DDT is metabolized to DDE and DDD, these metabolites persist in the body fat for many years after exposure. They can be detected at much lower levels in serum. The reported levels in fat vary among countries, the highest levels of DDT being found in countries where the compound is still used. Fat biopsies are not suitable for routine screening of pesticide exposure.

Some studies of the concentration of pesticides in human milk are summarized by Anwar (*26*). The contaminants found most frequently in human milk have been DDT, its main metabolite DDE, hexachlorobenzene, hexachlorocyclohexane, dieldrin, heptachlor epoxide, and the non-pesticide polychlorinated biphenyls (*33*).

ORGANOPHOSPHATES. Whereas persistent organochlorine pesticides tend to have relatively low human toxicity, the organophosphates (OP) are highly toxic to humans and are responsible for many deaths around the world. These compounds bind various cholinesterase enzymes, thereby interfering with transmission in the nervous system. A widely used biochemical biomarker is cholinesterase depression. It is frequently used in occupational health as a marker of OP exposure and is regularly used clinically as a marker of OP effect. With a specific antibody it is possible to measure the concentration of a particular esterase in plasma or serum. Diagnostic kits to measure specific activities of blood esterases are being developed for use in the field (*30*). However, it is essential that the workers with potential exposure have two baseline cholinesterase determinations, since cholinesterase activity varies greatly between individuals and within the same individual over time (*21,34*).

Further markers in pesticide application workers are described by Anwar (*26*).

Polycyclic Aromatic Hydrocarbons. PAH are usually present in complex mixtures of 3- to 6-ring compounds, of which hundreds of congeners may be present in various proportions. Analysis is usually by gas or liquid chromatography, where selected compounds, such as benzo[*a*]pyrene (B[*a*]P) are quantified.

Biological methods have been developed to measure 1-hydroxypyrene (1-HP), a metabolite of pyrene, in urine. 1-Hydroxypyrene is a prevalent species in PAH mixtures. It is excreted in the urine and is a good biomarker of exposure to PAH (*35*). An advantage is that urine can be stored for at least a year frozen at –20°C without preservative. After hydrolysis and cleanup, the 1-HP is extracted and analyzed on high performance liquid chromatography (HPLC). The result is expressed as µmols/mol of creatinine (*35*).

Øvrebø et al. (*36*) found levels up to 13.5 µmol/mol creatinine in coke oven workers compared to 1.54 µmol/mol in the surrounding community and up to only 0.2 µmol/mol in a control area.

Adducts of hemoglobin or albumin are measured after hydrolytic cleavage of PAH from the protein, followed by quantification by mass spectrometry. DNA adducts are measured by the ^{32}P-postlabeling technique and by immunoassay. Neither of these techniques quantifies individual PAHs.

Jongeneelen (*37*) studied the S9 supernatant fraction of liver samples from 15 kidney transplant donors for variation in PAH metabolism *in vitro*. 1-Hydroxypyrene was the major metabolite of pyrene and 3-OH-B[*a*]P was the major metabolite of B[*a*]P. The V_{max} for B[*a*]P and for purine were highly correlated. They found individual variation in the V_{max} and the apparent affinity for the substrates. The V_{max} for B[*a*]P ranged from 0.002 to 0.710, with a geometric mean of 0.012.

Volatile Organics. Ethylene oxide is discussed here as an example of a volatile organic compound. Ethylene oxide is used as sterilant in hospitals. It is also the principle metabolite of ethene, a precursor to polyethylene plastics and other synthetic chemicals. Ethylene oxide can be measured by gas chromatography in air or biological specimens. Ethylene oxide reacts in the body with hemoglobin; N-terminal valine may be released by a modified Edman degradation process and measured by gas chromatography–mass spectrometry. DNA adducts can be measured by a number of techniques [above; Hemminki (*11*)], including ^{32}P-postlabeling mass spectrometry and liquid chromatography/electrochemical detection.

Ionizing Radiation. Various cytogenetic techniques have been used extensively to study populations exposed to ionizing radiation, most notably the survivors of the bombing of Hiroshima and Nagasaki. Giemsa-banding techniques are labor intensive and provide a resolution of about 1 million base pairs. They give information on the frequency of chromosomal aberrations: breaks, dislocations, deletions, and translocations. The fluorescent *in situ* hybridization (FISH) technique (*38*) correlates well with traditional chromosomal aberration analysis and allows a more rapid screening of individual samples for dose reconstruction. This is also an effect marker. Reciprocal translocations are apparently permanent, hence are potentially useful for dose reconstruction even years after the exposure event. The use of hypoxanthine phosphoribosyltransferase (HPRT) mutants and other molecular biologic approaches to detect gene alteration, including radiation specific lesions in DNA (*39*) is dealt with below.

Sampling Considerations

Toxicokinetics: The Importance of Timing. Many endogenous chemicals and probably also xenobiotics do not maintain a constant level in the body but undergo diurnal variation, sometimes in a deterministic fashion. Therefore it is important to standardize techniques by sampling at one time of day. However, it is also important to understand the time course of excretion, which is not always a uniphasic half-life (negative exponential curve) but may be biphasic or triphasic with an initial rapid elimination followed by much slower elimination. If a substance is mainly excreted within 24 to 48 hr, it is possible only to monitor for acute exposure. Even for substances with a half-life of 6 to 12 months, it may be impossible to reconstruct the exposure that may have occurred in years past.

Conclusions and Recommendations

Biological monitoring of exposure can play an important role in the detection of exposed individuals or groups at an early stage before significant or irreversible adverse effects have occurred. Biological markers of exposure also play a role in quantifying or classifying exposure for epidemiologic studies. Biological monitoring is also useful in identifying variations in susceptibility among members of a population. It is important that any biological monitoring for exposure markers be conducted as part of a comprehensive framework that includes steps for intervening and reducing any hazards that are identified. A screening program should meet the criteria proposed by WHO. The information from such programs should be shared with the stakeholders.

Biological Markers of Effect

Definitions

The International Programme on Chemical Safety has defined a biomarker of effect as "A measurable biochemical, physiological, behavioral or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease" (*1*).

This is a very broad definition. Such biomarkers of effect can be elicited as a result of interaction of the organism with a host of different environmental factors (including chemical, physical, and biologic agents); this definition encompasses biomarkers of effect at the level of the whole organism, at the level of organ function, at the level of tissue and individual cells, and at the subcellular level. For example, the results of neuropsychologic tests may be considered biomarkers of behavioral effects of the organism which may be induced by solvent exposures. Spirometry results may be considered biomarkers of physiological effects on the respiratory system which may be induced by fibrogenic dust exposures. Sperm counts may be considered biomarkers of effect on reproductive cells which may be induced by exposure to synthetic estrogenic compounds. There are many other similar examples of biomarkers at these levels in almost all systems of the body, including biomarkers of hematological toxicity, nephrotoxicity, hepatotoxicity, immunotoxicity, neurotoxicity, pulmonary toxicity, and reproductive and developmental toxicity (*1*). These will not be considered further here, but rather this review will focus on biomarkers of effect at the subcellular level, particularly at the chromosomal and molecular level, which are particularly useful in assessing susceptibility. These include biomarkers at the genomic level, such as cytogenetic alterations and gene mutations, and biomarkers of gene expression, such as messenger RNA and proteins. Some assays of protein function, such as enzymatic activity, could be included in this category but could also be considered as biomarkers of exposure (e.g., cholinesterase activity in response to OP exposure), and thus they will not be included here. Biomarkers at the genomic level or at the level of gene expression have been most strongly associated with the risk for cancer, although in selected instances they may also be associated with other disease end points. Therefore, in general, we will be concerned here with subcellular biomarkers of effect that are believed to be associated with cancer. We will examine the methodologies employed with reference to the objectives of their use, their potential uses, and their advantages and limitations.

Because in many instances, these biomarkers of effect are believed to represent events in a causal pathway to disease, their occurrence may be viewed as indicative of an acquired susceptibility for that disease. This is in distinction to the biomarkers of susceptibility per se, which in many cases represent indicators of inherited susceptibility to disease due to their influence on steps in the causal pathway. Biological markers of effect, as indicators of acquired susceptibility for disease, have the theoretical potential to exhibit predictive value in identifying individuals who could develop disease at some point in the future or in determining the likely disease progression in terms of severity and prognosis once disease has occurred. In addition, biomarkers of effect, because they occur quite far along the pathway between exposure and disease outcome, essentially provide an effective integration of the influence of biomarkers of susceptibility for all preceding steps in the causal pathway and are thus a summary marker for susceptibility to that point.

Cytogenetic Markers

In humans, cytogenetic alterations are most often analyzed microscopically from peripheral lymphocytes after they have been stimulated to divide in culture by a mitogen (*40*). Lymphocytes are used as a surrogate for the actual target tissues of genotoxic carcinogens. Some cytogenetic end points (micronuclei, numerical aberrations) can also be studied in interphase cells from target tissues or proximate target tissues, such as exfoliated cells from buccal, nasal, urothelial, bronchial or esophageal epithelium (*41,42*). Specimens can also be obtained from hair bulbs. In special cases, for instance, in connection with accidental exposures, dividing cells from bone marrow may be available. Furthermore, cytogenetic biomarkers can be studied from samples collected for prenatal diagnosis (amniocytes or chorionic villus cells). Effects on germ cells can be examined from

semen specimens (*43*). Fresh samples are required in most cases, but preservation by freezing or fixation is possible for several of the cytogenetic techniques. The analysis of cytogenetic end points from such samples is used to show exposure to genotoxic carcinogens and may have value in the identification of groups of people at increased risk of cancer (*44,45*). Cells from different individuals can be challenged with genotoxic agents *in vitro*, which in some cases may enable the recognition of sensitive individuals (*46,47*).

Chromosome Aberrations. Chromosome aberrations (CAs) are structural alterations, breaks and rearrangements, in chromosomes, usually observed in metaphase-blocked cells using conventional microscopy (*40*). Chromosome-type rearrangements, such as translocations and dicentrics inspected in biological dosimetry of radiation, can also be analyzed using chromosome painting based on FISH with chromosome-specific DNA probe libraries (*48*). This approach has enabled the recognition of new types of complex chromosome rearrangements that have not been detectable with conventional cytogenetic techniques. Recently, a simplified FISH method to detect chromosome breakage and alterations of chromosome number, using tandem DNA probes specific for a region in chromosome number 1, was reported (*49*). FISH has also been applied to study numerical chromosome aberrations of specific chromosomes (*50*). Polymerase chain reaction (PCR)-based methods for the analysis of lymphocyte-specific illegitimate chromosome recombination involving human immunoglobulin or immune receptor loci and considered to reflect genetic instability have also been described (*51,52*). In two recent, independent reports, increased rates of CA in peripheral lymphocytes were shown to be associated with later development of cancer (*44,45*). Thus, the analysis of CAs is presently regarded as the cytogenetic method of choice in studies of human exposure to genotoxic carcinogens. *In vivo* inducers of CAs in humans include, among others, ionizing radiation, alkylating cytostatics, tobacco smoking, benzene, and styrene. Besides smoking, factors such as age, gender, and diagnostic and therapeutic X-rays are usually taken into account as possible confounders. *In vitro* challenging of lymphocytes from healthy individuals with genotoxins has revealed individual differences in CA response to some genotoxic agents (*53,54*). The reasons for such differences are unknown, except for 1,2:3,4-diepoxybutane, where an association probably exists with the deficiency of glutathione *S*-transferase T1 (GSTT1) (*46,47,55*). Among butadiene-exposed chemical workers, *GSTT1* null individuals also had an elevated rate of chromosome aberrations in peripheral lymphocytes *in vivo* (*56*). On the other hand, the lack of GSTM1 has been associated with elevated CA frequencies in smokers and in bus drivers (*57*).

Sister Chromatid Exchanges. Sister chromatid exchanges (SCE) represent symmetrical exchanges of DNA segments between the sister chromatids of a duplicated metaphase chromosome (*58*). In lymphocytes of humans, tobacco smoking, alkylating cytostatics, and ethylene oxide are well documented SCE inducers. Factors to be controlled in the analysis of SCEs include age, gender, and life style. Individuals with enhanced *in vitro* SCE response to some genotoxins have been described and, in some cases, this has been associated with certain metabolic genotypes or phenotypes (*46,47,50,59–66*). For example, individuals sensitive to *in vitro* SCE induction by 1,2:3,4-diepoxybutane were recently found to be deficient of *GSTT1* (*46,47,62,64,66*). Such people also show elevated baseline frequencies of SCEs in their peripheral lymphocytes (*47,62,67*). SCEs were not, however, elevated in *GSTT1* null individuals exposed to butadiene (*62*). Increased SCE rates have also been indicated in relation to the *GSTM1* null genotype and smoking (*68*) and deficiency of the low K_m aldehyde dehydrogenase and alcohol consumption (*69*).

Micronuclei. Micronuclei (MN) are small additional nuclei observable in interphase cells. Micronucleus induction can be triggered by either clastogens or agents that influence the mitotic apparatus, such as spindle poisons (*70,71*). Micronuclei can also be scored in exfoliated cells of buccal, nasal, or urothelial mucosa (*41*). The presence of whole chromosomes in MN can be checked by identifying centromeric DNA sequences (using FISH) or kinetochore proteins in the MN (*72,73*). *In vivo*, increased MN frequencies in lymphocytes have been associated with exposure to ionizing radiation, aging, and gender (*74*). In women, the influence of age on the frequency of MN in lymphocytes has been related to an increased inclusion of the X-chromosome in MN (*75,76*), while in men the Y-chromosome appears to be responsible for a part of this effect (*77*). In buccal or nasal mucosa, MN induction has clearly been shown for, e.g., various ethnic chewing habits (*3*) and exposure to formaldehyde (*78*). The MN analysis has given negative results in several studies in which another biomarker of genotoxic exposure has been positive. As MN can originate from chromosomal fragments and whole chromosomes, the number of cells presently examined may not be enough to reveal the relatively small effects usually expected in exposed humans. The identification of the centromeric contents of MN and automated analysis may improve the specificity and sensitivity of the MN assay of lymphocytes.

General Considerations. At the population level, structural CA appears to have predictive value for later development of cancer. As similar associations have not been detected for other cytogenetic endpoints, the analysis of CA appears to be, at present, the cytogenetic method of choice in studies of cytogenetic alterations in lymphocytes. However, the sensitivity of the assay does not allow evaluation of cancer risk based on individual values. Analysis of an *in vitro* cytogenetic response of human cells to genotoxins may, in some cases, be used to identify susceptible individuals, especially when genetic polymorphisms are taken into account. In biological dosimetry of radiation exposure, the analysis of chromosome-type aberrations has been used as the basis for individual dose estimates. Chromosome painting by FISH is becoming the new tool in the biological dosimetry of radiation; the applicability of techniques based on FISH and PCR for the identification of specific chromosome alterations for studies of chemical genotoxins should be evaluated. The MN assay combined with FISH analysis of centromeres in MN may offer an easier technique to score CA, especially in target tissues or proximate target tissue. Because each biomarker depicts a different phenomenon, the use of various exposure and effect biomarkers together is recommended in risk assessment. It is also a useful approach for detecting patterns of human susceptibility. The combination of cytogenetic parameters and information on metabolic genotypes or phenotypes is expected to increase the sensitivity of the cytogenetic assays and allow better understanding of the biological significance of genetic polymorphisms (*43*). As some polymorphisms have been shown to influence basic or induced levels of cytogenetic damage, information on the genotypes or phenotypes of the individuals studied is

becoming important when these end points are used as biomarkers. Automation and analysis techniques utilizing FISH offer faster and easier approaches to detect cytogenetic alterations.

Markers of Gene Mutations

Somatic Gene Mutations in Surrogate Tissues. The detection of mutations in the *HPRT* gene has been used in experimental mutagenicity studies of mammalian cells, and it is also the most extensively employed assay for human gene somatic mutations *in vivo*. In humans, *HPRT* mutations are examined in lymphocytes, and the standard assay involves T-lymphocyte cloning for phenotypic selection of 6-thioguanine-resistant mutant cells. With the development of PCR-based techniques, further molecular characterization of the mutations present in the T-cell clones has also become possible. Such studies have shown, for instance, a clear difference in mutation spectrum between "spontaneous" mutations occurring early in human development and mutations acquired later in life (*79*). Studies of human lymphocytes *in vitro* are also considered useful, as they provide information on *HPRT* mutation spectra obtained after exposure to specific carcinogens in controlled conditions. Several studies have been carried out on *HPRT*-mutant lymphocytes in human populations exposed to various genotoxic agents, as reviewed by Cole and Skopek (*80*) and briefly described by Hemminki (*11*). Only a few studies have thus far tried to correlate *HPRT* mutations with other measures of exposure and susceptibility. In a study of occupational exposure in a foundry, the frequencies of *HPRT*-mutant T-lymphocytes correlated with the level of aromatic DNA adducts (*81,82*). In a recent study on garage workers, a correlation was found between aromatic DNA adducts and mutation frequency at the individual level; genotypes of two xenobiotic-metabolizing genes (*GSTM1* and *NAT2*), alone or combined, did not influence *HPRT* mutation frequency (*83*).

The glycophorin A (GPA) assay is another *in vivo* method for the detection of somatic mutations to study the potential effects of exposure to chemical and physical mutagens (*84,85*). The assay is based on the autosomal GPA locus that encodes the cell surface sialoglycoprotein expressed in the erythrocytic lineage and responsible for the M,N blood group. It uses immunolabeling and flow cytometry to enumerate, in peripheral blood samples of M/N heterozygotes, erythrocyte variants reflecting mutations in the GPA locus. Most of the variants are considered to derive from mutations that occurred in bone marrow stem cells and are therefore permanent, depicting lifetime accumulation of mutations. The GPA assay has been automated and is thus easy, fast, and cheap to run. The main limitations are that only one half of the human population (M/N heterozygotes) can be studied and an expensive flow cytometer is required. As erythrocytes have no nuclei, the variant cells cannot be clonally expanded and the mutations cannot be characterized at the molecular level.

Both the *HPRT* and GPA mutation assays show a small number of people with exceptionally high mutant frequencies in healthy subjects (*86*). While at least part of such high frequencies are due to clonal expansion of a few original mutations, some of them might indicate individuals of enhanced susceptibility. In a study of GPA mutations in reinforced plastics workers, the high frequency individuals were primarily smokers or ex-smokers (*87*). Recently it was shown that in smokers significantly elevated NN variant frequency was associated with *GSTT1* null genotype (*88*).

Gene Mutations in Target Tissue. Knowledge on gene mutations from other tissue than peripheral blood and lymphocytes is sparse. This relates to problems in availability of samples and difficulties in developing methods suitable for detecting mutations occurring at a low frequency. Growing cells under *in vitro* conditions circumvents the problem with lymphocytes. Somatic mutations have been detected in genes related to human disease and particularly in cancer-related genes (protooncogenes and tumor suppressor genes). In most cases somatic mutations in disease-related genes do not give rise to any functional change in the cell, which would allow its isolation or expansion *in vitro*. In the case of cancer tissue, malignant growth involves clonal expansion of cells, which allows detection of mutations by the methods presently available. The methods appear to work best and most reproducibly on fresh frozen tissue, but in many instances only preserved tissue is available.

Mutations in oncogenes and tumor suppressor genes are most common in many types of human cancer. In the context of external exposure to carcinogens, the *ras* genes have been shown to be mutationally activated in a number of environmental cancers in humans (*89*), and in a carcinogen-specific manner in animal studies (*90*). Since the *p53* tumor suppressor gene is among the most frequently altered genes in human cancer, molecular analysis of its mutational spectra has been used as a clue to cancer etiology and mechanisms of carcinogenesis (*91,92*). In the *p53* gene, DNA sequences corresponding to the highly conserved domains of the protein have been identified as a "hot spot" region for mutations. A majority of mutations identified in these genes in human cancers are missense mutations, making them suitable targets for such analysis. The exposure-related nature of *p53* mutations has been proposed in many studies on various forms of human cancers (*91–94*). Well-known examples of this are nonmelanoma skin cancer associated with exposure to sunlight (UVB) as well as hepatocellular carcinoma related to dietary exposure to the mycotoxin aflatoxin B_1 (AFB_1) (*92*). With the highly sensitive mutation detection assays it has been possible to investigate whether the suggested causative carcinogens (AFB_1, UVB) induce similar kinds of mutations in human cells *in vitro*. In the case of AFB_1, the results were in agreement with the etiological role of AFB_1, although other types of mutations were also seen (*95*). In nonmalignant human liver tissue, the frequency of the specific AGG to AGT mutation at codon 249 was found to parallel the level of AFB_1 exposure in the geographical areas where the patients lived (*96*). For UVB exposure, the experimental work on human skin fibroblasts did not find the tandem double CC to TT mutations seen frequently in skin cancer (*97*). A relationship between certain types of *ras* mutations or *p53* mutations and occupational exposure to agents like solvents, vinyl chloride monomer, some bladder carcinogens, and radon has been suggested (*98–100*). In addition, smoking has been associated with increased prevalence of *p53* mutations in some tumors, and certain types of base substitutions have been linked with exposure to tobacco smoke (*101,102*). Recently, two studies have reported a higher frequency of *p53* mutations in lung cancer patients with the at risk genotype of *GSTM1* gene (*103–105*).

Studies on prognostic significance of p53 protein overexpression (determined immunohistochemically) or mutation have suggested that accumulation of the mutant protein may predict poor survival in many cancer types (*92*).

Methodological Considerations. An array of methods has been used for detection of point mutations in unknown positions of the target gene. Most of them initially

used radioactive labeling but alternative techniques have been developed. One of the first techniques available was the RNase mismatch cleavage analysis method (*106*). Methods relying on chemical modification of mismatched nucleotides, by either carbodiimide or hydroxylamine and osmium tetroxide (chemical cleavage of mismatch), have been developed; they use mutation detection mainly in genes of inherited diseases (*107*). Two assays used widely in mutation detection in association with inherited diseases, genetic polymorphisms, and also for somatic mutations in tumor tissue are single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE). SSCP assay is based on conformational changes in DNA due to sequence alterations (*108,109*). SSCP has been successfully used for detection of DNA alterations of many cancer-related genes in human cancer (*ras, p53, RB,* for example). It is estimated to yield over 90% efficiency in detecting single base substitutions in sequences of 300 bp or less in length (*109,110*). It is also well suited for detection of polymorphic alleles of various genes (*109*). DGGE separates DNA molecules based on their sequence-determined ability to melt (separate partially) in longer fragments (low- and high-temperature melting domains) (*111*). DGGE separates physically wild-type DNA from mutant molecules, and under appropriate conditions all single-base substitutions, frameshifts, and deletions less than about 10 bp can be resolved from the wild-type DNA (*112*). DGGE, or its application called constant gradient gel electrophoresis (CDGE) (*113*), has been used to study both germline and somatic mutations in many human genes. These include mutations in cancer-related genes, human *ras* and *p53* genes, as well as chemically induced mutations of *HPRT* gene in human lymphocytes *in vitro* (*112*). More recently, a capillary electrophoresis technique has been applied for CDGE (*114*).

For detection of known mutations in certain sites of a gene, two much-applied methods include allele-specific oligonucleotide hybridization and genotypic mutation analysis by restriction fragment length polymorphism (RFLP)/PCR method. Allele-specific oligonucleotide hybridization uses labeled probes which are hybridized to PCR-amplified genomic DNA. It has especially been used in studies on *ras* gene mutations in various human cancers (*89,115*). More recently, many assays relying on recognition of a certain restriction site which either is present in the wild-type sequence or results from a mutation have been developed. Of these, one of the most sensitive and perhaps most promising for molecular toxicology purposes is the RFLP/PCR assay for detection of codon 12 mutations in the human H-*ras* gene and codon 247 to 250 mutations in the human *p53* tumor suppressor gene (*116,117*). The method is very sensitive and suitable for detection of very low frequency mutations, such as those in premalignant or in normal cells after chemical treatment *in vitro*. They are, however, limited to a single base substitution at a certain codon.

DNA sequencing based on dideoxy-mediated chain termination reaction (*118*) can be performed also after *in vitro* amplification of the DNA template by PCR. Direct sequencing of PCR amplification products is thus one method of choice for mutation analysis; it is perhaps more labor intensive when performed manually but has been automated (*119*). Methods using solid phase support in sequencing are being used increasingly (*120*). Sequencing has of course the advantage of giving precise information on the sequence alterations unlike some other methods (SSCP, DGGE). For targeted use, an application called solid-phase minisequencing has been developed (*121*). However, direct sequencing of genomic DNA of poor quality may be problematic (fixed and paraffin-embedded tissue samples) and other methods are better suited in such instances.

Markers of Gene Expression

mRNA Expression. Markers of gene expression include assays for detection of mRNA or for detection of proteins. Many of the assays for mRNA employ methodologies similar to those used for DNA analysis noted above. In most cases, the assays have been used for the examination of diseased tissue (e.g., cancer tissue) in the target organ. This poses problems of accessibility. In addition, mRNA has very limited stability, and, therefore, tissue samples must be obtained fresh and processed quite rapidly so that the mRNA is preserved.

With the appropriate oligonucleotide probes, the presence of mRNA in tissue can be detected by *in situ* hybridization. For example, differences in mRNA for oncogenes may be detectable between cancer tissue and adjacent normal tissue (*122*). Other disease end points may also be relevant. For example, *in situ* hybridization can detect increases in mRNA for growth factors, such as platelet-derived growth factor and transforming growth factor-β, in relation to the stimulation of cell growth and the development of fibrosis resulting from asbestos (*123*). Alternately, tissue can be analyzed for mRNA expression by isolation of message and amplification by quantitative PCR followed by Northern blot analysis (analogous to the separation techniques of Southern blot analysis for DNA by gel electrophoresis) with appropriate oligonucleotide probes. Quantitative PCR is considerably more difficult to perform reliably compared to qualitative PCR, adding further complexity to this approach.

None of the approaches to mRNA expression have been well developed for routine application. Thus, among other problems, there is generally little standardization of assay techniques, little definition of normal values or values of pathophysiologic significance, and little examination of reproducibility, sensitivity, specificity and predictive value of the tests in terms of the occurrence of or prognosis for disease state (i.e., indication of susceptibility for development or severity of disease). Given these limitations and the other difficulties noted above, it is probably unrealistic to expect that gene expression by mRNA analysis will be very useful as a biomarker of effect. At any rate, the true index of gene expression from the point of view of effect in the cell is at the protein level, not the mRNA level, and it is not even clear that the two always correlate. As noted below, detection of protein expression has the potential to avoid some of the problems inherent in mRNA detection and is thus the more attractive candidate for a biomarker of gene expression for many reasons.

Protein Expression. Methodologies for examining gene expression by the detection of the encoded proteins all depend on the development of antibodies (either polyclonal or monoclonal) that are theoretically specific for the identification of the protein of interest. In many cases, however, the specificity is only theoretical because there is cross reactivity of the antibody with similar epitopes in other, unrelated proteins, raising the possibility of false-positive tests. Conversely, sensitivity of the antibody detection (related to many factors, including avidity and affinity for the epitope, accessibility and stability of the epitope, and the sensitivity of the secondary reaction system used to identify the occurrence of the antigen–antibody interaction) may be insufficient to detect the antigen, leading to false-negative tests. Monoclonal antibodies

that are produced from a single cell that is clonally expanded and producing an antibody for only a single epitope are more easily standardized. Polyclonal antibodies can be variable and are thus more difficult to standardize but may have the virtue of reacting with several different epitopes on the protein of interest, increasing the likelihood of detection. Modifications of antibody applications that can improve the results include confirmation of the molecular weight of the identified protein as consistent with what is expected and the use of more than one antibody probe to demonstrate that the protein identified reacts with all expected positive antibodies; it does not react with negative antibodies.

For protein identification of gene expression, antibodies can be used in tissue analysis or in assays of surrogate sites such as extracellular fluids. As with mRNA analysis, tissue analysis for protein expression usually relies on diseased tissue (e.g., cancer tissue) in the target organ, although in some cases the same analysis can be applied to exfoliated cells, for example, from the urothelium or the bronchial epithelium (*124*). Tissue analysis can be either *in situ* or in aggregate. *In situ* analysis relies on the application of the appropriate antibody in immunohistochemistry either to fresh-frozen tissue sections or to fixed, paraffin-embedded tissue sections. The latter approach raises issues of the stability of the antigen with fixation and with storage over time, which can vary with the different proteins. Immunohistochemistry allows for the localization of proteins of interest within cells (e.g., the question of whether the protein is identified at the subcellular site one would expect) as well as within the histology of the specimen (e.g., if the protein is identified in cancer tissue, in precursor lesions, in apparently normal adjacent tissue). Aggregate analysis of tissue can be accomplished with fresh tissue specimens by lysis of the cells and analysis of the appropriate cellular fraction of the lysate (*125,126*). Analysis of cell lysate can be performed with antibodies applied in Western blotting (separation of the proteins by gel electrophoresis followed by antibody probing), which has the advantage of allowing molecular weight determination as a confirmatory indicator but which can be laborious, difficult, and hard to standardize. Alternately, analysis of cell lysate can be performed with antibodies incorporated into an antigen-competition or an immunosorbent assay, such as radioimmunoasays or enzyme-linked immunosorbent assays (ELISA), which are generally easier, more rapid, and more easily standardized. Stepwise application of assays can be employed, for example, in which an ELISA may be used to screen samples and Western blotting may be used to confirm positive results.

Protein identification in extracellular fluids can be similarly accomplished with antibodies employed in Western blotting or ELISA or related assays. This approach is obviously more accessible and convenient than relying on tissue, but it raises issues such as whether extracellular fluid protein levels accurately reflect cellular protein levels. Identification of certain proteins in extracellular fluids, such as tumor-associated antigens, has been well standardized in convenient assays and widely applied. Prostate-specific antigen (PSA) is a good example (*127,128*). Even in these cases, however, many important issues remain unresolved. Tumor-associated antigens like PSA may be useful monitoring the course of disease once identified and treated, but the relationship to the pathophysiology of the disease process remains unclear and thus its utility in predicting future occurrence of disease that will be clinically significant in an individual is unproven. Other proteins that represent expression of genes in the presumed causal pathway for disease, such as growth factors and oncoproteins, can also be analyzed with antibody-based techniques in extracellular fluids, as described in depth by Brandt-Rauf (*129*). Unfortunately, these tests are not as well developed as the others mentioned. In general, they are not standardized; normal values and values of pathophysiologic significance are not well defined; confounders have not been identified; and sensitivity, specificity, and predictive value as indicators of susceptibility for disease occurrence need to be more closely examined. Thus, they are clearly not ready for routine use at this time. As with all biomarkers of gene expression, additional research will be necessary to more clearly define their advantages and limitations and to determine their potential uses.

Conclusions and Recommendations

Considerations for Developing Countries. Little work has been done to monitor the level of exposures and their effects on human populations in developing countries (*130*). Although trials have been done for cytogenetic monitoring of populations at risk (*42,131*), more effort should be made to improve the information concerning human exposure possibilities and the identification of exposure-related diseases.

Simple cytogenetic techniques (such as CAs, SCEs and MN) and simple gene expression techniques (ELISA for protein levels) can be applied for monitoring in developing countries. More advanced molecular biomarkers (e.g., based on PCR and gene sequencing) may be considered only if the appropriate resources are available.

General Considerations. Most of the biological markers of effect described here are still experimental tools whose utility remains to be established. For these biomarkers, issues of assay validation, QA/QC, and convenience and cost need to be addressed. As noted above within the context of susceptibility, one objective of their use would be the identification of individuals or groups who, by virtue of the acquired susceptibility to the effect, are likely to develop disease. Some examples, such as the evidence that increased rates of chromosomal aberrations are associated with later development of cancer, suggest that this may be possible, but in most cases of biomarkers of effect, the predictive value remains unproven.

Even if predictive value is ultimately established, problems will remain. The utility of establishing such susceptibility without any way to alter it is of questionable value. In some cases, the effects detected may be irreversible, so effective secondary prevention would be one important option. In other cases, the effects detected may be reversible (as with the reversion of SCE rates following removal of exposure to cytostatic drugs), so that primary prevention may be effective. At any rate, primary prevention through the reduction or elimination of the exposure producing the effect is always good policy, and secondary preventive interventions or the use of biomarkers to eliminate individuals from ongoing exposure situations are not a substitute for primary preventive efforts.

Other important social, legal, and ethical implications also derive from these aspects of biomarkers of effect. For example, the knowledge of truly predictive biomarkers of effect could obviously be misused to detrimentally affect the employability and insurability of the individual concerned, compounding the suffering induced by the psychological burden of the knowledge. Furthermore, such knowledge having a potentially adverse psychological effect raises the issue of whether the detection of a biomarker of effect is itself an impairment and perhaps a disability, even without other demonstrable clinical

effects. Could such an impairment/disability be compensable under tort or workers' compensation laws? Would such an individual be entitled to special considerations under laws for protecting the disabled in employment and other societal circumstances? Prior to the general use of biomarkers of effect, such questions will have to be addressed. Some general ethical aspects of these issues are considered in more detail elsewhere in this volume.

Other potential uses of biomarkers of effect are in monitoring of disease progression and prognosis, and as adjuncts to other biomarkers in providing refinements of epidemiology and risk assessments. At the very least, biomarkers of effect, as well as other biomarkers, offer the opportunity to provide scientific confirmation of proposed exposure–disease pathways *in vivo* in human populations. Biomarkers of effect may be particularly useful for demonstrating the biologic influence of preceding susceptibility factors (e.g., genetic polymorphisms of xenobiotic-metabolizing enzymes). It should be noted once again, however, that at this time these biomarkers of effect should only be employed in the context of carefully designed studies with all due attention to the rights and concerns of the human subjects involved. Nevertheless, we believe that research on biomarkers of effect is worth pursuing because of the potential benefits for disease prevention.

Biological Markers of Susceptibility

Definition of Susceptibility Markers

A biomarker of susceptibility is defined as an indicator or a measure of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance (*1*). Biomarkers of susceptibility are concerned with factors in kinetics and dynamics of uptake and metabolism of exogenous chemicals. Thus the concept encompasses enzymes of activation and detoxication, repair enzymes, and changes in target molecules for toxic chemicals. Many of the latter conditions include factors that confer highly increased risks to predisposed individuals (i.e., repair gene defects, "fragile" DNA conditions, etc.). Susceptibility factors occur along a continuum from "near-obligatory" determinants of high risk to contributory low risk factors (such as metabolic polymorphisms). In this document, we focus on those factors that involve a major contribution of gene–environment interaction to become manifest.

Monogenic traits are inherited in a Mendelian fashion. A genetic polymorphism refers to a monogenic variation that occurs with at least two phenotypes with sufficient frequency (> 1%) to cause population differences. Genetic variations occur as either germline inheritance or somatic cell mutations. Rare inherited metabolic diseases such as Crigler-Najjar syndrome occur as a germline mutation (*132*); a well-known example of a somatic cell mutation is hepatocellular carcinoma caused by dietary exposure to AFB_1 (*91*).

Nongenetic factors such as age and sex are obviously important but need further refinement in terms of molecular mechanisms and their interplay with environmental factors before being useful for any intervention. Acquired factors (for example physiological changes, disease-induced changes, induction and inhibition of enzymes by dietary factors, etc.) are also critical but difficult to study because of their individual nature. Such changes may not be permanent and thus may be impractical to take into consideration in any long-term or retrospective study.

Interindividual variability is observed for practically all diseases and toxic responses. It is certainly related in part to different qualitative and quantitative environmental exposures, but differences in susceptibility between individuals in response to the same level of exposure are also common. In most chronic diseases, a long sequence of events leads to a final response, which is seen as a result of an interplay of multiple genetic and other host factors and environmental factors, each contributing to an overall risk of having a manifest response.

Although biomarkers of susceptibility identify those individuals in a population who have a difference in susceptibility to the effects of chemical exposure, only in some circumstances can they predict an individual's risk with any confidence. It is important to not generalize inappropriately from *in vitro* evidence of susceptibility, as has occurred in inferring that individuals with red blood cell glucose 6-phosphate dehydrogenase deficiency are particularly sensitive to inhaled ozone (*133*).

Objectives of Use of Susceptibility Markers

Interindividual variation occurs as a result of different genetically inherited background modified by dietary and environmental exposure and revealed by genotypic and phenotypic variation. Susceptibility markers are useful because they can partially explain interindividual variation inherent in the general population and thus provide a biological rationale for investigation of inherent vulnerability prior to exposure to environmental hazards.

The objectives of the use of susceptibility markers are the following:

- To evaluate interindividual variation of risk associated with environmental and dietary exposure to hazardous chemicals. These methods cannot easily identify all individuals at risk in a hazardous environment due to lack of understanding of the interaction of compensatory genetic and cellular mechanisms and complex environmental influences. While individual susceptibility may only occasionally be specifically linked to a single defective gene (e.g., *CYP2D6*) where the specificity of substrate structure has been better characterized, it currently is our best approach to assess populations at risk.
- To determine the role of genetic variations to explain interethnic differences associated with susceptibility to chemical exposures and to predict population vulnerability.
- To improve the detection of environmental hazards by increasing the sensitivity of epidemiological studies which in turn will result in *a*) reduction of risk through avoidance or limitation of chemical exposure *b*) changes in dietary and social habits to improve health or reduce risk *c*) improved drug treatment to maximize response and minimize toxicity. Better knowledge of xenobiotic metabolism and pharmacokinetics of elimination of toxins will speed progress of this work.

Methodology in Studying Susceptibility-related Genes

The approaches and methods described in this section will focus on studies of polymorphisms in xenobiotic-metabolizing enzymes. However, most of these methods, in particular the molecular techniques, can be used for studying polymorphisms of other susceptibility-related genes, such as DNA repair enzyme genes or disease genes.

In addition to the polymorphisms of susceptibility-related genes, other genetic alterations such as "mini- and microsatellites" are also believed to be important in human carcinogenesis and are involved in individual susceptibility (*134*).

There are two important parts in studies of genetic polymorphisms. The first is how to identify a new polymorphism and its

possible functional significance. The second is how to define the role of a known polymorphism in human susceptibility to environmental toxicity in which the frequency distribution of the polymorphic genotypes or phenotypes should be determined. In both cases the PCR technology has greatly increased our ability to study genetic polymorphisms. In the PCR reaction specific stretches of the DNA can be amplified exponentially by thermostable DNA polymerases with a pair of specific primers. Several methods are widely used in studies of genetic polymorphisms and interindividual variations in chemical metabolism.

Methods for Studying Phenotypic Expression. USE OF PROBE DRUGS *IN VIVO.* When an appropriate probe drug (with specificity and safety) is available, metabolic polymorphisms can be identified by determining the metabolic ratio, i.e., the ratio of the blood or urinary concentration of the parent drug over its metabolite in different individuals administered the probe drug. Metabolic polymorphism is usually indicated in a population if the frequency distribution of the metabolic ratio is shown to be bimodal or trimodal. For example, a bimodal distribution of debrisoquine 4-hydroxylation (catalyzed by CYP2D6) is observed due to the existence of "poor" and "extensive" metabolizers (*135,136*). The role of gender-based CYP1A2 variability in susceptibility to bladder cancer has been explored using the caffeine breath test (*137*).

USE OF PROBE DRUGS *IN VITRO.* The probe drugs can be used for *in vitro* metabolism studies to look for possible polymorphisms of the metabolizing enzymes, which are usually indicated if large interindividual variations in the activities are observed. Diagnostic probe drugs for individual CYP enzymes have been developed such as coumarin for CYP2A6 and caffeine for CYP1A2 (*138*).

OTHER METHODS. Polymorphisms of xenobiotic-metabolizing enzymes can be determined at other phenotypic levels. Enzyme protein levels can be measured by immunological methods such as immunoblotting and immunohistochemical analyses. mRNA levels are quantified by different nucleic acid hybridization techniques (Northern and slot blotting, RNase protection, and *in situ* hybridization). Quantitative RT–PCR (reverse transcription of the mRNA to cDNA followed by PCR amplification) has been developed for detection of mRNA in a small amount of tissue samples. Expression of *CYP1A1* mRNA in human lymphocytes and its regulation by tetrachloro-dibenzo-*p*-dioxin (TCDD) have been successfully determined by this method (*139*).

Most genotoxic chemicals need to be metabolically activated to exert their effects. Therefore variations in the activities of the xenobiotic metabolizing enzymes by polymorphic changes can influence the genotoxic effects. Biomarkers of effect, such as cytogenetic damage caused by a genotoxic compound, can be used as an indirect measurement to evaluate the metabolic activity of cells from donors with different polymorphic genotypes or phenotypes. The commonly used cytogenetic parameters include CAs, SCEs, and MN (*46*).

Methods for Studying Genetic Polymorphisms. IDENTIFICATION OF NOVEL GENETIC POLYMORPHISMS. Once a metabolic polymorphism (or a polymorphism at other phenotypic expression levels) is demonstrated, various molecular biology techniques including cloning and DNA sequencing can be used to look for possible genetic changes of the enzyme. A successful example is the discovery of *CYP2D6* genetic polymorphism (*140*). After cDNA cloning and DNA sequencing, it was demonstrated that a mutant *2D6* allele is responsible for the majority of "poor metabolizers." Further work established that a mutation at a splicing site caused the production of defective 2D6 mRNA and a total absence of 2D6 protein (*141*).

The DNA sequence alterations can be screened by SSCP analysis, which detects the mutation-caused mobility shift of the DNA fragments on gel electrophoresis (*109*), or by DGGE, in which DNA molecules are separated based on differences in their melting temperatures due to the sequence alterations (*113*). In some cases, the variant alleles are detected by sequence comparison with no knowledge of the related phenotypic polymorphism. Most of the polymorphisms will not have a functional significance. Functional analysis is then critically important in establishing the phenotypic significance of these variant alleles. Catalytic activity of enzymes can be studied by expressing different variant proteins with the cDNA expression systems if the polymorphic changes are localized in the coding region, such as in *CYP2A6* polymorphism (*142*). If the polymorphic loci are in the noncoding region, their effects on the transcriptional regulation can be studied by linking the mutated sequence with a reporter gene, such as in *CYP2E1 Rsa*I polymorphism (*143*).

GENOTYPING OF POPULATIONS. It is rather simple to determine an individual's genotype with respect to a specific locus by current molecular biology techniques, when the polymorphic sites of a xenobiotic-metabolizing enzyme gene are clearly identified. If a polymorphic site changes the recognition sequence of a restriction enzyme, or the polymorphic alteration involves a large deletion, the genetic polymorphism can be identified by RFLP analysis in which DNA is subjected to Southern blotting after digestion with appropriate restriction enzymes and hybridized with specific probes.

PCR technology has greatly increased our ability to detect genetic polymorphisms. DNA amplification can be carried out with specific PCR primers for any particular sequence of a polymorphic gene, with small amounts of human tissue or cell samples. The DNA source can be from blood leukocytes, buccal epithelial cells, hair roots, and exfoliated cells such as bladder epithelial cells in the urine. The PCR-amplified DNA sequence containing the polymorphic sites can be analyzed by RFLP with restriction digestion and visualized on a stained gel after electrophoresis. If the genetic polymorphism results in a loss, or in some cases a gain, of a restriction site, the band pattern on the gel will be different from the wild-type samples, such as in *CYP2E1* polymorphisms (*143*). If the PCR primers are designed to be within the missing sequence of a deletion polymorphism, no PCR product will be formed.

Obviously, the RFLP method cannot be used to screen the genetic polymorphisms in which the sequence alterations cause no changes at suitable restriction sites. In this case, genotyping can be carried out by creating a restriction site by mismatch primers (*144,145*) or by allele-specific PCR (*146*). If necessary, the results from PCR-RFLP and allele-specific PCR can be confirmed by PCR-direct sequencing (*147*).

Advantages and Limitations of Genetic Susceptibility Markers

Determinants of the Quality/ Appropriateness of the Approaches. ASSESSMENT OF AN EXPOSURE. The reliable characterization and assessment of the level of exposure(s) in the population studied is an important task. However, there are difficulties in measuring exposure because assessment methodologies frequently depend on personal recall (*148,149*). In many studies published so far on metabolic polymorphisms and susceptibility to

environmentally induced diseases, the exposure data are either very scarce or not available. This may contribute in part to the divergent findings reported for potential host factors in individual responses to toxicants. Knowledge of the substrate specificity of the polymorphic enzyme studied is needed before any interpretations concerning its potential effect on individual response to a given exposure can be made.

SELECTION OF THE STUDY GROUP. The cases and controls should have comparable exposures in studies on individual responses to environmental agents. Age and gender matching should also be performed. If these potential confounding factors are not controlled, the effect may be nullified (*150*). Where matching is not possible, logistic regression can be used to evaluate the individual contributions by multiple factors and to identify confounding factors (*151*).

SAMPLE COLLECTION. The sample collection is one important aspect of the appropriate assays. The proper treatment and storage of the samples is a prerequisite for obtaining good results. For instance, blood samples for DNA analyses can be stored in a refrigerator for several days and subsequently may be transported on ice or frozen for transportation or for further storage. In contrast, the samples collected for RNA analyses have to be frozen immediately, preferably in liquid nitrogen, and stored at –80°C before use. Blood, which has been the most used source of DNA samples, should not be collected in heparin tubes for DNA extraction. Interference with the PCR assay occurs when compared to the DNA extracted from blood collected in EDTA tubes. Although the DNA-bound heparin can be removed using heparinases, they are far too expensive to be used in studies involving large sample sizes. The inhibition can be decreased by extensive dilution of the samples (*152*).

In future studies, increased use of alternative sources of the DNA for genotyping studies, such as cells from buccal mucosa (*153*), is expected. These samples are easily collected by scraping or mouth washing and will thus overcome some of the problems associated with blood sampling. Aside from the samples collected for "prospective" studies, the DNA can also be from stored pathological tissue sections, which provide great advantage for retrospective studies but have additional problems due to the small amount of DNA obtained and due to possible DNA degradation.

NUMBER OF MARKERS STUDIED SIMULTANEOUSLY. Given the number and variability in expression of the xenobiotic metabolizing enzymes, assessment of a single polymorphic phenotype or genotype cannot be expected to be sufficient for evaluating individual susceptibility to environmental agents. The ultimate goal should thus be to concurrently assess individual phenotypes or genotypes for all the metabolic genes relevant for a given exposure (*154*). This may raise an urgent need for software to aid in the interpretation of the overall risk contributed by several different host factors.

Phenotyping versus Genotyping. Phenotyping has been widely offered as a more appropriate method compared to the new DNA-based techniques in studies on individual metabolic capacity. Determination of the actual phenotype of an individual, although more time consuming, is justified when no genotyping methods are available or when the correlation between the genotype and phenotype is very poor. However, in most cases the correlation is fairly good. Moreover, phenotyping is easily affected by confounders such as food or drug intake prior to testing, which do not affect genotyping analyses.

Protein and mRNA levels, although they give some indication of the expression level of a given metabolic enzyme, do not necessarily reflect the metabolic activity (*155*). On the other hand, the power of PCR to detect only a few copies of the target sequence makes it particularly vulnerable to errors caused by contaminating DNA. Both approaches will be discussed in more detail below.

PHENOTYPING ASSAYS. *Use of probe drugs* in vivo. The identification of polymorphic traits has repeatedly been by *in vivo* probe and involves the collection of blood or urine usually over a specific period. The use of *in vivo* probes gives a picture of the whole body capacity for a specific enzyme to metabolize a given substance. Probes are not yet available for all known polymorphically expressed enzymes. In many cases the probe is metabolized by other enzymes as well, which may complicate interpretation. It is also important to have a probe that is both specific and safe for use in humans.

Use of probe drugs in vitro. Because of drug/chemical toxicity, the use of an *in vitro* probe may be the only safe alternative to perform phenotyping. A simple *in vitro* assay can be developed, if the polymorphic enzyme is expressed in blood cells. The use of other tissues, such as liver biopsies, may provide an opportunity to measure the expression level of the enzyme as well. Concerns for patient safety limit the availability of these tissues for study.

GENOTYPING ASSAYS. *DGGE and SSCP.* DGGE and SSCP methods are widely used for detection of alterations in oncogenes and tumor suppressor genes, but they could also be used to track down sequence variations in the genes coding for xenobiotic-metabolizing enzymes. Recently they have also been used as a genotyping methodology (*156*), but at present the RFLP- and PCR-based analyses may be more useful in these studies.

RFLP assays. The early RFLP-based genotyping studies by Southern blotting were time consuming and labor intensive and usually incorporated the less desirable use of radioactive probes. Southern blotting has been mainly replaced by PCR-based analyses, which have greatly facilitated studies of individual susceptibility. This is best exemplified by the rapid expansion of the studies on *GSTM1* polymorphism after elucidation of PCR-based methods (*157*) for detection of the gene deficiency (*158*). Although several PCR-based methods are available for detection of homozygous gene deletions (*157,159*), amplifications (*160*) or heterozygous deletions (*158*) of the genes are still detectable only by the Southern blotting analyses.

PCR-based methods. As mentioned above, the PCR methods have mainly replaced the genomic DNA methods in genotyping studies. The RT–PCR is also used to quantitate tissue-specific expression of the metabolic genes (*161*). While rare mRNA transcripts can be detected by the RT–PCR approach, determination of relative or absolute copy number may be more problematic (*162*). Consequently, there is debate about whether RT–PCR is suitable for these studies.

One advantage of PCR methods is the requirement of only nanogram quantities of the DNA or RNA template. The need for starting material can be further diminished by employing a multiplex PCR that gives the genotype of several metabolic genes from the same sample. However, optimization of the multiplex PCRs may be time consuming and the number of assays one can add to one reaction is relatively limited.

The evident disadvantage in PCR methods is their extreme sensitivity to contaminating DNA. Consequently, special emphasis has to be put on controlling the contamination (*163*). This can be achieved by minimizing the possibilities for contamination to occur, by UV radiating the reaction mixture before adding template

DNA, and by using carefully selected positive and negative internal controls in all amplifications. An additional limitation, specific for RT–PCR, is that measurement of mRNA levels does not necessarily reflect metabolic activity.

Sequencing. Sequencing is the only method that provides the actual DNA sequence of interest. Consequently, it is still used mainly to identify novel allelic variants and to confirm the applicability of new genotyping methods. As the methodology improves, sequencing may be more commonly used to determine genetic markers (*164*).

Ethical considerations. Ethical review of all studies regarding human risk is appropriate. Specific issues arise with the use of archival samples for which donor permission may not have been obtained for the current research investigation. Current practice is variable regarding the need for consent. A second issue is the type and quantity of information derived from the project that should be provided to the subject [see below and Soskolne (*15*)].

Conclusions and Recommendations

There are a large number of different methods to measure susceptibility factors, ranging from the activity of an enzyme to the detection of mutant alleles of a gene associated with a modified phenotypic trait. In an era of molecular biology, exceedingly sensitive and specific methods to detect alleles and expression of genes of interest have taken center stage; but one has to keep in mind that it is actually the phenotype that is of importance for the final response to the hazardous insult.

One serious problem in phenotyping studies is lack of knowledge of the levels of enzymes in the target cells and organs relevant to the whole body metabolism and the correlation of genotype with *in vivo* phenotype. In humans, practical considerations dictate to a large extent that one has to resort to surrogate tissues.

Genotyping has some advantages, such as unequivocal identification and lack of interference with confounding factors; but a modified sequence within a gene (polymorphism) must be demonstrated to generate a different phenotype.

In vivo phenotyping has the advantage in some cases of assigning a value with one assay that might require determination of multiple genotyping assays when several alleles are present in the population. *In vivo* methods to measure phenotype may lead to misclassification of different genotypes, which in the case of rare alleles can have a large effect on the frequency.

To fully exploit the genetics of xenobiotic metabolizing enzymes as risk assessment tools, much more information on the structure/function relationships and regulation of these enzymes correlated to the *in vivo* phenotype must be elucidated.

On the basis of the current knowledge of the field and of the above conclusions, the following recommendations are made:

a) Phenotyping should be continued along with genotyping, until we gain experience with large numbers to enable interpretation of more complex relationships.

b) The combined impact of all relevant genes for a given exposure (as far as they are known) needs to be assessed through population-based studies using multiple markers. New insights into the physiologic function of genes encoding xenobiotic metabolizing enzymes will be revealed by studies of transgenic "knockout" or "insert" models.

c) Better kinetic characterization of enzyme substrate and inhibitor specificity must be determined through studies of human tissues, as well as through detailed metabolic studies of whole organisms. The future availability of crystal structure of drug metabolizing enzymes in combination with computational chemistry will provide better information on structure/function predictions.

d) A greater understanding of the regulation of enzyme expression by environmental agents is needed.

e) The value of intermediate markers of exposure such as DNA adducts, should be studied and related to other end points of disease, e.g., cancer, by cohort studies of individuals who have had unintentional exposure.

f) Widely available software for interpretation of the overall risk contributed by several different host factors is needed.

g) Better methods of DNA sample collection should be devised to facilitate storage and transport and to reduce costs because of the need to perform complex DNA investigations at remote laboratories.

h) A future approach will be to use information obtained from analysis of populations of interethnic groups, in an attempt to modify risk by reducing exposure to potentially hazardous environmental and dietary factors linked to disease. The effects of these actions must be evaluated to validate proposed interventions.

i) All of these areas need further exploration before we will be able to assign individual specific prevention strategies.

Factors That Determine the Susceptibility of Organisms, Species, Communities, and Ecosystems

Introduction

There are both commonalities and differences between the sections on human and nonhuman organisms in this report on susceptibility. Many of the techniques are the same, but whereas for human health the main consideration is the protection of susceptible individuals, in environmental terms we are more concerned with susceptible species and communities. Even this concern is double edged, as there are species for which human efforts to control have led to the formation of highly resistant forms. Studies of the mechanisms whereby this resistance has occurred have given much valuable information on the differences between susceptible and resistant populations.

While this section of the joint report follows the sequence of the human health sections, namely, biomarkers of exposure, effect, and susceptibility, emphasis is given to the last category as in general the same biomarkers of exposure and effect are used in human and nonhuman biota. Examples of how these biomarkers are used in the environmental context will be given.

Here we use the term biomarker as a biological change that is a measure of exposure and sometimes of toxic effect caused by environmental chemical(s) at the level of the individual or below. Changes occurring above the level of the individual are referred to as biological indicators.

The discussion here on susceptibility covers a broad canvas. There is first a discussion of biomarkers of susceptibility at the cellular and molecular level, followed by those at the individual level, both long and short term. Then we move up the organizational scale discussing biological indicators of sensitivity at the population, community structure, and ecosystem levels. We conclude with two case examples of individual chemicals or chemical classes to illustrate these points. Finally, we note that humans are part of ecosystems, and the biomarkers of ecosystem susceptibility can also indicate and predict effects of chemicals on humans. The best biological indicators will both warn humans of potential danger and alert us to severe ecosystem damage (Figure 2).

Biomarkers of Exposure and Effect

We regard biomarkers of exposure and effect as essentially a continuum (Figure 2).

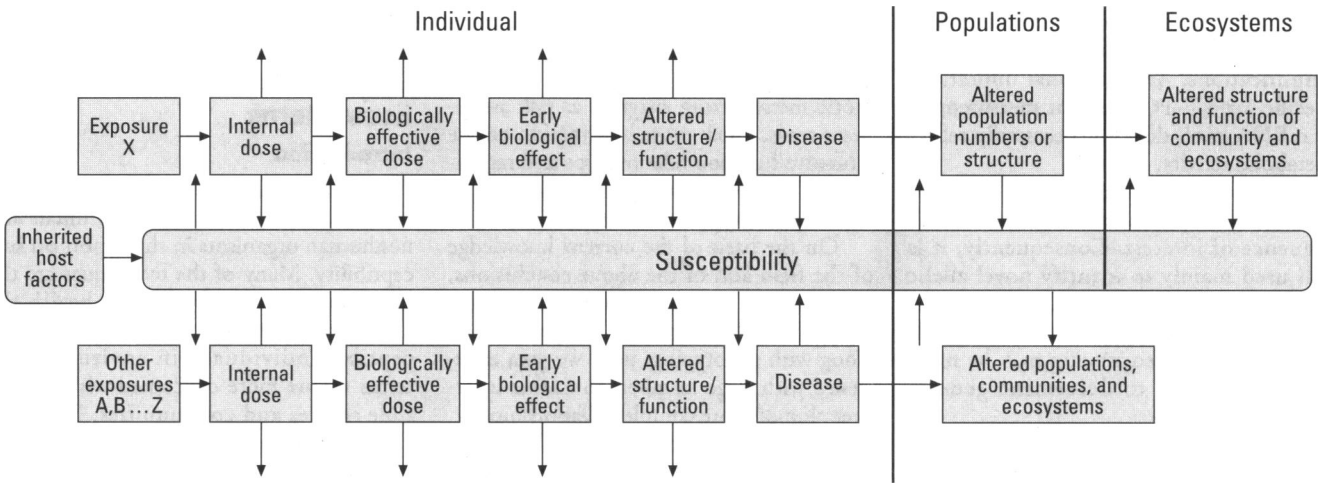

Figure 2. The role of susceptibility in individuals, biota, populations, and ecosystems.

Whether a biomarker can be used as an indicator of effect as well as of exposure often depends on the depth of our knowledge of a particular case. Thus, we have not made any effort at making this rather artificial separation.

Biomarkers and higher level biological indicators are important tools in ecotoxicology. Their application to studies of chemical effects on nonhuman biota are well documented. However, as this does not fall within the bounds of this discussion, the readers' attention is drawn to a number of authoritative texts (*165–167*).

Among the hierarchy of biomarkers and biological indicators, representing pollutant-induced changes in biota at all levels of organization (from the molecular to ecosystem), lie the means to identify, classify, and ultimately quantify, the susceptibility of individuals and populations to the detrimental effects of chemical exposure. At the levels of molecule, organelle, and cell, there are a number of changes we may use in our studies of susceptibilities. In the case of DNA adducts, for instance, we can measure the quantity and rate of adduct formation in one individual and compare it to others in the same population. Interspecies variations could also be measured. Similarly, there are a number of important cellular regulation and defense proteins that may be quantified for comparative purposes.

Detoxification proteins such as the mixed-function oxidases (MFO) and the cytochrome P450 enzymes may be measured, as well as the multidrug/multixenobiotic resistance proteins. The levels of such molecules in individuals may give some important clues to the susceptibility of an organism to toxic insult.

Efficient DNA repair has been shown to be an important determinant of chemical and radiation-mediated damage in cells. We may therefore consider the cellular levels of the various repair enzymes as indices of overall susceptibility. Other important molecules are the proteins involved in cell regulation mechanisms. Proteins such as the oncoproteins may be useful, along with the functional analogues of proteins such as p53, coded by the tumor-suppressing gene.

Other indicators of susceptibility at the subcellular level include changes in organelle structure and function, chromosomal aberrations, and the formation of micronuclei to genotoxic agents, as well as the functional integrity of lysosomes. These cellular/molecular indicators of susceptibility may be considered in higher animals and plants to be only components of the overall picture of organism response. Thus they may be used in conjunction with other higher level markers. However, for a large number of single-celled organisms (and simple organisms) they may play a more important role.

Chemical-induced changes in many types of cells, tissues and organs can also be seen as markers of susceptibility. A high level of cellular dysfunction results in distinct morphological and biochemical consequences. An example is apoptosis or programmed cell death. The function of a cell may be so impaired, or damage to its DNA so great, that a "suicide" sequence is initiated, resulting in degeneration and death. Such a process may be measured histologically, and the levels of apoptosis measured in a target tissue or organ. Cellular dysfunction may also be measured in a biochemical manner, and tracing increases and/or decreases in tissue specific molecules such as hormones and enzymes may be a key indicator of individual susceptibility.

Finally, physiological parameters are potentially important markers, as they relate directly to fitness and consequently have higher level effects. Also included are homeostatic processes such as thermo- and osmoregulation, alteration to respiration, and changes in cardiac function.

DNA Adducts

^{32}P-postlabeling has been used for a number of environmental studies. Some of the most detailed are those carried out in Puget Sound in the State of Washington. Varanasi et al. (*168*) used the 1-butanol adduct enhancement method to measure the level of DNA adducts in English sole (*Parophyrs vetulus*) that were exposed to high concentrations of sediment-associated chemical contaminants and exhibited elevated levels of hepatic neoplasms. The level of DNA adducts in contaminated sites averaged from 17 to 26 nmol/mol nucleotides compared to < 0.2 from a control site. The finding that the levels of aromatic hydrocarbons in the sediments of Puget Sound were positively correlated with prevalence of hepatic neoplasms and related lesions in English sole were compared to those found for the starry flounder (*Platichthys stellatus*) (*169*). These workers found that the starry flounder had a lower prevalence of hepatic neoplasms and studies suggested that biochemical differences in the metabolism of carcinogenic PAHs can explain the lower susceptibility of the flounder to chemical-induced hepatocarcinogenesis.

The HPLC/fluorescence spectrophotometry approach has been used to show

the presence of B[*a*]P adducts in a population of beluga whale (*Delphinapterus leucas*) in the St. Lawrence River, whereas no such adducts were found in the brains of whales from the Arctic population (*170*). The finding of B[*a*]P adducts in the brains of belugas in the St. Lawrence can be correlated to the high incidence of tumors (cause of death in 18% of the 61 whales post mortemed) in this population.

The formation of DNA adducts has been considered a key step in the initiation of carcinogenesis. However, the presence of DNA adducts in aquatic invertebrates that rarely or never develop neoplasms may obviously be linked to genotoxic end points other than tumorigenesis. Based on good correlations between the induction of DNA adducts and gene mutations, it was hypothesized that, in natural species, a variety of manifestations of the mutational event may actually prove much more biologically and ecologically important than the induction of neoplasia (*171*).

Strand breakage of DNA has also been used to study the effects of environmental pollutants. Although strand breakage can lead to cell death, in many cases it is repaired before serious damage occurs. Strand breakage as measured by the alkaline unwinding assay is one of a suite of biomarkers used to study the effects of pollution on the liver DNA of bluegill sunfish (*Lepomis macrochirus*) in rivers of the eastern United States (*172*). The basic prerequisite for the successful use of DNA adduct and DNA strand breakage in environmental risk assessment studies, however, is the recognition of the fact that lower invertebrates may be incompetent in forming DNA adducts from some highly prevalent pollutants such as PAHs (*173*).

Some indicators of chemical exposure and effects methods are shown in Table 2.

Biological Indicators of Susceptibility at the Individual Level

The phenomenon of resistance is a striking example of differential susceptibility of organisms to environmental chemicals. There are a number of mechanisms whereby organisms can resist the action of toxic chemicals. These include decreased uptake, increased efflux, increased rate of metabolism of the toxicant, sequestration of the toxin, and increase or repair of target sites within the cell. In some cases more than one mechanism is involved and changes made against one chemical can protect against another; hence, the phenomenon of cross-resistance.

The factors that influence the onset of resistance fall into three broad categories: genetic, biological, and operational. In the first category is the frequency and dominance of the resistant gene. Dominance is important; if the gene is normally recessive because it has disadvantages, such as lower reproductive capacity, the greater the genetic disadvantage, the slower the resistant strain takes over. The shorter the generation turnover and the greater the number of offspring per generation, the more rapidly resistance can occur. For pesticide resistance, the operational factors include the actual pesticide used and the mode and area of application. It is in the area of disease resistance in insects that we have the greatest control (*174*).

The degree of resistance can be large. For example there are houseflies for which the LD_{50} for DDT is 3000 times normal, the main mechanism that defends the organism being increased levels of a dehydrochlorinase (*175*). Resistance among vertebrates is not so evident, although it has been reported in several species of fish, and the case of warfarin resistance in rats is well known.

Resistance can cause considerable changes in the community structure of invertebrates. Man's attempts to control the pests that affect cotton in northeastern Mexico and the southern United States is a good example of the problems that occur with resistance and the alterations of community structure caused by differential susceptibility of organisms to environmental chemicals (*176*). During the period 1945 to the mid-1950s there was almost complete control of the main pests—the boll weevil and the cotton fleahopper—by OC pesticides and there was a spectacular increase in cotton production. In the mid-1950s it was necessary to switch to OP compounds which increased the costs but cotton was still profitable. Former minor pests, the bollworm and tobacco budworm

Table 2. Some biomarkers, the pollutant, and their utility for study of environmental susceptibility.

Biomarker	Measure of toxic effect?	Pollutant	Organization level	Reliability index
Inhibition of δ-ALAD	Yes	Lead	Organ, intact animal	Sufficiently reliable to replace chemical analysis; can be related to mechanisms of action
Induction of metallothionein	No	Cadmium	Organ	No advantage over chemical analysis; not related to mechanism of action
Eggshell thinning	Yes	DDT, DDE, Dicofol	Intact animal, population	Wide variation in sensitivity; related to reproductive success
Anticoagulant clotting proteins	Yes	Rodenticides	Intact animal, population	Has been related to mortality; can be assessed from blood protein
Porphyrin profiles	No	Several OCs	Organ	Levels of porphyrins found in samples are well below those causing adverse effects
Depression of plasma retional and thyroxine	Yes	3,4,3', 4'-TCB	Tissue, intact animal	Dermal and epithelia lesions to specific protein have been shown
Inhibition of AchE	Yes	OPs, carbamates	Organ, intact animal	Easier and more reliable than chemical analysis
Induction of MFOs	Yes[a]	OCs, PAHs	Organ, population	Analysis of TCDD-EQ[a] has been linked to reproductive success; P450s related to specific pollutants
DNA and hemoglobin adducts	No	Largely PAHs	Organ	Good monitor of exposure, for PAHs, relation to effects
DNA integrity	?	Metals, OCs, PAHs	Organ	DNA damage is serious to the individual but relationship to effects on reproduction is unknown
Other serum enzymes	No	Metals, OCs, OPs	Organ	A considerable number of enzymes altered by pollutants, but effects are not clear
Immune responses	?	Metals, OCs, PAHs	Organ	Proper functioning of this is critical to health but there is considerable reserve
Stress proteins	No	Metals, OCs	Organ	Difficult to separate effects from nonchemical stresses

[a]TCDD-EQ, TCDD equivalents.

were becoming major pests. By the mid- to late-1960s all the pests were highly resistant to all insecticides and cotton production dropped markedly. The introduction of integrated pest management was needed to solve this problem.

Individual Susceptibility. Susceptibility of individuals to chemical stress, or their sensitivity to chemicals, is hard to define. Pragmatically, susceptibility may be defined as a lack of resistance, or tolerance, to chemical stress. Such a definition enables the recognition of molecular, biochemical, or physiological mechanisms that constitute a biological defense mechanism against chemical pollution. These include mechanisms dependent upon the activity or levels of mixed-function oxidases, *N*-acetyltransferases, glutathione *S*-transferases, glutathione, glucuronidation and sulfation, heat-shock proteins, and metallothioneins. The basic characteristic of these biologic defense/detoxication mechanisms is their inducibility. The induced state of these enzymes, often expressed in thousands of percent increase over natural state, serve as useful biomarkers of exposure/effect. Their activity and inducibility directly influence the outcome of exposure to toxic chemicals (induction of DNA adducts, induction of single strand breaks in the DNA, induction of chromosomal aberrations, modulation of fitness and the frequency of diseases, lethality, extinction of species). In resistant organisms both the natural level and more importantly the inducibility of one or more of these mechanisms is higher than in susceptible individuals. Generally, better response of detoxication/defense mechanisms is inversely proportional to susceptibility. Thus, biomarkers of exposure may be used as short-term indicators of biological susceptibility of individuals to chemicals: low response in these biomarkers will denote a high level of susceptibility. For example, lower inducibility of 7-ethoxyresorufin *O*-deethylase activity in carp will denote their higher susceptibility to toxic effects of pollutants.

Using the same pragmatic definition of susceptibility as a lack of resistance, one can utilize a recently discovered multixenobiotic resistance mechanism (MXR) as a long-term biomarker of susceptibility of individuals to chemicals. The MXR represents a general biologic first-line defense mechanism, it is taxonomically broadly distributed, and its expression is species dependent. However, there are considerable variations in its expression on an interindividual basis (*177*). The low expression of activity of *P*-glycoprotein, a dominant feature in the MXR mechanism (*178*), in, for example, the snail *Monodonta turbinata* specimens living in a pristine marine environment, indicates high susceptibility for accumulation of xenobiotics and, consequently, higher likelihood of effects (*179*). In contrast, specimens of snails living at polluted sites, expressing higher activity of *P*-glycoprotein due to its induction, are less susceptible to toxic effects of xenobiotics due to a lower level of its accumulation. Thus, the level of expression of MXR may be a good long-term indicator of susceptibility to chemicals. Similarly, species with high levels of MXR (*P*-glycoprotein) expression, such as mussels (*Mytilus galloprovincialis, Mytilus edulis, Crasostera gigas*) (*180*), clam (*Corbicula fluminea*) (*181*), marine snail (*M. turbinata*) (*179*), marine worm (*Urechis caupo*) (*182*), or four marine sponges (*183*), in contrast to a fresh water mussel (*Anodonta cygnea*) and a fresh water snail (*Paludina vivipara*) (*179*), are not susceptible to chemical pollution. Thus, the low, or nihil, expression of MXR in *A. cygnea* and *P. vivipara* are biomarkers of their long-term susceptibility.

Excellent methods are available for measurement of a functional state of MXR (*184*), as well as for measurement of concentration of inhibitors of MXR, the chemosensitizers, in environmental samples (*185*).

Several species or groups can be good indicator species. Very good bioindicators are the water insects, especially species and groups of species of the order Ephemerophera. Ecological niches and needs of their larvae are well known. By feeding on detritus they accumulate in their bodies xenobiotics that can be reliably identified (*186*).

Behavioral Mechanisms. Individuals can clearly be exposed to chemicals in situations in which they cannot escape or move away from the exposure: then they must rely on biochemical detoxifying methods to avoid effects. Sessile organisms that make up most species in the aquatic world are unable to move away from exposure. However, many organisms, particularly vertebrates, have a number of behavioral and life-history strategies that enable them to avoid exposure to chemicals. These mechanisms make them less vulnerable or less susceptible to the effects of these chemicals.

The most obvious mechanism to avoid chemicals is to move away from exposure; and many animals do this by simply flying, swimming, or walking away. This mechanism functions in many species when they are exposed to high doses of chemicals such as occurs during an oil spill. Birds, many marine mammals, and fish will swim or fly from advancing oil when it is possible to do so (*187*). However, other fish and some marine mammals will not do so until it is too late to avoid exposure. Most exposures to chemicals, however, occur at such low doses that animals do not, or cannot avoid them.

The impairment of locomotion or abnormal behavior of animals is being used to monitor pollutants. Fish have been used commonly—monitoring the frequency of coughing (*188,189*), loss of rheotaxis, or disturbance of schooling. These methods are linked with automatic water sampling systems to serve pollutant analysis. Direct measurement of respiration through blood vessels in fish was devised to monitor contaminants (*190*). Monitoring of mussels' activity is also practical since mussels respond to hazardous chemicals by closing their bulbs. Abnormal web spinning by caddisfly larvae or failure to make nests by other aquatic insects are also useful methods to monitor pollutants. Feeding activity can be another biomarker. A short-cut test with *Daphnia* using toxicant-induced inhibition of feeding is being studied.

For showing the changes caused by long-term exposure, it is most important to observe changes in the populations of individual species. Changes in populations that are useful include the changes in population dynamics, fertility and fecundity, physiological resistance to external factors, behavior, levels of parasitism or pathogen infestation, and changes in the structure of communities of social insects (*186*).

Life History Parameters. In addition to behavior, some life history strategies allow animals to avoid exposure, thus rendering them less susceptible to exposure and subsequent effects. Many species migrate during part of the year, removing them from low-level chronic exposure; other species hibernate beneath the ground where they also reduce exposure and thus their vulnerability.

The two life history strategies that render animals most susceptible to the effects of chemicals are a sessile life style and a long life span. Species that are relatively long lived, such as most vertebrates and trees, are more susceptible to chronic exposure to chemicals than those that have a short life span. This factor functions with chronic, low-level exposure rather than

acute, toxic levels of chemicals. Species that have a life history strategy that involves short life spans have a reduced susceptibility to chemicals because the potential for bioaccumulation is less and their reproductive potential is higher. Chemicals such as dioxins, PCBs, DDTs, and mercury, which bioaccumulate in internal tissues and are thus available for remobilization into the bloodstream, can have severe effects on organisms that live a long time.

Of the invertebrate bioindicators, insects are the most valuable because of their quantities and diversity, and because of our sufficient knowledge of their taxonomy, morphology, physiology, and ontogeny. Their living areas may be rather limited, their life cycles short, and their reproductive potential enormous, with great variations that can be regulating. These properties are useful for bioindication. The diversity of insect communities is distinct and cumulation of substances is specific; it is not difficult to observe changes in population dynamics and in individual development because the life cycles are short. The reproductive system of insects is very sensitive to biologically active substances (proliferation of follicular cells, change in structure and function of nutritive cells, etc.) This provides the opportunity to use the reproductive system of insects as a model for testing the effects of toxic substances in the environment.

Taxa Variation. In the environmental field we have to look not only at susceptible populations of any one species but also at interspecies variations that can cause a differential effect. In this section we examine a number of biomarkers that vary widely in their sensitivity from one group of animals to another.

MIXED-FUNCTION OXIDASES. MFOs are a major component of the defenses of organisms against toxic chemicals in their environment. Originally evolved to handle naturally occurring toxic compounds, they now play an important role in the detoxification of man-made chemicals. Nebert et al. (*191*) consider that the ancestral cytochrome gene is probably two thousand million years old. The major divergence occurred eight hundred to one thousand million years ago when animals began using plants as food and self-defense mechanisms against toxins in plants evolved. Later, additional families of cytochromes evolved in response to the necessity to metabolize combustion products.

There is some variation between taxa: in general terms, the relative activity of the MFO enzyme epoxide hydrase follows phylogenetic lines with mammals > birds and amphibia > fish, with little overlap between the groupings. Levels in invertebrates are much lower. Glucuronyltransferase activities were much higher in mammals than fish with virtually no overlap. Aldrin epoxidase activity follows the same trend, but in this case there is considerable overlap. A linear log relationship was found between relative activity and body weight for mammals, with man being the outlier having activity lower than would be expected by body weight.

Fish-eating birds show a high correlation coefficient for the regression of activity against body weight, with the values for relative activity being approximately an order of magnitude lower than for mammals of comparable weight. Other species of birds tend to have values intermediate between fish-eating birds and mammals, but the correlation of activity with body weight is considerably weaker. Low values for fish-eating birds are considered to be due to the fact that until xenobiotics contaminated aquatic food chains, the birds had less need of these defense mechanisms. Regrettably no data appear to be available on fish-eating mammals.

The low level of activity in fish has been considered to be due to the fact that until historical times this group of organisms has found the excretory route across the gills effective for removing most xenobiotics. It is clear that this mechanism is quite inadequate to deal with highly liposoluble organochlorine molecules that have been released into the environment by humans in the last few decades.

The relationship between feeding habits and MFO activity has also been demonstrated in insects. A detailed review of the data for aldrin epoxidase has been made by Brattsten (*192*). He found that aldrin epoxidase activity is lowest in monophagous species, considerably higher in oligophagous, and some 10-fold higher in the most polyphagous species.

The comparison of Phase I and Phase II hepatic transformation in quail and trout to that of a number of mammalian species commonly used in toxicity testing has been made by Gregus et al. (*193*). They found that the overall metabolism of xenobiotics could vary several hundredfold between species.

TOXICITY OF DIOXINS. The toxicity of dioxins to various mammalian species varies greatly. The most sensitive species known is the guinea pig; the LD_{50} for the male is 0.6 µg/kg and the female 2.1 µg/kg, the value for the male rat is 22 µg/kg, for the female rat 45 µg/kg and for the rabbit (mixed sex) 115 µg/kg, whereas the least sensitive species is the hamster (1200 µg/kg) (*194*).

EGGSHELL THINNING. The phenomenon of eggshell thinning, although confined to only one order (Aves) does illustrate some interesting points in susceptibility. Eggshell thinning is caused by DDE and the sensitivity varies greatly. A diet of only a few parts per million will cause 20% thinning (the degree of thinning that causes eggshell breakage and thus reproductive failure) in raptorial birds and some species of fish-eating birds such as the pelican and cormorant. Other species such as gull, terns, and ducks are only moderately sensitive with diets of 40 to 50 ppm being needed to cause biologically significant eggshell thinning. Still other species, such as quail and chicken are completely insensitive and it is impossible to achieve more than a few percent thinning even at the highest dosage that can be used without mortality. One important point from this variation is that the common test species are insensitive and thus even if the measurement of eggshell thinning had been included in test protocols for new pesticides this phenomenon would not then have been recognized. Another point is that the species exposed to the greatest amount of DDE, due to bioaccumulation, are those that are the most sensitive. DDE-induced eggshell thinning caused the decline of many species of raptorial birds, such as the peregrine falcon (*Falco peregrinus*) and european sparrow hawk (*Accipter nisus*) over wide areas of the northern hemisphere. These declines have been reversed in areas where bans on DDT have been imposed.

Biological Indicators of Susceptibility to Chemicals at the Population Level

Biological indicators for assessing susceptibility at the population level measure parameters that are a consequence of low activity of mechanisms of resistance detected by molecular and physiological biomarkers. For example, the induction of DNA adducts and DNA strand breakages found in individuals by molecular biomarkers predicted the increase in mutational events in population. Consequently there is a higher susceptibility of populations to diseases, especially viral, bacterial, and parasitic, as the consequence of impairments in the immunosystem. These populations also are more highly susceptible to decreased

fitness of population, to impairments in reproduction, to sickness and increased lethality. All these indicators of susceptibility of populations to chemical stress will predict the expected consequences on the level of population such as alterations in adaption, survival and succession. On the ecological level, the most severe effect—extinction of a species—can occur.

Several examples illustrate the use of implications of susceptibility at the population level. In salt marsh ecosystems of North America, salt marsh grass (*Spartina alterniflora*) is the primary producer; susceptibility of this species to specific chemicals, which results in lowered reproductive output, would have a greater effect on community and ecosystem structure than would susceptibility of other, less important plant species (*195*). *S. alterniflora* is susceptible to PAHs from oil pollution; the sensitive vegetative propagules can be killed and the species may take years to recover. Similarly, differences in susceptibility to chemicals among species of algae in salt marsh communities would also have a critical effect on community and ecosystem structure since algae productivity is critical to the overall productivity of the animal communities in the marsh.

Equally important to remember when using cellular and molecular biomarkers as measures of the susceptibility of communities and ecosystems to chemicals, is that changes in molecular and cellular organization must have effects at the population level to be important for community and ecosystem structure.

In many cases, chemical, physical, and biological stressors occur together; and the communities and ecosystems must contend with them at once. Since communities and ecosystems are assemblages of organisms, and thereby are populations that make up the system and are responsible for the stability and repair of that system, the relative susceptibility of that system depends on the susceptibility of individuals and populations within the system.

Biological Indicators of Susceptibility to Chemicals at the Community Level

Within any community or ecosystem the species are not necessarily of equal importance with respect to the maintenance of stability. That is, some species have a keystone role (*196*). Keystone species include species that provide key nutrients or energy at the lower trophic levels, or species that regulate competition and predation at the higher trophic levels. Where molecular and cellular biomarkers are used to indicate potential susceptibility differences among individuals or species, care must be given to select indicator species that have a key or pivotal role in community structure (*197*). Chemical disruptions to species that are essential for either the structure or the function of the system will have a greater effect on the susceptibility of the ecosystem than those that are less critical.

Several measures of the structure and function of communities and ecosystems (Table 3) can be used to measure change (*167,197–201*). The degree of change in ecosystem structure and function that occurs is a measure of susceptibility. That is, if a community or ecosystem undergoes few changes in either structure or function in response to a chemical, biological, or physical perturbation, then it is relatively resistant or can be said to be less susceptible to the effects of a given chemical or class of chemicals.

The methods to evaluate community and ecosystem change are often costly and time consuming, but they can sometimes be used with less technology and equipment. That is, in places or situations where extensive molecular and cellular laboratory facilities are not available, the community and ecosystem measures outlined in Table 3 can be effectively used to determine differences in susceptibility. On the other hand, where such laboratory facilities exist, some of these techniques can be used effectively as surrogates to determine whether individual reproductive potential is compromised, thereby affecting population levels, and by extension, community structure. Table 3 also gives an indication of the difficulties of each methodology, as well as its usefulness for evaluation of the effects of chemicals.

Table 3. Methods to measure differences in susceptibility at the community and ecosystem level.[a]

Characteristic	Ease of measurement[a] Aquatic	Ease of measurement[a] Terrestrial	Usefulness for chemical assessment[b]
Biodiversity	1	5	A
Relative density	1	2	A
Relative abundance	1	1	A
Dominance	5	5	C
Food web characteristics	8	8	A
Genetic diversity	10	10	A
Primary productivity	1	1	A
Secondary productivity	5	3–7	B
Decomposition	1	1	A
Energy flow	5	3	C
Mineral cycles	8	3	A
Nutrient cycles	8	5	B
Keystone species	5	5	A
Index of biotic integrity	10	10	A
Guild structure	10	8	C
Biomass	10	10	A
Erosion	5	5	C

Revised from Burger and Peakall (*195*); and several articles in this volume. [a]Ease of measurement is scored on a continuum of 1 to 10, 1 being the easiest to measure, 10 the most difficult. [b]Usefulness for assessment is scored on a continuum, A being most useful, C the least useful or indicative.

One of the most detailed studies at the community structure level was made in Canada to determine the effects caused by acidification in an experimental lake situation (*202*). In this study the pH of a poorly buffered lake in northwestern Ontario was reduced from pH 6.8 to pH 5.0 over 8 years. Fish populations started to collapse due to lack of reproduction when the pH reached 5.9, and there were marked changes in the phytoplankton composition although primary production was not decreased. At pH 5.6 thick mats of filamentous algae appeared, which persisted throughout the study. When the stable value of pH 5.0 was reached the species composition of phytoplankton was completely different although primary production remained high; no fish reproduction was occurring; crayfish, leeches, and mayflies were absent; but there was a considerable increase in chlorophyll and no changes in nutrient concentration were observed.

Case Studies

Lead. Lead is an example of how biomarkers can be used at different levels of organization. Concentrations of lead in tissues such as blood, liver, and kidney are measures of exposure. Analysis of levels of lead and other heavy metals, however, is costly and requires technical knowledge and sophisticated equipment. However, δ-aminolevulinic acid dehydratase (δ-ALAD) activity can also be measured in blood as a biomarker of exposure and effect (*203*). It is a cheaper and easier method of analysis than of lead itself.

Additionally, the effects of lead can be measured with a number of neurobehavioral assays, since one of the primary nonlethal effects of lead is on cognitive, psychomotor, and other neural processes in both human (*204*) and nonhuman biota (*205,206*). Lead can cause mortality directly, or can cause lowered reproductive success through depression of clutch and egg size, mortality of embryos, depression of growth, and disruption of reproductive behavior in a variety of species, especially

on birds, the work on which has been key (*206*). There are differences in susceptibility among birds: seabirds such as gulls and terns are more susceptible than chickens, ducks, and passerines (*207–209*).

Lead shows the classic trophic level relationships with respect to vulnerability: species that are higher on the food chain have higher levels of exposure than those that are lower on the food chain (*206*). However, susceptibility will also vary with such factors as developmental maturity at hatching, at least in birds. That is, lead disrupts parental recognition, making those species dependent on recognition (i.e., precocial birds) more susceptible to the effects of lead than species that do not depend on this recognition (*210*). Humans are at a relatively high trophic level and clearly show effects of lead. These effects, especially the neurological and behavioral ones, are particularly apparent during development, and persist for many years in humans (*204*).

Lead can also have disruptive effects at the population and community levels. The abnormalities caused by lead can reduce survival and lower reproduction success, resulting in lowered population levels for impacted populations (*206*). The effects in humans have the potential to severely alter social structure and behavior of populations that are severely exposed (*204*). As early as the 1950s, Bellrose demonstrated that lead poisoning could depress population levels by differentially affecting those wounded by hunters, and the response was dose related (*211*). Similarly, birds nesting along roadways are heavily exposed to lead from gasoline, while those in more remote areas are not. Animals near smelters are also heavily exposed through the aquatic food chain. By differentially affecting different species, the species composition of communities can be affected by lead, leading ultimately to changes in ecosystem structure. Because organisms higher on the food chain are more susceptible, this end of the system would be more affected.

While the mechanisms whereby lead affects hemoglobin formation (e.g., inhibition of δ-ALAD) are well known, the mechanisms disrupting neural systems, development, and behavior are not well established. These phenomena have been quantified, without clarifying the mechanisms. Phenomena include differences in cell growth and neuronal projections.

PCBs and Dioxins. The mechanism of action of PCBs, polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenozo-*p*-dioxin (PCDDs) is considered to proceed via initial binding to a high affinity, low capacity cytosolic receptor protein. The identification of the *Ah* receptor (*212*) with stereospecific, high affinity binding to 2,3,7,8-TCDD was a key finding in bringing molecular biology into the realm of toxicology. Examining the toxicological and receptor binding data, Poland and Knutson (*213*) concluded that it was likely that these compounds exert their toxicity through the cytosol receptor.

The ability of specific PCBs, PCDFs, and PCDDs to induce the P450I system is greatly influenced by the degree of chlorination and the chlorine substitution pattern. The most toxic PCBs are those that are unsubstituted in the ortho positions, i.e., 3,3′,4,4′- tetrachlorobiphenyl (TCB), 3,3′,4,4′,5-pentachlorobiphenyl, and 3,3′,4,4′,5,5′-hexachlorobiphenyl, which allows the molecule to assume a co-planar configuration. There is a close relationship between aryl hydrocarbon hydroxylase (AHH) induction and body weight loss, and AHH induction and thymic atrophy, although the interactions of enzyme induction, receptor binding, and toxicological manifestations are very complex and our knowledge is far from complete.

Problems that have to be faced before one can use this approach for wildlife toxicological investigations in the field are extrapolations from species to species and extrapolations from cell culture to the intact animal. Brunstrom and co-workers have carried out studies on avian embryos. Marked differences were found in the sensitivity of the chicken, pheasant, turkey, duck, and gull (*214–216*). They found that the pheasant was 50 times less sensitive than the chicken, and other species were even less sensitive. This emphasizes the difficulties of interspecies comparison since the chicken and pheasant both belong to the order Phasianidae.

The application of this complex biochemistry to field investigations has been based on expressing the complex mixtures of PCBs, PCDFs, and PCDDs as dioxin equivalents (TCDD-EQ). Based on their affinity for the *Ah* receptor, the activity of the individual congeners are assigned a value relative to the most active compound (2,3,7,8-TCDD) which is given a value of 1. These toxic equivalent factors are multiplied by their concentration to give TCDD-EQ for each compound. Although even the co-planar PCBs are a good deal less active than 2,3,7,8-TCDD, their concentrations are much higher and thus they contribute more than the dioxins to the total TCDD-EQs. Now it is possible to do the process in reverse and use the degree of enzyme induction to estimate the TCDD-EQs. This bioassay approach is rapid and inexpensive compared to the conventional chemical analysis by gas chromatography–mass spectrometry.

Good correlations have been found between TCDD- EQ of egg contents and both the reproductive success and incidence of deformities in fish-eating birds in the Great Lakes of North America. In terms of sensitivity, it is of note that the cormorant is some 20 times more sensitive than the Caspian tern (*Hydroprogne caspia*). In the most contaminated areas of the Great Lakes, the productivity success has been linked to population declines.

Ethical Issues and Ecosystem Susceptibility

There are at least three areas where ethical considerations impact methods evaluation for ecological systems: scientist versus all other stakeholder views, government actions and ecosystem vulnerability, and, the conflict between using biomarkers in ecosystems for understanding the ecosystem itself and using ecosystems as indicators for human health [see section on ethics below, and Soskolne (*15*)]. Increasingly, the responsibilities of scientists toward stakeholders (including scientists) are obscure with respect to how well the methodologies actually measure the phenomena in question. It is unclear, therefore, how soon scientific "findings" should be released or made available, how stringent the criteria for acceptance of an effect must be (given the accuracy of the methods), and how to resolve conflicts between scientific viewpoints that develop as a result of methodology differences.

Certain communities and ecosystems are more susceptible to damage from exposure to chemicals by virtue of their species diversities, unique species assemblages, or presence of endangered species. In these cases, ecologists and ecotoxicologists have a responsibility to make the susceptibility known, and where possible, to affect decisions to reduce the potential for exposure to chemicals.

For example, the National Research Council examined the susceptibilities of offshore communities in the United States to oil pollution. They determined that the coast of Florida was very vulnerable because of the presence of subtropical coral reefs and manatees (both limited in the

continental United States). As a result, the U.S. government decided not to allow offshore oil exploration in Florida.

There is a conflict between the use of biomarkers as indicators of ecosystem health for itself, compared to using these biomarkers only as indicators of human exposure. We would argue that both are important uses of biomarkers, and biomarkers should be developed that fit and evaluate the effects of chemicals in both. We have an ethical responsibility to preserve ecosystem integrity on a worldwide basis.

Conclusions and Recommendations

The following steps should be undertaken to protect organisms, species, communities, and ecosystems:

a) development of biomarkers at the cellular and molecular level that cross-cut taxonomic levels, including the vast diversity of invertebrates

b) study of molecules and mechanisms conserved across the animal kingdom; these highly conserved mechanisms may also have implications for higher animals including humans

c) development of biomarkers that integrate across all levels of biological organization because some chemicals may have a greater effect at lower trophic levels while others will be more apparent at higher levels

d) development of biological indicators that identify ecosystems susceptible to chemicals

e) Development of biomarkers that are rapid and inexpensive and thus capable of being widely used.

Ethical, Social, and Legal Issues Surrounding Studies of Susceptibility

Introduction: Ethics as a Methodology

Growing Attention to Ethics. Since the early 1980s, there has been a growing wave of concern about the ethics of studies on biomarkers of susceptibility (*217–227*). Technological advances continue to challenge our sense of what may be deemed "right" and "wrong" or "morally appropriate." These concerns have escalated since the beginning of the 1990s (*228–248*).

Education in the formal theories, principles, and rules of ethics generally has not been an integral part of graduate training among risk scientists. Even though scientists have tended to focus on "the scientific method" in their work, there are prominent examples of concern with the ethical dimensions of their speciality (*249*). Notions of "peer review" are well developed and mechanisms for achieving "informed consent" are firmly in place. However, the social consequences, including both potential benefits and harms, have been relatively neglected in many areas of science (*250,251*).

Utility of Ethics. A brief overview of ethical theories in relation to scientific technologies is provided, with special attention to their application in the field of biomarkers of susceptibility. It will be shown that the discipline of moral philosophy, like the scientific specialty areas comprising the risk sciences, provides us with methodologies for analyzing decisions (e.g., whether or not to implement a new technology) and a philosophical basis for our actions. In practical terms, ethical analysis provides us with the tools through which decisions we make can be tested against ethical theories (and their attendant principles and rules) as a basis for explaining or justifying (moral) actions (*252*).

Ethical Theories and Principles. Analogous to other disciplines, ethical analysis has a theoretical basis. Stemming from each theory are principles and associated rules, providing a framework for ethical analysis. Empirical data then are testable against these theoretical frameworks. Several theories from the discipline of ethics warrant review in the context of biomarkers of susceptibility.

Deontology, a duty-based theory that specifies obligations to be upheld by members of the profession, is perhaps the most commonly evidenced ethical theory among health professionals. The "scientific ethic," in fact, is deontological (duty based), requiring of scientists, among other things, to be objective, honest, and unbiased in the use of appropriate methods related to their subspecialty area of practice. Physicians, too, are bound by duties that derive from the principles of autonomy (i.e., the right of the individual to make independent choices), beneficence (i.e., the obligation to do good), nonmaleficence (i.e., the obligation to do no harm), and distributive justice (i.e., social equity). The preeminent duty among physicians is to not cause harm to their patients. It is through this duty that the physicians' role extends to that of patient advocate. Autonomy, as more recently interpreted, extends to include involving the patient as a partner in decisions about care. Distributive justice is manifest in the principle of equal access to care, regardless of ability to pay. These brief glimpses of ethics relate to physician–patient relationships (*253*).

Other relationships among health professionals exist. For example, those engaged in public health have the community's interests to protect, and usually adhere more to the utilitarian theory of ethics requiring that the greatest good be done for the greatest number of people. This practice does not preclude the inclusion in public health of other principles such as autonomy, nonmaleficence, and equity. However, the libertarian ethic has less utility within public health because it holds the individual more important than the community.

The egalitarian ethic holds all community members equally important and upholds the principle of solidarity. It measures the well-being of the group by that of the least well off. As an example of the use of egalitarianism, the U.S. Clean Air Act sets regulatory standards to protect the most susceptible members of the population. The principle of justice that flows from the egalitarian theory provides for equal access to the process of susceptibility assessment; by the same principle, the environmental risks should be fairly distributed across social classes, ethnic groups, and races. Equal outcomes cannot be expected because of the nonegalitarian characteristics of inherited genetic traits. The egalitarian philosophy, however, would strive to compensate for those inequalities through biologically based treatment or by social means.

It becomes evident that, depending on the ethical theory that one draws upon, one can rationalize apparently disparate conclusions. This is where normative professional practices need to be defined as an aid to individual practitioners faced with dilemmas, ethical conflicts, or tensions among various principles deriving from the respective theories. The recently formulated ethics of postmodernism uphold the principle of social specificity. This implies that local, subjective, and sometimes fragmented narratives may be more appropriate in guiding the actions of both scientists and the community than traditional overarching or absolutist theories.

The relational ethic, for those more familiar with moral philosophy, provides a basis for making rational comparisons among the various theories. While it is often heard that "ethics are value neutral," in practice it is generally agreed that ethics are, in fact, value laden. Hence, relational ethics can be especially helpful for identifying the theories and principles most appropriate to the issues in studies of biomarkers of susceptibility.

Distinguishing between "Research" and "Practice." A distinction needs to be drawn between the professional engaged in research and the professional engaged in clinical or public health practice. The latter is often governed by legal requirements especially in the area of public health practice where, for example, tensions can arise between the principle of autonomy (in terms of the right to privacy) and the public's right to know about, for example, a potentially contagious condition that requires isolation or quarantine.

Research is that area of professional pursuit that develops new approaches to prevention, treatment, and cure. Adequate testing is required to assure that significantly more good than harm will result before any new product or technology is made available for general practice or commercial application. Where concern is focused on establishing the sensitivity, specificity, and predictive value of a new test, this constitutes research. Here, access by the individual to his/her findings in the absence of any clear interpretation would be inappropriate and volunteers for such research ought to have been so advised at the time that informed consent was obtained. Any person, including the one who participates in research, who would wish to know the results of the tests then would be required to await the conclusion of the scientific investigation and the availability of the test for general use or practice.

Legal and Regulatory Perspectives. While new technologies may have appeal as "magic bullets" to people with concerns for potential benefit, it is government's role to protect the public from harmful effects. Therefore, the involvement of government in regulating the use of technologies is appropriate in those instances where public exploitation or harm could arise from access to inadequately tested products, promoted in the absence of adequate review. It is the scientists' role to pronounce the time when they deem products or technologies to have been adequately tested and hence safe, reliable, and accurate enough for public use.

There is, however, often a fine line that separates "safe" from "unsafe." No product or technology can be said to be absolutely safe, so regardless of where that cut point is set, more or fewer untoward (and unintended) effects will be seen. Society has to be involved in the decisions that define "acceptable levels of unintended effects" as a consequence of any new technology. It then becomes the burden of those in risk communication to ensure public understanding of any risk associated with a new technology used for identifying susceptibilities or for building risk management policies around susceptibility issues.

Ecosystem Perspective. Aside from the ethical concerns of professionals engaged directly in human-health-related disciplines, indirect concerns that are intricately tied to the ecosystem also must be considered. Whether animal species are appropriately used as sentinels (i.e., to serve as indicator species) for exposure effects on humans, the ecosphere should be seen as life sustaining in itself. Hence, damage to nonhuman species or the ecosphere should be considered as potentially harmful to human life. There is also the deontological ethic that assigns to humans stewardship responsibility for the natural world. Therefore, broader concerns than anthropocentric ethics need to be considered in any ethical analysis.

Context of Macro Nature: Ethical, Social, Legal

Relational. The ethical, social, and legal frameworks of biomarker research will differ depending on the professional and contractual relationships of the participants. Individual relationships, such as between physician and patient, lawyer and client, and researcher and participant, are governed predominantly by a deontological ethic whereby the providing professional has the primary obligation to look after and protect the interest of the individual over all other considerations, including social benefits. This obligation is recognized in law that protects the privacy and confidentiality of the physician–patient and lawyer–client (and, by implication, the researcher–participant) relationship against undue intrusion.

This protection may not be complete, and will vary under different legal, social, and cultural conditions. For example, the patient–physician contract may be influenced by the ethics of the payor of services. This ethic places constraints on the primacy of the deontologic relationship because the utilitarian-based principles seek the use of fiscal resources for the maximum good of the larger population. An additional threat to confidentiality rests in the potential vulnerability of computer-based records in which security may be breached by technological intrusion potentially to the detriment of the patient/client/participant.

The significance of these considerations for studies on biomarkers of exposure, effects, and susceptibility rests in the inherent conflicts and tensions that occur when the payor, courts, employer, or public health laws impose requirements on biomarker data that intrude on the principles of privacy and confidentiality in a way that is beyond the power of the original contractees to prevent, and with consequences potentially detrimental to both parties. In this way, data on genetic susceptibility may be used to exclude workers from certain job opportunities, or patients may find themselves unsuspectedly constrained by public health laws. There is also the consideration that the worker in possession of information on genetic susceptibility or biologic effect may be motivated, in the absence of a full understanding of the limitations of the methods, to seek compensation for injury; this could occur in the absence of documented evidence of exposure to a substance in the workplace because of the lack of accompanying technology to identify exposure.

The prevention and resolution of these difficulties are complex and various depending on the social, legal, and cultural environment. The overriding ethical principle is, however, that the participants in these professional/contractual relationships be made fully aware of these possibilities in agreeing to participate in biomarker studies, and that the legal frameworks adjust to the potential negative outcomes if they stand in the way of accomplishing a desired social goal. The scientist, professional, ethicist, and lawyer should work in concert to address these issues, recognizing the adversarial nature of the process and the desirability of engaging the professional organizations in identifying capable experts to provide testimony.

Social Context. Another level of analysis needed to broaden an understanding of ethical concerns on a macro level is the social context. The implication is that the technologies used to identify susceptibility are shaped by a variety of social processes. On this level of analysis, questions surrounding the intended and unintended social consequences of dealing with susceptible individuals and populations need to be raised. For example, will the results of identifying relevant biomarkers in an individual, community, or population have a beneficial or harmful effect upon current or future employment; compensation status in terms of both private insurance and public social security systems; and, more generally, existing forms of social inequality? In this sense, ethical discussions must consider that these consequences may be beneficial as well as

harmful and this should be recognized within the scientific community.

Another aspect within the social context that needs consideration is the interface between the public health needs of a specific community and the duty of professionals to identify as well as to provide for some of these needs. Simply, what ethical demands emerge when professionals are confronted with susceptibility in a particular community? This may expose a tension between utilitarian and deontological theories. For example, on the one hand a public health perspective, driven by utilitarian ethics, upholds the principle of the greatest good for the greatest number. On the other hand, the deontological ethic, which governs health professionals, emphasizes their duty to serve those susceptible in the community as well as to protect a community's right to health.

An intervening ethical theory may be the libertarian theory of rights, which demands that any health decisions taken, whether by professionals or the community, will be absolutely respectful of individual autonomy. Here the emphasis is that to act ethically, decisionmakers need to assume that their future actions enable all persons to attain benefits (on their own initiative).

It is worthwhile in an ethical context to consider the impact of legislative and regulatory measures on communities. In light of the issue of susceptibility, what biological markers appear as important in determining whether a disease should be reportable or notifiable or considered communicable? How does susceptibility status affect regulatory measures or official disease classifications in the field of public health? Answers to these questions should be informed by clear ethical principles. But a tension emerges if a community's rights for distributive justice (i.e., social justice or equity) under the law overrides the social need for trust in the public health professional.

Still another area of concern is the role of the expert in the formation of public health policy. Here, a key issue emerges: at what stage in the production of knowledge about susceptibility factors can experts agree collectively that this knowledge will contribute to more accurate methods of prevention? In this way, the ethical principle of beneficence is upheld. Hence, meeting the need for sensitivity, specificity, and predictive value in this field has meant that an effective contribution to prevention should be able to be recognized. However, other ethical principles besides beneficence need to be considered for experts to be successful in having an impact on the formulation of appropriate prevention policies.

Most definitely, those involved in studies on biomarkers of exposure, effect, and susceptibility should be cognizant of the fact that ethics guidelines may vary across cultures. The sorts of ethical principles upheld by the scientific community of a particular culture may differ significantly from those upheld by their counterparts in another culture. For example, the basic tenets of both contemporary medical and public health ethics are derived from major conceptual developments in Western cultures. Scientists and public health officials from the "developed world" need to recognize this fact when they attempt to compare studies or assess the utility of biomarkers in the "developing world."

Time-related Factors. A third factor in the context of the application of biomarker technology is the evolutionary nature of social, ethical and legal concepts and norms. The fairly recent institution of the principles and practices of informed consent is an example of evolution in the ethical conduct of science. What is considered ethically acceptable or legally permissible at the time an understanding is reached or a contract negotiated may become questioned over the life of the outcome of that contract, years or decades later, because of the maturation of legal, social, or ethical philosophy. Research studies on captive populations or using potentially injurious substances for worthy scientific goals are no longer considered ethically justifiable or legally permissible. Indeed, tort action is proceeding retrospectively.

The status of legal protection of confidentiality is in a state of flux, becoming more secure in some societies and less secure in others. Since it is not possible to foretell at this time the full implications of a biomarker of exposure, effect, or susceptibility, the potential for unforeseen outcomes (detrimental or beneficial) is very real. There is a need to provide legal protection to the participants in the future, provided the contemporary criteria are fulfilled. The alternative is to withhold or delay the use of biomarker technology until greater certainty is achieved.

Process and Content

Self-Regulation. ACCOUNTABILITY. Governments usually relegate control of science to the subspecialty scientific professions. It therefore falls on the shoulders of the scientific organizations to ensure that guidelines exist against which members of the subspeciality groups of scientists can be held accountable (*250*). Ethics guidelines, standards of practice, and the development of good laboratory practices are designed to help maintain objectivity and scrupulous honesty, so necessary for the advancement of knowledge. Adherence to good practices of record-keeping facilitates the auditing of laboratories and thereby minimizes the chances of misconduct in terms of data handling (i.e., falsification, fabrication, and plagiarism) (*254*).

CAPTIVE POPULATIONS. It is currently recognized that captive populations should not be included in research because the prior voluntary consent needed for their participation can have little meaning in such circumstances. Furthermore, the scientific validity of findings derived from captive populations may be of questionable generalizability in the context of the imposed constraints under which such participants may live (*255,256*).

PRIVACY. The privacy of findings from research on biomarkers of susceptibility has perhaps a higher level of personal concern than other personal data. Because such research can have profound ramifications, not only for the research participant him/herself, but also for his/her family (i.e., siblings and offspring), special attention must be given to respecting the participant's right to privacy. In research settings generally, as well as in practice, some level of uncertainty is associated with the findings from susceptibility studies. The confidence with which highly sensitive information can be shared with the person to whom it directly relates is not always optimal. The question, therefore, of whether to share this information (together with its uncertain interpretation) must be raised. Current deliberations around the principle of autonomy suggest that the informed consent process should include the option of whether or not the research participant would want to know his/her results in the presence of no clear interpretation.

Furthermore, whether results for which no treatment can be offered should be provided to research participants is a topic that extends to the underlying principles of screening, where screening should not be undertaken unless something can be offered to remediate the condition. In genetic marker susceptibility studies, genetic counseling can be offered; inheritable conditions are viewed as "treatable" through recommendations of the option of abstention or, more extremely, of sterilization, an option

that in the presence of uncertainty, could result in substantial harm to the individual.

SECURITY. The degree to which information is not only to be protected (secured) by researchers, but also the degree to which it is to be shared with research participants should be effectively addressed in applications for ethics review to institutional review boards, or human subjects/research ethics committees. Studies of biomarkers of susceptibility require special attention to these details because of the heightened sensitivity associated with such findings. Indeed, owing to the heightened sensitivity associated with the information gleaned from studies into biomarkers of susceptibility, special care by scientific reviewers might include the question of whether or not the hypothesis or question being proposed by the study warrants being addressed. The latter point flows from the social values that may or may not permit such questions to be addressed from the public purse. The question that then follows is how to handle the private funding for research of a highly sensitive nature.

When research is funded, researchers need sufficient funds to ensure data security and to conduct a study of adequate statistical power. Pilot studies, while necessary for the formulation of a major study proposal, need to be undertaken with as much attention to data security issues as if they were full-scale studies.

Concern about the public demand for tests that are not scientifically validated is especially serious for biomarker studies. Whereas syphilis testing remains a required premarital test in many countries, in part because a treatment is available, this is not the situation for many markers of susceptibility, especially genetic markers. Where genetic markers are not clearly interpretable, more harm than good could result from access to such testing; certainly, in the current state of development, few cures or treatments are possible. Until such time as society recognizes genetic aberrations as a part of the normal range of biological diversity, the desire to eliminate that which can be eliminated within the constraints of respect for life will continue.

RIGHT TO KNOW AND NOT TO KNOW. Do we have the right to know our personal genetic characteristics? The peculiar and highly sensitive nature of such knowledge indicates that certain limits and precautions may need to be considered in view of the serious potential consequences of such disclosures for the individual, for relatives, and for children. Knowledge of an individual's genetic characteristics can, in some cases and, with some limitations, provide knowledge about the genetic characteristics of his/her relatives.

We also can consider that we have the right *not* to know our own genetic characteristics, the right to a carefree life, and to remain ignorant about our own lot. Because genetic screening could lead to fatalistic or pathological behavior, some people might prefer to remain ignorant of this information. Such an attitude would deserve the same respect as the one that demands the most exhaustive information about one's state of health. If, however, offspring are planned and, say, a 50% chance of transmission of a serious genetic defect exists, should the noncarrying spouse and, for that matter, the carrier, be forced to know or be provided with the information?

SCIENTIFIC INTEGRITY. The importance of ensuring absolute integrity in scientific studies involving highly sensitive information is apparent. Hence, methods of laboratory procedures that minimize the risk of data falsification and fabrication are all the more important. Greater scrutiny (oversight) of these studies is in order.

Conflicting interests must be protected against. These could arise in biomarker studies in which premature results are published and the public demand for the "new" test serves the interests of the manufacturers (and its shareholders or stockholders) at a time when the test results may cause more concern through the inability to interpret the results.

When population-based studies are undertaken to determine the prevalence of any susceptibility factor, unlinked studies might be preferable by virtue of their total anonymity. In drawing biological specimens for these or other studies, however, informed consent dictates adherence also to the principle of veracity and fidelity in honoring commitments to, for example, privacy and to the withholding of, or the communication of, results.

CONTINUING EDUCATION. Because professions are expected to be self regulating, the production of this document is one mechanism by which the continuing education of researchers engaged in studies of biomarkers of susceptibility, as well as their students, can be kept abreast of advances in the field. Constructive criticism of any of the ethics guides presented herein should be encouraged, given that societal values and technology differ and change over time. The need to engage the public and stakeholder interest groups in this discussion, while difficult, cannot be overstressed.

Communication. Communicating and campaigning with different categories of scientific bodies, nonscientific bodies, and other concerned organizations, are as important as the discovery of genetic tests themselves. Satisfying the concerns of those bodies will facilitate the approval and application of those methods, rendering them more effective.

The current practice of genetic techniques offered by researchers for protection, selection, or surveillance of susceptible persons to chemical environmental exposure needs to be scientifically sound with honest information and validated standards. Then these techniques can be presented for peer review.

Researchers also should inform and explain such methods to important groups such as health authorities, general or family physicians, occupational health physicians, trade unions, legislators, and administrators. They should explain the benefits of the work, as well as the risk assessment findings and possible risk management approaches to be considered. Other scientific bodies and researchers can be informed through scientific journals, periodicals, lectures, conferences, and special symposia and workshops.

Information to the general public using mass media techniques can be handled through the public press, radio, and television applying simplified, understandable, and uncomplicated language. Risk communication with the general public should be provided for in lay terms. People who undergo such tests can be informed or not informed depending on their wish to know or not to know. Information to family members, children, and siblings depends mainly on local values and on legal requirements.

Stakeholder Involvement. As a key stakeholder in the development of biomarker technology, the scientist is socially accountable. The whole question of determining biomarkers of exposure, effects, and susceptibility leads to assessments of the health of individuals and populations as well as considerations of the use of biology in social relationships. For example, ethics, as a guiding narrative for those making scientific claims, is situated at the interface between the scientific discourse on susceptibility and the general, social discourse on professional ethics. Within ethics, ethical principles and basic human rights become visible. While ethical principles include autonomy, beneficence, nonmaleficence and distributive justice, basic human rights such as the right to health,

the right to work, and the right to privacy emerge concurrently.

As these principles and rights become established as collective values, it is the duty of all stakeholders involved to ensure that they indeed are upheld. One way to ensure this is to maintain open lines of communication among the various groups of stakeholders. Here, the process of implementing the ethical principles of veracity and fidelity become clear.

Science is not value neutral in that the direction of scientific pursuit is determined by a synthesis of the value of knowledge for its own sake and the value of knowledge for social benefit. All stakeholders should be made aware of the nature and limitations of the scientific method: that it functions by observation, hypothesis, and experiment and requires a tightly controlled methodology for it to provide reliable results. By the same token, although investigating a universe that is governed by the absolute laws of nature, science can never provide an absolute result but phrases its conclusions on the best available evidence at the time. These conclusions are likely to change in the future as new hypotheses and subsequent data about the laws of nature are gathered. Science strives to minimize—but can never eliminate—uncertainty. For these reasons, the process of science must be transparent to all participants, including the research participants themselves, witnesses, and communicators of results. Furthermore, in the interests of reliability, credibility, and durability, the understandable demands for the premature release of data, drugs, and technologies must be resisted according to the ethical principle of nonmaleficence.

In advocating for a position favorable to science, medicine, or public health, the scientist should retain the identity and adhere to the standards of science—objectivity, impartiality, and stating limitations and uncertainties—because the audience always will lend the scientist the credibility of science. To abandon these principles may endanger the stature of science in terms of the scientists' obligation to improve the state of humankind. The need for scientists to convey an attitude of "healthy scepticism" should support the role of science in the public interest.

Case Studies

The recognition of biomarkers in combination with the rapid development of new methods in molecular genetics and analytical biochemistry have facilitated the screening of individuals for genetic variations of direct relevance for susceptibility to environmental factors and diseases. These biomarkers can be analyzed at different levels, indicating differences in metabolic conversion of chemicals, differences in uptake and exposure to DNA-binding chemicals, differences in genetic effects of chemicals, and hereditary differences predisposing to diseases. Several case studies are used to exemplify these applications. The concluding section of each case study attempts to draw out relevant ethical tensions or to highlight particular ethical principles.

Metabolic Variation. Chemicals to which people are exposed are primarily detoxified in the liver by essentially two systems—one that converts chemicals with low water solubility to soluble metabolites (cytochrome P450) and one that causes conjugation to glutathione (glutathione *S*-transferases) and to some other compounds.

Of relevance in the present context is the fact that both enzyme systems embrace genetic variants that affect the efficiency with which potentially harmful chemicals are metabolized or conjugated to innocent or less toxic chemical components. Individuals lacking or carrying variants of some of the genes for metabolism and conjugation exhibit an increased susceptibility to carcinogenic chemicals in the environment. This increased susceptibility has been indicated by the observation of an increase in the binding of the chemicals to DNA, the increase of cytological effects such as chromosome breakage, sister chromatid exchange, formation of micronuclei, and an increase of point mutations (*46*).

The recognition of individuals who are subjected to a potentially increased risk of cancer from this exposure poses the ethical dilemma common to much of the present development of biomarker applications: how to prevent susceptible individuals (particularly those occupationally exposed) from being exposed to these chemicals. In this context, a tension appears between the human right to work and the ethical principle of nonmaleficence. To resolve this tension, scientists should consider more fully the principle of solidarity. In addition, scientists should work in conjunction with public health officials who, at the same time, tend to uphold the principle of social justice.

Mutations Predisposing to Human Diseases. In the last few years, the characterization of genetic factors involved in human disease has undergone a dramatic development. A great number of genes now have been localized and the DNA to a great extent has been sequenced. The human genome project, HUGO, which implies the sequencing of the total human genome can be expected to provide much new material in this respect. A shortcut in this procedure has been performed with "expressed sequence tag" through which mRNA is used instead of DNA to identify human coding genes. DNA of the coding genes is collected by enzymatic conversion of mRNA to cDNA. With this technique, the work can be focused on the 3 to 4% of the human DNA giving rise to genes. To date, about 50,000 of the 70,000 to 100,000 genes have been identified. This material already has played a crucial role for the characterization of the mismatched repair genes particularly involved in human colorectal cancer (below). This will put the person concerned, the physician, and the administrator into a dilemma on how to handle such information and how to protect the person. If the ethical rules will be applied and the test with its pros and cons explained to the person beforehand, this should provide reasonable resolution to potential dilemmas.

Repeated DNA Sequences. Among genetic variants in the human population giving rise to diseases, repeated DNA sequences have attracted a great deal of attention in recent years. Repeated DNA sequences of relevance in this context of biomarkers concern amplification of coding genes as well as short repeated sequences, minisatellite and microsatellite DNA (*134*).

Amplification resulting in overexpression of coding genes is a regular phenomenon under certain circumstances (*257*). The gene for metallothionein protects against heavy metals, and exposure to heavy metals can cause an induction and an amplification of this gene (*258*). The subsequent increase of the protein therefore can be a biomarker for exposure to heavy metals like cadmium and mercury.

Of pathological importance is the occurrence of amplification particularly of nuclear oncogenes such as c-*myc*. Such amplification has been suggested as a biomarker of some prognosis value in breast cancer patients (*259*).

An essential part of the noncoding DNA is built up of short, repeated sequences and some of that DNA exhibits a pronounced instability, particularly involving length alterations. The function of this DNA has been and remains obscure, but in recent years several serious

human diseases have been associated with short, repeated DNA sequences, minisatellites (10–100 bp) and microsatellites (2–4 bp).

One case of pathological connection with minisatellites concerns a minisatellite associated with the oncogene *ras*. Some rare variants of this minisatellite are associated with multiple forms of human cancers. It is thus possible to identify carriers of these rare minisatellite alleles that result in a significantly increased risk for cancer.

Microsatellites are particularly important as biomarkers and the cause of some serious human neurological disorders. The microsatellites are linked to the actual genes involved in the disorders and an extension of the repeated DNA sequences above a certain number causes the disease. An important aspect of this process is the fact that the amplification tends to increase from one generation to the next, making the disease gradually more serious with earlier onset—"genetic anticipation."

These microsatellite-linked diseases involve, for instance, fragile X, which is one of the most prevalent mental retardation conditions, and the well-known Huntington's disease. Huntington's disease causes a neurological disintegration with onset usually in the age range of 40 to 60 years.

The genetic predisposition for these diseases is passed on to 50% of the offspring and appropriate testing will predict disease in the offspring. Only occasionally has it happened that the number of repeated DNA sequences has diminished from one generation to the next. The fact that Huntington's disease (as well as other neurological diseases) is incurable poses many ethical problems at the individual and family level, such as testing the offspring and other family members; offering prenatal diagnosis; or deciding whether or not abortion should be performed.

Traditional debates in this area expose major tensions between the ethical principles of autonomy, expressed by those upholding the right to choose, and nonmaleficence, implicit in those opposing abortion. While this tension has not yet been adequately resolved, scientists as well as public health professionals would benefit from considering how developments in biomarker technology may have the potential to modify this traditional tension. If all stakeholders involved would agree collectively about what is meant by "human life" and when it starts, this awareness would shift the traditional debate into a different ethical arena. Here, deontology guides the scientist to stimulate the public's awareness of autonomy.

Repair Mutations. It has been known for over 25 years that a deficiency of DNA repair can cause cancer. The classical case is the recessive autosomal mutation Xeroderma pigmentosum, which causes a serious skin disease. Patients with the disease lack the ability to repair the DNA lesions caused by ultraviolet light irradiation and they invariably develop skin cancer.

In recent years, several genes involved in the repair of mispaired nucleotides, mismatched repair, have been characterized and localized (*260*). Mutations in these genes are particularly linked to an elevated risk of colon cancer. The mutations occur as heterozygotes and the tumors are induced as the result of the loss of the wild-type allele. It has been estimated that this mutation is carried by 1 in 200 people, and it thus constitutes one of the most prevalent human disorder mutations. Screening for this mutation is likely to be recommended, at least in families that exhibit a high rate of this specific colon cancer-type linked to deficiency of mismatched repair. Such screening will fulfill the purpose of avoiding malignant growth of tumors by regularly checking the colon. In addition, it is evident that exposure to mutagenic and carcinogenic agents can be expected to increase the risk of cancer to a greater extent in mutant carriers, with possible implications for occupational and lifestyle choices.

The fact that susceptibility studies of colon cancer implicate family members poses the tension already discussed in the preceding section "Right to Know and Not to Know." Nevertheless, varying attitudes about genetic information—and the genetic information itself—should be seen as equally important by both the public health professional and the scientist.

Reliability of Laboratories and Methods. Many of the analyses of genetic variants and genetic disorders at the molecular level require sophisticated laboratory techniques and professional knowledge to properly interpret the data. It is of paramount importance that laboratories involved in such analyses are subjected to quality control to avoid mistakes. For instance, it is critical that analyses of mini- and microsatellite patterns in forensic medicine to identify criminals is performed without any error—the consequences could otherwise be disastrous. It also has been pointed out that, although the chance of two unrelated persons having a similar genetic pattern is very remote, relationship has to be excluded in the analysis (*261*). On the other hand, correctly performed, these techniques for characterizing individuals genetically constitute invaluable tools in forensic medicine, paternity determination, and epidemiological and population analysis. The proliferation of these techniques and tools, and their use with individuals and populations, demand that the collective scientific value of quality assurance be maintained. In ethical terms, the principle of scientific honesty is most relevant in laboratory settings.

Impact of Genetic Monitoring or Screening on Society. We are doubtless only in the beginning stages of the evolution of the genetic characterization of individuals, but already we can foresee many practical and ethical problems for society. Solutions must be developed with the ethical and moral implications defined explicitly. When social costs are involved, the right of autonomy over such personally significant data should be balanced against the interests of society according to, for example, principles derived from utilitarian, libertarian, or egalitarian theories of ethics.

Among the pressing problems is the question of who will have access to the genetic information of individuals. The initial governing principle here would seem to be autonomy. The principle of autonomy requires respect for the individual's right to privacy. However, a tension emerges when the individual's own actions can have a negative impact on the group. The tension is between the individual's right to privacy and the group's (or the community's) right to know.

For example, the fact that people can obtain information about mutations that are likely to shorten their life expectancy may become important in securing life insurance. Attempts to prevent genetic data from reaching insurance companies are not likely to be successful in that insurance companies can require the right of access to medical records as a condition to considering insurability.

If egalitarian theories are to be followed, the cost of this insurance risk would be shared without penalty across the pool of insured people. If either utilitarian or libertarian theories are adopted, susceptible persons might be excluded from the pool of insured, or they might be charged higher premiums. It could be foreseen that persons who know that they carry a life-shortening genetic condition might purchase large

amounts of life insurance and that, in turn, could cause an economic deterioration of the insurance system. Prior consideration of these possibilities might help society to focus on the risks associated with the adoption of technological advances.

Genetic Screening of Workers. The recognition that certain genetic polymorphisms could be identified in humans (e.g., hemoglobin S, G-6-PD deficiency, α_1-antitrypsin deficiency) and could be related to differential susceptibility, led Stokinger (*262*) to advocate their application in screening for so-called "hypersusceptible workers." Omenn (*223*) cautioned against the blanket application of such techniques as inadequately predictive of risk, and several critics have noted the importance of controlling workplace exposures instead of removing susceptible workers from the workplace (*263*). Although unusually susceptible individuals would benefit from not being exposed to the agents that are likely to make them sick, this intent should not be used as an excuse to avoid reducing exposures in the workplace; nor can it be invoked to avoid taking differential susceptibility into account in risk assessment (*264*). Moreover, none of the genetic screening programs thus far proposed are sufficiently predictive of risk. Hence, any exclusion by virtue of membership in a genotypic class would be discriminatory, at least under United States law.

The elimination of women of childbearing age from certain occupations is now deemed illegal in the United States and represents an example of what is essentially genetic screening; namely, the elimination of persons with a particular genotype (XX) without regard to their actual susceptibility status.

Ecological Ethics. The emission of harmful chemicals into the environment also should be considered from an ecotoxicological point of view. Particularly, the contamination of the environment with persistent chemicals like some chlorinated hydrocarbons and heavy metals can have serious effects on the ecosystem through bioaccumulation along the food chain. Organisms at the top of the food chain run the risk of acute intoxication, reproductive inability, and behavioral disturbances. These effects on the ecosystem are relevant for the preservation of biological diversity in accordance with the convention of biodiversity accepted by the United Nations conference on environment and development in Rio de Janeiro, 1992. But besides our moral obligation to take into account effects on the ecosystem and biodiversity, the monitoring of ecotoxicological effects often can have relevance to human health.

The human species is also at the top of the food chain and is often exposed in the same way as other species at this trophic level. The recent focus on chlorinated compounds like PCBs, DDT, and tetrachlorodioxin, because of hormonal effects, may serve as a further warning signal for human beings. The deontologic duty of humans to serve as stewards of the environment for their own self interest and that of future generations is highlighted in this context. This duty enhances the right to life of other species.

Ethical Components of Biomarker Project Proposals

Parties responsible for biomarker studies need to ensure that the broad range of persons involved in the planning, implementation, and outcome of projects (the stakeholders) are provided the protection of explicit ethical principles under which a project will be conducted. Persons involved include the study participants, the beneficiaries (including both participants and nonparticipants), the community (special and general), those gathering the data (researchers and surveyors), employers, trade unions, regulators, sponsors, and other potential recipients of the results.

The ethical issues to be addressed include the following:

- autonomy of the participant in participating, including fully informed consent
- beneficence of the project for the persons and institutions affected
- absence of maleficence toward the participants and institutions affected
- assurance of justice (equity among participants and nonparticipants)
- test validity as a function of the target population, including consideration of the degree of risk, exposure, and other factors
- explicit provisions to ensure equal access to participation across race, social class, and gender as appropriate to the attribute under study
- nonabridgement of deontologic (duty-based) obligations of the participants
- scientific integrity and soundness
- confidentiality and security of data, including anonymity and nonlinkage to the individual
- referral for treatment and counseling for a significant incidental medical finding
- assurance that a recognized public health or legal risk will be reported
- formulated response to anticipated questions about biomarker findings
- identification and allocation of legal liability
- formats and pathways for communicating study results, including publication, media and employer
- sponsorship, conflict of interest; clear understanding of deliverables warranted for funding received
- action to be taken in the event of an untoward finding, including employment status
- future mutual obligations of participants in the event of new developments.

Conclusions

A number of conclusions can be drawn, particularly the following:

a) Because the issues contained herein are from the scientist's perspective, the attention of other stakeholders is called for.

b) The positive aspects of biomarker technologies must be emphasized without failing to recognize the potential for their misuse.

c) There is a need to continue research to produce better evidence concerning susceptibility markers while simultaneously protecting against any misuse of premature or tentative evidence.

d) The active participation of the public is to be sought in a partnership capacity to ensure the advancement of knowledge concerning biomarkers for identifying susceptible people and populations.

e) It must be recognized that science is imperfect. Uncertainty is inherent to the scientific method and varies as a function of the evolution of scientific knowledge.

f) The imperfections of science require humility on the part of scientists. Science is but one part of a number of inputs for decision making.

g) For every decision that is made, the tension between risk and benefit should be considered.

h) Good science should be tied to good ethics and vice versa.

i) An unintended consequence of biomarker technology is that individuals and populations could be discriminated against.

j) Until the scientists involved with developing a new susceptibility biomarker technology have declared it adequately reliable and accurate and after full peer review, the public should be protected from commercial interests that wish to prematurely release the technology into the market place.

Recommendations

Participants in preparing this report make the following recommendations:

a) Because a multidisciplinary group assessed the ethical dimensions of each respective subspecialty discipline, this report ought to carry some authoritative weight in the context of professional ethics.

b) Professionals and their students need to engage in an ongoing dialogue concerning applied ethics.

c) Legislation needs to be developed to protect all interests against liability that could be judged in hindsight arising from changes in ethical standards and scientific knowledge over time (i.e., against retrospective tort action in soundly conducted studies because of intervening changes in social and ethical standards or advances in scientific knowledge).

d) There is a need for guidelines concerning biomarker susceptibility studies for use by researchers and practitioners alike, which also would be of use in the training of students. Other agencies that have engaged in related deliberations (such as those concerning the Human Genome Project and the U.S. Office of Technology Assessment), should be sought out for collaboration since they have developed guidelines in the area and related areas.

e) Every research proposal in the area of biomarker susceptibility should address the ethical dimensions of the proposed study. In addition, strategies for communication, from peer review through individuals and the public, should be documented. Studies in biomarker susceptibility should be audited for adherence to proposed intentions.

f) Scientists must be vigilant in developing ethics guidelines in that they should ensure equity across all segments of society. All guidelines should be sensitive to cultural differences.

g) Because of the extremely sensitive (i.e., intimately personal) nature of the information gleaned from biomarker susceptibility studies, extraordinary precautions relating to the privacy of the information are to be exercised.

h) Specific guidelines on how industry and insurance companies might deal with individuals discovered to have a susceptibility are beyond the scope of this text. However, attention is drawn to this since guidelines in these specific areas may be aided by the discussion contained in this section.

Appendix

Analysis and Quality Assurance

Laboratory results are only useful if they are accurate and precise, and are reported in a responsible manner. Accuracy refers to the ability of the analyst to determine the exact quantity present in a sample. Precision refers to the ability to provide a consistent result (which may not necessarily be accurate, if for example, there is a failure in calibration). QA represents the sum total of activities that are required to produce reliable results (good precision and accuracy). In terms of laboratory analysis it includes setting data quality objectives on the one hand and evaluating quality control measures on the other. Stating a data quality objective consistent with the goals of the screening program or study is a necessary first step. Lack of resources or power sufficient to meet these objectives is grounds for not doing the study. Excessive power increases the cost.

Standard QC procedures have been well documented. Most published methodologies include QC procedures which must be individualized for each laboratory and each analytic instrument and procedure. Each laboratory must have a written QA/QC statement and protocol. These should be very detailed, including, for example, assurance that the deionized water is free of analytes in question, at least within the limits of detection required. Similarly, the purity of the solvents and reagents and the source of all standards should be specified.

Calibration procedures should use blanks and fresh standards within the range of anticipated concentrations. (One can only assume that a method yields linear results within the range of the calibration curve). The method detection limit should be specified for each analyte in each matrix (blood, urine, hair, etc). Field and method blanks should assure that there is no contamination at any point in the collection and analysis of samples. Spiked samples should assure that the technique can recover the analyte from the matrix. This allows one to document the percent recovery of the spike from the sample. Unless otherwise specified, it is customary to require that each analytic run produce a recovery between 85 and 115% of the actual spike. For some analytes it is difficult to achieve this level of accuracy on certain instruments, while for others tighter limits are routine. If recovery lies outside a certain range of values, then the entire run is rejected, and the stored samples are redigested and reanalyzed. Alternatively, some procedures allow the correction of the analytic results by the recovery percent. Thus if the recovery is only 80% of the spike, the results can be multiplied by 1.25 to correct for the deficiency. This approach is much less desirable and should be avoided, unless there is no further material available for analysis or unless the method repeatedly results in a similar recovery.

Each laboratory should participate regularly in a proficiency testing program involving the blind analysis of unknown samples provided by a reference laboratory. The results of such testing should be maintained with the QA/QC documents and should be available for inspection.

Challenges for Susceptibility Monitoring in Developing Nations

In many cases through international cooperation, developing nations are able to mount sophisticated screening programs for environmental as well as infectious agents. For example, the Mexican government, with the support of several international agencies such as United Nations Environmental Program, has been able to evaluate the exposure and possible effects of chemicals on human health for both acute and long-term exposures (Table A1). However, such programs can only touch the surface of the environmental pollution problems present throughout the world. Moreover, at the same time that industrialization is increasing in areas with minimal environmental regulation and enforcement, the developed nations are cutting back on funding for international cooperative programs.

Guidelines for the Implementation of Biomarker Studies

In any program the number of guidelines should be considered in implementing any

Table A1. Assessment of exposure using various approaches: examples from Mexico.

Exposures	Monitoring level	Test
Acute		
Chlorine gas tank leak, Mexico City	Clinical	Spirometry
Smelter emissions, El Paso, Texas	Blood level	Lead, cadmium, zinc
Metal exposure (10-year followup) conduction	Neurological	Peroneal nerve
	Blood level	Lead, cadmium, zinc
Cows raised on farmland with high natural arsenic content exposure	Food chain	As in milk
Chronic		
Studies of air pollution prevalence,[a] Mexico City	Questionnaire symptom	
Studies of air pollution, Mexico City	Clinical	Vital capacity in children
International 10-city study of lead exposure in schoolteachers	Blood lead level	
Study of schoolteacher exposure, Mexico City	Environmental media	Air, food, water, soil, cooking pottery
Organochlorine pesticide exposures in representative sample of women, Mexico City	Breast milk	DDT and other organochlorines
Carbon monoxide levels in workers exposed on streets, Mexico City	Blood	Carboxyhemoglobin

[a]Daily frequency of respiratory symptoms in elderly or chronically ill people.

screening program for biomarkers. These guidelines should include the following:

Overall Quality Planning

a) The economic resources available for the program must be identified.
b) The benefits of the program must be justified to assure adequate funding.
c) The intervention must be specified and funds identified in advance, if possible.
d) Where resources are not available for the "best" methodology, the use of alternative tests must be evaluated.

Quality Assurance

a) The screening protocol should include a statement of the data objectives and the required detection limits, the anticipated range of values, and the acceptable limits for precision and accuracy.
b) High quality laboratories should be identified.
c) All participating laboratories should have a written QA/QC document which should include documentation of participation in some Laboratory Proficiency program.
d) The protocol should allow for an internal QC program including blind replicates and confirmation of some percentage of the samples by an external reference laboratory.
e) Data quality evaluation should be completed prior to data analysis.

Standards for Collection of Samples

a) The population to be studied should be clearly identified and the people to be screened should be representative of the population of interest (minimize confounders).
b) All personnel should be selected carefully and should be conscientious and responsible.
c) All personnel (registrars, interviewers, phlebotomists, etc.) should be carefully trained and periodically evaluated.
d) Field sampling conditions should be made as ideal as possible. Use extreme precautions to avoid contaminating samples of blood, urine, etc. at the time of collection.
e) Care must be taken in the transportation of samples from the field to the central laboratory, particularly if samples require freezing or refrigeration.
f) All samples must be carefully and accurately labeled and a chain-of-custody form may be desirable for certain study situations.

Laboratory Standards and Practices

a) High-quality laboratory facilities are essential.
b) Where possible, a laboratory that is already functioning and of proven quality should be used.
c) Whether old or new, laboratories should be designed to eliminate both external and internal sources of contamination. This requires high standards of design and maintenance.
d) Laboratory equipment should be appropriate for the analyses being performed.
e) The appropriate preservation of samples is important, so facilities should have reliable refrigeration, preferably with a backup source of emergency electricity (gasoline generator).
f) All laboratory personnel should be trained; usually this will require training at an analytic center in a major city or outside the country. Training should embody the general principles of laboratory QA/QC and should also be relevant to the equipment available for use in the laboratory.
g) All results should be carefully checked for internal consistency. Deviant results should be repeated.
h) Laboratory supervision should be strict and consistent to assure high quality data.

Results

a) There should be adequate computing facilities and trained personnel for analysis of data.
b) All individual results should be treated as confidential and individuals should be informed of their own results with adequate information as medically appropriate.
c) The suppression of results for economic or political reasons is strongly discouraged.

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