CHEMICAL MANUFACTURERS ASSOCIATION

November 28, 1994

Charles M. Auer
Director
Chemical Control Division
Environmental Protection Agency
401 M Street, SW
Washington, D.C. 20460

Christopher T. DeRosa, Ph.D.
Director
Division of Toxicology
Agency for Toxic Substances and
Disease Registry
1600 Clifton Rd.
Atlanta, GA 30333

Dear Mr. Auer and Dr. DeRosa:

I am writing this letter on behalf of the Chemical Manufacturers Association Vinyl Chloride Panel to express our intent to work with the Agency for Toxic Substances and Disease Registry to develop a voluntary testing program that will satisfy the two data needs for vinyl chloride identified by ATSDR and referred to EPA. The Panel also would like to discuss with EPA the rationale for a neurotoxicity study and how to address EPA's concerns. The Panel represents all U.S. manufacturers of vinyl chloride.

ATSDR identified final priority data needs for 38 priority hazardous substances, including studies of the reproductive and development toxicity of vinyl chloride by the inhalation route. 57 Fed. Reg. 54150 (Nov. 16, 1992). More recently, ATSDR solicited voluntary research proposals to meet the identified data needs, and indicated that it had referred the two data needs described above to the EPA for addition to its master list, the first step in test rule development under Section 4 of the Toxic Substances Control Act. 59 Fed. Reg. 11434 (March 10, 1994).

EPA now has issued a notice inviting manufacturers and processors of nine of the chemicals referred by ATSDR to negotiate enforceable consent agreements with EPA for testing to fill those data needs. 59 Fed. Reg. 49934 (Sept. 30, 1994) (the "solicitation notice"). In certain cases, EPA has included testing to satisfy requests from its own program offices or from other agencies that are additional to the data needs referred to it by ATSDR. For vinyl chloride, EPA has added an inhalation neurotoxicity study to the data needs referred to it by ATSDR.

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2501 M Street, NW, Washington, DC 20037 Telephone 202-887-1100 Fax 202-887-1237

On November 18, our counsel, Mr. Caffey Norman, received from you a policy statement concerning the respective roles of ATSDR and EPA with regard to voluntary testing for four of the chemicals that are the subject of the solicitation notice, including vinyl chloride. This statement has been quite helpful to us in responding to the solicitation notice.

To begin our negotiations with ATSDR and EPA to develop a voluntary testing program, we propose a meeting between scientists from our member companies and ATSDR scientific staff in the first half of December to discuss the design of a two-generation reproductive study by an inhalation route. In light of the existing two-species inhalation developmental toxicity study, and other available data concerning the developmental toxicity of vinyl chloride, we would like to discuss how this study and other available data might be enhanced to include measures of developmental toxicity as an alternative to the two-species developmental toxicity study referred to EPA. Once we have reached agreement on the testing program, the Panel would propose to move forward with ATSDR to negotiate and execute, by May 31, 1995, a memorandum of understanding to address these data needs for vinyl chloride.

We understand that, should EPA decide that our voluntary research agreement with ATSDR will not address all the testing needs for vinyl chloride identified in the solicitation notice, EPA may proceed to negotiate an ECA or propose a test rule to meet such unaddressed testing needs. According to the policy statement, EPA will proceed with development of an ECA or test rule for vinyl chloride if an MOU between the Panel and ATSDR has not been executed by May 31, 1995.

If possible, the Panel would prefer that any voluntary testing program it develops addresses the data needs for vinyl chloride that have been identified by EPA as well as the two studies proposed by ATSDR. As the rationale for a neurotoxicity data need is not identified in the solicitation notice, we propose that scientists from our member companies also meet with EPA scientific staff to discuss EPA's concerns and how the neurotoxicity data need identified by EPA might be addressed.

According to the policy statement, ATSDR will within 30 days notify EPA of the terms of any voluntary testing agreement it might enter into with the Panel on vinyl chloride, and EPA will then decide whether the agreement addresses all the testing needs identified in the solicitation notice. We are not at this time in a position to judge whether EPA would decide that our voluntary research agreement with ATSDR will address all the testing needs for vinyl chloride identified in its solicitation notice. As described above, it will be necessary in this regard to have further discussions with EPA and ATSDR scientific staff and to see how our voluntary testing program develops. Accordingly, we request that the time for submission of a proposal to EPA to conduct testing of vinyl chloride under an ECA for any testing needs identified in the solicitation notice but not included under a voluntary research agreement with ATSDR be extended until June 30, 1995.

I will call you in the next several days to determine your response regarding the negotiations with ATSDR and EPA on a voluntary testing program for vinyl chloride and to discuss meeting schedules. If you have any questions or need additional information, please call me at (202) 887-1192.

Sincerely,

Has Shah

Hasmukh C. Shah, Ph.D.
Manager, Vinyl Chloride Panel

cc: Brian P. Riedel, Esq.
W. Caffey Norman, Esq.
EPA Docket OPPTS-42052;FRL-4756-5

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Practical Applications of Biomarkers in the Study of Environmental Liver Disease

Carlo H. Tamburro
John L. Wong

Essential Background

Introduction

The concern over adverse health effects caused by exposure to environmental toxins and unique role of the liver in the metabolism of these toxicants makes biomarkers of liver metabolism and disease clinically very important. Hepatic biomarkers are needed to assess exposure, identify subclinical hepatic injury, monitor for chronic disease, assess long-term risk, and allow for preventive intervention before liver injury progresses to an irreversible stage. Examination of these hepatic biomarkers in the clinical, occupational, and environmental settings will verify their ability to detect specific exposures or adverse health effects. Also, under proper conditions, they can provide the means to reassure exposed individuals of no future adverse health risk.

Liver

Structure

Hepatic biomarkers, especially enzymatic ones, are biologically related to the anatomical architecture of the liver. The *architectural unit* of the liver has been described classically as subunits of hexagonal lobules, 1–2 mm in diameter, situated about a central vein. The boundaries of these lobules are demarcated by portal tracts, composed of two blood supplies (arterial and venous) and the biliary excretory system. These portal tracts approximately

Molecular Epidemiology: Principles and Practices

follow the angles of the hexagons (Figure 20.1A). Blood flows to the parenchymal tissue from the portal triads at the periphery of the classic lobule and exits via the central veins. About one-third of the blood supply to the lobules is provided by the hepatic artery; the remainder is supplied by the portal vein. Because hepatic blood flow is such an essential component of hepatic function, the portal triads are considered the center of the *functional unit,* called the asinus. The parenchymal cells surrounding this vascular distribution are divided into three zones: Zone 1, closest to the arterial and portal blood supplies; Zone 2, between Zones 1 and 3 in the center of the parenchyma; Zone 3, surrounding the central vein region (Figure 20.1B).

Function

The liver has multiple functions. The primary role is metabolic, involving uptake of substrates for storage, metabolism, and distribution via the blood and bile. Its second major role is conversion of xenobiotic agents and endogenous materials into excretable compounds. This second metabolic function can sometimes convert otherwise harmless compounds into toxic ones. Third, the liver is a major site for clearance of bacterial and other ma-

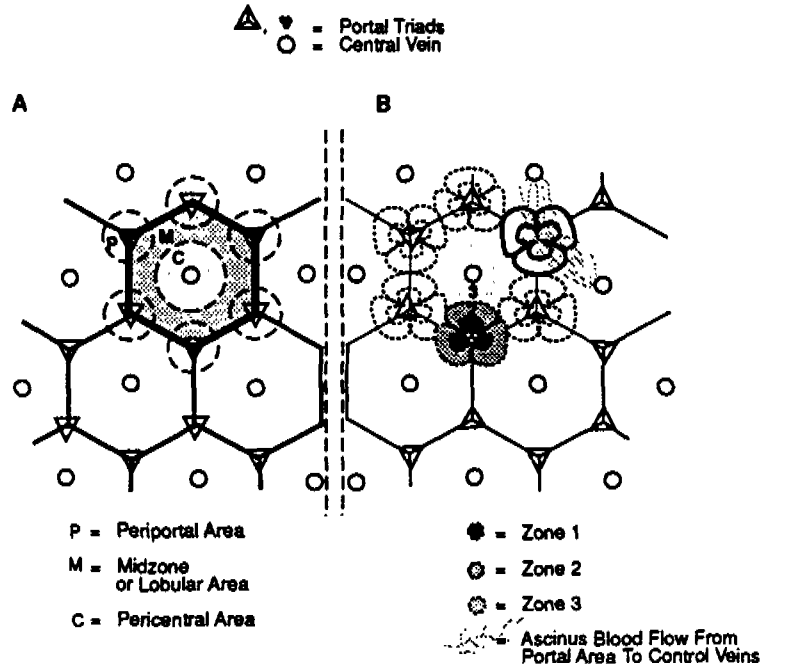

FIGURE 20.1 Normal architecture of the liver illustrating the structural concept of hepatic lobule (A) and the functional concept of hepatic ascinus (B).

TABLE 20.1 Useful Biochemical Markers of Liver Function and Injury[a]

Enzymes
Alanine aminotransferase (ALT)
Aspartate aminotransferase (AST)
Gammaglutamyl transpeptidase (GGT)
Lactic acid dehydrogenase (LDH)
Alkaline phosphatase (AP)
Proteins
Albumin (Alb)
Prothrombin (PT)
Bile acids (BA)
Cholyglycine
Total bile acids
Bilirubin (TB)
Conjugated/direct (DB)
Unconjugated/indirect (IB)
Metabolic/Physiologic
Aminopyrine breath test (ABT)
Indocyanine green clearance (ICG)

[a] Referred to as biochemical liver tests (BLTs).

terials by its phagocytic activity via the reticular endothelial system (RES), which also encompasses parts of the immune system. The metabolic role of the liver makes it vulnerable to toxic injury from exposure to a variety of metabolic and xenobiotic insults. Hepatic biomarkers can identify this injury and, in many cases, characterize the location, severity, and nature of the damage. Toxic exposure may manifest itself in three forms: enzyme induction, hepatocellular damage, and cholestasis. Currently useful biochemical markers (clinical tests) that identify these liver responses to toxins are shown in Table 20.1.

Biomarkers Currently in Use or under Consideration

Exposure Detection

Cellular Enzymes

Xenobiotics are known to undergo hepatic biotransformation and produce bioactive, rather than detoxified, metabolites. Although most bioactivation has been demonstrated in animals, acetaminophen, aflatoxin B_1, arsenic, carbon tetrachloride, halothane, isoniazide, and vinyl chloride have been shown to produce acute or chronic disease and malignant transformation in humans. The striking characteristic of biotransformation is the enhancement of cellular enzyme activity. This activity may be used to determine acute and chronic exposure to xenobiotics that exceed the background enzyme induction caused by natural products in the diet and inhaled air. These

cellular enzyme markers, as presently characterized, are generic in response to xenobiotic exposure.

Oxidative induction: MFO-P450 Cytochrome P450, the monooxygenase system, is a family of mixed-function oxidase (MFO) enzymes with unusual versatility because of the multiplicity of forms. In humans, Wang *et al.* (1983) have identified six P450s shown to metabolize different compounds. As shown in Table 20.2, these human P450s have different but overlapping broad substrate specificities. Chemical induction of P450 has been divided arbitrarily into two classes, the PB type (phenobarbitol-induced) and the 3MC type (3-methylcholanthrene-induced), on the basis of the induction of characteristic P450 isozymes and the mechanism of induction. For example, a dioxin derivative, TCDD, belongs to the 3MC class (Le Provost *et al.*, 1983). This type of cellular enzyme biomarker may be used to identify specific xenobiotic injury for which organ tissue is available. Indirect measurement of these types of enzyme biomarkers can be done by metabolic clearance tests that are surrogate measures of oxidation.

Surrogate measure of oxidation: aminopyrine breath test More applicable means of indirect measurements of the hepatic P450 oxidation system are metabolic clearance tests. There are a number of such tests, such as the aminopyrine breath test (ABT) or caffeine clearance (Baker *et al.*, 1983). ABT has had the most extensive use in xenotoxic assessment. Aminopyrine is administered orally and oxidized primarily in the liver, liberating formaldehyde, which undergoes subsequent metabolism to CO_2. This carbon atom is labeled with either ^{14}C or ^{13}C and recovered in the breath, allowing an indirect measurement of P450 activity as a reflection of the functional liver mass.

TABLE 20.2 **Human Hepatic Microsomal P450 Induction in Xenobiotic Metabolism**[a]

Xenobiotic	P450 −2	P450 −3	P450 −4	P450 −5	P450 −7	P450 −8
Acetanilide	L	M	L	L	L	L
Benzo(*a*)pyrene	L	M	M	M	M	M
d-Benzphetamine	M	H	H	H	H	H
Trichloroethylene	L	L	L	L	L	L
1-Naphthylamine	L	—	L/M	—	—	L/M
2-Naphthylamine	L	—	L	—	—	L

Source: Adapted from Wang *et al.* (1983).

[a] Relative rates of metabolism (nmol/min/nmol P450): L, low (≤ 0.09); M, medium (0.1–0.99); H, high (≥ 1.0).

Lidocaine clearance Like ABT, lidocaine clearance is an induced measurement of P450 activity which, in turn, reflects the functional hepatic mass. Lidocaine is aminoethylacetanilide, which undergoes rapid *N*-deacylation via the hepatic cytochrome P450 system to yield several metabolites, principally monoethyglycinexylidide (MEGX). The concentration of MEGX in serum before and 15 min after iv administration of 1 mg/kg lidocaine can be determined by a fluorescence polarization immunoassay system.

Detoxification induction: glutathione Glutathione (GSH) is the major endogenous protective substance that participates in covalent binding of reactive electrophilic metabolites and in reducing peroxides. The enzymes (transferase) involved in catalyzing the glutathione detoxification effects are also potential biomarkers. These transferases comprise a family of enzymes with overlapping but distinct substrate specificities (Vander Jagt *et al.*, 1985). For example, glutathione *S*-transferase (GST) is an enzyme involved in catalyzing the detoxification of potential diol-epoxide carcinogenic or mutagenic metabolites of polycyclic aromatic hydrocarbons (Glatt *et al.*, 1983).

Metabolic and Physiologic Tests

Clearance tests: indocyanine green clearance Hepatic function and reserve are related to the ability of the liver to clear substances from the blood. Hepatic extraction (intrinsic clearance) is a determinant of bioavailability that can be measured by the systemic clearance of liver specific substances, for example, iodocyanine green (ICG), galactose, or bile acids. There is good correlation between systemic clearance of ICG and early xenotoxic liver injury. The measurement of hepatic extraction is highly correlated to hepatic blood flow. These substances are given intravenously and their clearance rate is determined by small serial blood samples over 10–15 min (Tamburro and Liss, 1986). Lower clearance of these substances results from lower extraction due to intrahepatic blood flow changes secondary to toxic liver injury.

Bile acids Bile acids are naturally produced hepatic substrates, cleared solely by the hepatocytes, and have shown good correlation with early xenobiotic liver injury. The advantage of this study is that no injection of any substance is required. Bile acids are measurable in serum by radioimmunoassays.

Proteins: Antigens and Antibodies

Some environmental hazards are biologic, for example, viruses that can occur concomitantly with xenobiotic exposure and act as confounders or cotoxins to the liver. Specific antigen–antibody markers are available for identification of exposure and active hepatocellular injury due to a major hepatic virus (Tamburro, 1991). The human virus (e.g., hepatitis B and C)

plays a very important role in the causation of liver cancers associated with natural and synthetic xenobiotics (e.g., aflatoxin).

Hepatic fibrosis is the alternative repair mechanism (as opposed to regeneration) for hepatic injury. It is the key indicator of serious hepatic injury after xenobiotic exposure. The detection of hepatic fibrosis is especially important in low-level chronic and subclinical exposure. Noninvasive serum markers of hepatic fibrosis showing early promise include *N*-terminal propeptides of Type III procollagen and Type IV collagen fragments. These markers of serum concentration correlate well with gene expression (messenger RNA levels) in dimethylnitrosamine- and carbon tetrachloride-induced hepatic fibrosis (Hayasaka *et al.*, 1988; Salvolainen *et al.*, 1988). Further studies are needed to establish baseline variations and to characterize their course in various forms of human liver injury.

Adducts

Assays for xenobiotic binding to GSH, various proteins, and DNA are under development and field application. Antibodies, polyclonal and monoclonal, have been in development for a number of hepatic xenobiotics, for example, aflatoxin B_1 (Sabbioni *et al.*, 1990) and acrylonitrile (Wong *et al.*, 1990). Immunoassays for these xenobiotic antibodies are being developed to detect adducts to GSH, albumin, hemoglobin, and DNA. Clinical trials for each type of adduct provides different information with respect to degree, duration, and dose of exposure. Monoclonal antibody assay for specific hepatic xenobiotics can be applied in many ways, as shown in Table 20.3 (Perera and Weinstein, 1982; Perera *et al.*, 1986).

Genetic Markers

Restriction fragment length polymorphism Gene susceptibility for the development of alcohol liver injury is suggested, since only a minority of alcoholics develop cirrhosis. Using restriction fragment length polymorphism (RFLP) testing of the Type I collagen gene, the collagen type most prevalent

TABLE 20.3 **Useful Molecular Monitoring of Hepatic Xenobiotics by Adduct Formation**

Biologic site	Adduct	Half-life
Metabolic	GSH Conjugates	Hours
Protein	Albumin	Days
Cell	Hemoglobin (RBC)	Weeks
Nucleus	DNA	Months/Years (*if no repair*)

in human cirrhosis (Winer *et al.*, 1988), specific agent identification can be made. A RFLP exists when two different but normal nucleotide patterns exist at the same site in the genomic DNA. This difference can be recognized by digestion with a bacterial restriction endonuclease. White blood cell DNA is obtained from exposed individuals, digested with two restriction enzymes, and hydridized with two Type I collagen DNA probes to reveal an RFLP. Six haplotypes or patterns of polymorphisms have been found in alcohol-exposed individuals, based on the presence or absence of these two polymorphisms. One haplotype is found more frequently in cirrhotic alcoholics than in alcoholics without cirrhosis or in controls. If family studies confirm such a linkage, this type of identified polymorphism could provide a means of identifying individuals at risk of developing liver disease (cirrhosis) when exposed to alcohol. The ability to identify various capabilities of metabolism of xenobiotics, as well as the propensity for formation of collagen after injury, can be used as a generic molecular marker for low-dose xenobiotic hepatic exposure or injury.

Activated proto-oncogenes and inactivated tumor suppressor genes Activated transforming genes (oncogenes) have been found in a number of human tumors by use of assays in which transformed foci result from transfection of tumor DNA into NIH3T3 cells. One striking fact that has emerged from screening transfecting DNA is that, for both human and rodent tumor DNA, the transforming genes are virtually all related to the *ras* oncogene family. Activation of *ras* proto-oncogenes by a carcinogenic agent often involves base substitutions at codons 12 and 61. Some examples of activated *ras* oncogenes found in liver tumors are given in a review by Harris (1991): aflatoxin B_1-induced $G^{34} \rightarrow T$ and $G^{35} \rightarrow A$ mutations in Ki-*ras* of rat; benzidine-induced $C^{181} \rightarrow A$ mutation in Ha-*ras* of mouse; and urethane-induced $A^{182} \rightarrow T$ mutation in Ha-*ras* of mouse. In addition, Wogan and coworkers (McMahon *et al.*, 1990) reported the presence of Ki-*ras* oncogenes ($G{\cdot}C \rightarrow A{\cdot}T$ or $G{\cdot}C \rightarrow T{\cdot}A$ in codon 12) in the liver of flounders with hepatocellular carcinomas that were taken from a contaminated site in Boston Harbor. DNA samples from histologically normal liver of flounder from a less polluted site showed only wild-type DNA sequences at codon 12 of Ki-*ras*. Since the *ras* oncogene is involved in early, late, and metastatic stages of carcinogenesis, determination of *ras* mutation in a liver biopsy sample may be used as a biomarker of susceptibility (in the absence of liver impairment) or of effect (in conjunction with liver tumor).

In contrast to proto-oncogenes, tumor suppressor genes are cellular genes that regulate cell growth, induce apoptosis (programmed cell death), and maintain genomic stability. Inactivating the normal allele will cause dysregulation of growth and differentiation pathways, enhancing cell transfor-

mation. In this sense, it is far more likely to disable a gene than to activate a proto-oncogene by point mutation. For example, the *ras* gene is activated by mutation in a few specific codons only. Further, some mutated forms of *p53* are transforming oncogenes. In sum, the *p53* tumor suppressor gene has shown the best association with human liver cancers (Harris, 1991). The majority of the mutations in human tumors occur in exons 5–8 of the *p53* gene, where the hot spots are grouped in the coding region 248–282. A *p53* hot spot mutation in hepatocellular carcinoma has been linked to aflatoxin exposure and hepatitis B virus. Codon 249 mutation of the *p53* gene is strongly associated with high aflatoxin exposure and identifies an endemic form of hepatocellular cancer (Ozturk *et al.*, 1991). Detections of mutations in *ras* and *p53* in small tissue samples are now made possible by polymerase chain reaction (PCR) technology. PCR rapidly is becoming the preeminent area of diagnostic hepatology with respect to environmental hazards, including viruses. Often, exposure to environmental xenobiotics is complicated by latent hepatotoxic agents, especially hepatitis types B, C, and D. Especially relevant is the ability of hepatitis B to become integrated into human DNA and no longer be identifiable by standard immunologic markers. PCR application of specific hepatic viral RNA and DNA species allows detection of such latent confounders, provides more accurate classification of the exposed individual, and has the ability to determine whether any synergistic effect may occur due to the dual hepatotoxin exposure. PCR allows vast amplification of specific DNA species of interest (10^6-fold increases are routine). DNA can be detected from nucleotide samples of less than 10 pg; therefore, the technique is applicable to human liver biopsy samples.

Analytical Techniques

Metabolites in body fluids Oxygenated derivatives of environmental carcinogens, such as benzo[a]pyrenes and aflatoxin in blood and urine, are direct exposure markers. Analytical techniques involving gas chromatography, mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC) with fluorescence detection are sensitive to nanogram levels. However, blood and urinary metabolites of volatile chemicals (e.g., vinyl chloride and acrylonitrile yielding water-soluble thio acids) are not readily quantitated under low-level exposure conditions. Direct analysis of hepatotoxin metabolites is most useful in heavy metal (e.g., iron, arsenic, copper) dose–response study. Although atomic absorption spectroscopy is widely used to determine metals at ppb concentration in biologic specimens, this method does not provide species information because the analyte is determined at the atomic state. To this end, absorptive stripping voltammetry is being developed for metal speciation, for example, speciation of nickel(II)-histidine as a biomarker for nickel exposure (Wu and Wong, 1991).

Effect/Diagnostic

Application of Biomarkers of Hepatic Effects Caused by Xenobiotic Exposure

Hepatic biomarkers of environmental exposure can identify three major outcomes: acute, chronic, and latent. Chronic and latent diseases (e.g., cancer) are complex multistepped processes. Therefore, any single biomarker is likely to identify only one or a few of the various steps. However, two major steps are essential to all chronic and carcinogenic processes: tissue injury and cellular repair or regeneration. In acute or chronic hepatic exposure, only incomplete cellular repair of toxic injury allows identification of the exposure. Without detectable injury, there is no clinically meaningful exposure. In the carcinogenic process, malignant transformation cannot occur without both tissue injury and cellular regeneration (i.e., dead cells or cells unable to replicate cannot become malignant). Therefore, the most useful hepatic biomarkers assess injury or identify cellular replication.

Presently, the most frequently used markers of hepatic injury are enzymatic or biochemical ones (Table 20.1). These markers are relatively nonspecific with respect to etiology, but are the clinical standard for the absence or presence of hepatic injury. Depending on the degree (level) of hepatic injury, these markers have relative diagnostic usefulness in the detection of hepatotoxic exposure (Table 20.4).

Level 1: adaptive response At this level, clinical exposure is followed by metabolic or biologic changes that result in no injury, for example, gamma glutamyl transpeptidase (GGT) enzyme induction after alcohol exposure or P450 induction (via abnormal ABT) after synthetic hydrocarbon exposure. Enzyme induction is a physiologic or structural adaptation. There is no cellular damage or death. All other biochemical liver tests (BLTs) and tests of synthetic function are normal.

Level 2: acute injury, mild This level of clinical exposure causes cellular changes that are nonprogressive, reversible, without disruption of cellular

TABLE 20.4 **Diagnostic Effect: Biomarkers for Various Outcomes**[a]

Adaptive response: Biological change, no injury (P450s, GGT, ABT)
Acute injury: Mild (AST/SGOT, ALT/SGPT, ICG)
Acute injury: Severe (total bilirubin, albumin, PT, transferrin)
Chronic injury (cholyglycine, alkaline phosphatase, procollagen III)
Disease
Nonmalignant: Cirrhosis (albumin, PT, cholyglycine, alkaline phosphatase)
Malignant (α-fetoprotein, lactic dehydrogenase)

[a] Markers in parentheses represent those that are more characteristic of a condition.

function, and without evidence of residual injury, for example, alcohol-induced fatty liver with cellular enzyme leakage (shown by increased alanine aminotransferase (ALT)/aspartate aminotransferase (AST), indocyanine green (ICG) clearance). All other BLTs are normal. At this level, there is structural adaptation without functional impairment or permanent architectural damage, even though some histologic changes are identifiable.

Level 3: acute injury, severe Clinical exposure to this degree causes disruption of cellular function and leaves residual evidence of liver injury, for example, carbon tetrachloride exposure causing cellular necrosis (shown by increased ALT/AST), disrupted function (by elevated bilirubin), and synthesis (by lowered albumin). There are specific histologic changes (by pericentral necrosis) and later fibrosis and scarring (residual injury shown by elevated alkaline phosphatase). True cellular injury has occurred, with repair. Even with repair, there is residual evidence of damage without major architectural changes. Genetic injury may have occurred but is unlikely to be clinically significant or permanent.

Level 4: chronic injury Clinical exposure under these circumstances causes cellular disruption and architectural changes that reduce functional hepatic capacity, for example, vinyl chloride-induced fibrosis (shown by procollagen III, IV) and portal hypertension (evidenced by elevated cholylglycine and alkaline phosphatase with decreased ICG clearance). The clinically significant injury is permanent, often with characteristic structural changes. Genetic injury can occur with risk of cancer development.

Level 5: disease At this stage, clinical exposure has caused permanent structural damage, impaired organ function, and reduced capacity. Genetic injury can cause disruption of cellular control and, with active regeneration, may ultimately lead to malignant transformation, for example, chronic viral hepatitis with cirrhosis (shown by HBV–DNA, HBsAg), primary hepatocellular carcinoma, vinyl chloride fibrosis, peliosis hepatis, and hepatic angiosarcoma. Changes in these molecular and BLT markers correlate with the degree and type of hepatic tissue response to various environmental hepatotoxins (Liss *et al.*, 1985).

Susceptibility

Tests of susceptibility to hepatic xenobiotic injury are governed by the ability of the liver to metabolize and detoxify reactive metabolites. Therefore, markers that identify the oxidative pathway of a xenobiotic or the degree of punitive metabolite detoxification are the best ones to use to assess individual susceptibility. Although some markers (e.g., P450 levels) may indicate in-

creased oxidation and others (e.g., GSH) indicate detoxification, none of the presently available hepatic markers are sufficiently specific or sensitive to identify the metabolic capabilities of an individual. Monoclonal antibodies or adducts with GSH, albumin, or hemoglobin are able to identify exposure or reactive metabolites of specific hepatotoxins. However, tests of future risk must be able to identify specific hepatic changes that will make an adverse outcome more likely. Such hepatic changes include scar formation (fibrosis, i.e., incomplete repair), active regeneration, DNA adduct formation, and oncogene activation. These changes are all related to increased risk of cancer development.

In exposed individuals, for example, identification of *p53* and *ras* gene activity requires concurrent assessment of the putative metabolite (e.g., by adduct occurrence) with the histologic and clinical markers (BLT and serologic) of hepatotoxic injury. Without this form of combined assessment, the differentiation of an exposed individual with subclinical hepatic injury and competent reparative capability from a susceptible individual with genetic injury and high-risk outcomes cannot be accomplished effectively.

The detection of viral confounders (hepatitis B, C, and D), which enhance susceptibility, has improved vastly with the use of PCR. These biomarkers provide proper classification of individuals with chronic liver disease who have exposures to various hepatotoxic chemicals and allow causal differentiation [e.g., Vietnam veterans with viral hepatitis B and dioxin exposure (Tamburro, 1992) and alcoholics with viral hepatitis C (Mendenhall *et al.*, 1991)].

Case Studies

Aflatoxin (Hepatocellular Carcinoma): Natural Environmental Toxin

Human hepatocellular carcinoma (HCC) has been causally associated with chronic active hepatitis (CAH), secondary to hepatitis B virus (HBV) and moldy food grain contaminated by aflatoxins (AF), mycotoxin metabolites of the *Aspergillus* fungus (Harris, 1990). HCC is prevalent in certain regions of Africa and Asia, where HBV carriers and dietary AF, typically AFB_1, are common. AFB_1 has been shown to be a potent carcinogen; its activity depends on the balance of AFB_1 metabolism between oxidation by specific cytochrome P450 phase I isozymes, that produce either the less toxic hydroxylated AFB_1 products or the carcinogenic 2,3-epoxide, and conjugation by glutathione. This balance has been shown (Schrager *et al.*, 1990) to shift with nutritional modulation and chemical intervention, both of which may enhance or diminish liver cancer induced by AFB_1 in rats. The AFB_1 epoxide covalently binds to DNA at the N^7-guanine site, as well as to proteins, for example, via lysine ϵ-amino groups (Figure 20.2). Such chemical reactions

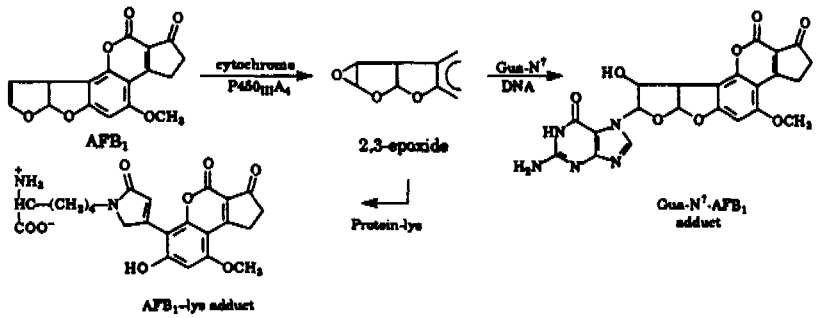

FIGURE 20.2 AFB_1 epoxide covalently bonding to DNA, which might lead to heparocellular transformation.

may lead to transformation of hepatocytes whose clones may expand during the regenerative phase of CAH. CAH behaves as a "viral partial hepatectomy," liberating endogenous proliferative factors.

In addition to environmental monitoring of food contaminants by AFB_1 using TLC and HPLC, noninvasive biologic screening of populations to determine the "internal dose" of AFB_1 in HCC etiology have been carried out. Immunoassays of the major AFB_1–serum albumin adduct, aflatoxin–lysine, have been applied to human populations (Sabbioni *et al.*, 1990). Quantification of this adduct in human serum is achieved by combined immunoaffinity chromatography and HPLC with fluorescence detection. For this method, serum is digested by pronase and the adducts are purified by monoclonal antibody (MAb). The MAb was obtained from a hybridoma of mouse SP-2 myeloma cells with spleen cells of mice immunized with a synthetic antigen of AFB_1 epoxide covalently bound to bovine gamma globulin (Sabbioni *et al.*, 1990). One MAb isolated (2B11) was found to be a high IgM antibody with an affinity constant for AFB_1 and derivatives of about 1×10^9 liter/mol. A significant correlation coefficient of 0.82 was obtained between the aflatoxin–lysine adduct levels and AFB_1 consumption for an epidemiologic study in China. The human data revealed an average aflatoxin–lysine adduct level of 0.38 ng adduct/μg AFB_1 from the diet, or a daily albumin adduct burden of 2.9% of the AFB_1 daily intake.

The MAb 2B11 also showed significantly cross-reactivity for the major aflatoxin–DNA adducts, the N^7-guanosyl, and the corresponding imidazole-ring opened derivative, suggesting that these adducts share a common antigenic determinant. The antibody was applied by Groopman *et al.* (1985) to quantify AFB_1–N^7-G in urine. The MAb first was bound covalently to Sepharose 4B, which made a reusable preparative column for isolating aflatoxin derivatives from human urine. As a measure of MAb sensitivity, a competitive radioimmunoassay (RIA) showed a 50% inhibition value of approxi-

mately 300 fmol for AFB_1. When this methodology was applied to human urine samples, the aflatoxin metabolites detected were AFB_1–N^7-G and the hydroxylated aflatoxins M_1 and P_1 in individuals exposed to AFB_1 through dietary contamination at levels of 10–250 ppb.

Although antibody technology facilitates isolation and detection of urinary metabolites of aflatoxins, some studies of aflatoxin exposure may be subject to criticisms. In a cross-sectional ecological survey in China of possible risk factors for primary liver cancer (PLC; Campbell *et al.*, 1990), multiple regression analyses for various combinations of risk factors were attempted that showed that aflatoxin exposure consistently remained unassociated with PLC mortality. In contrast, HBsAg and plasma cholesterol were associated. This unique comprehensive survey included 48 county sites, approximately 600-fold aflatoxin exposure range, a 39-fold range of PLC mortality rates, a 28-fold range of HBsAg carrier prevalence, and estimation of other life-style features. The aflatoxin exposure was determined from 4-hr urine samples, which were analyzed by isolating oxidative aflatoxin metabolites such as AFM_1 (excluding nucleic acid adducts) on an antiaflatoxin MAb affinity column and quantifying them by a competitive ^3H–base RIA. This analysis procedure, however, was faulted (Wild and Montesano, 1991) for not being representative of aflatoxin intake; the aflatoxin–albumin adduct was suggested as the proper biomarker for determining recent past exposure to AF. The counterargument (Campbell *et al.*, 1990) is the strong correlation between the intake of AFB_1 and the urinary excretion of AFM_1, as well as the correlation between serum aflatoxin–albumin adduct levels and urinary AFM_1. Since the null effect of aflatoxin in this study contrasts sharply with other surveys, the new provocative conclusion makes it imperative to confirm that the aflatoxin exposure measured during the survey period can represent past intakes when PLC was forming.

The larger question is how to relate aflatoxin exposure to oncogene activation in the etiology of liver cancers. Evidence of such a relationship has appeared. McMahon *et al.* (1987) showed that AFB_1–N^7-G adducts were distributed nonrandomly in tumor-derived DNA of aflatoxin-induced HCC in rats. Such liver tumors also were found to contain activated c-Ki-*ras* oncogenes as identified in NIH3T3 mouse transformants. A single G·C to A·T base mutation in codon 12 was found to activate the *ras* gene. In view of an accumulating body of evidence concerning single base mutations in codons 12, 13, or 61 that arise in cellular *ras* genes after administration of chemical carcinogens, this AF activation of a *ras* gene may not serve the purpose of an exposure biomarker for aflatoxins. However, a combination of positive immunoassay of AFB_1–N^7-G in a dose–response manner, with the presence of multiple c-Ki-*ras* oncogene alleles, will make a compelling case for carcinogenesis induced by aflatoxins. Further, studies have elucidated a significant mutation in the *p53* gene during the development of liver tumors. The *p53* nuclear phosphoprotein appears to function as a cell cycle regulatory mole-

cule, controlling cell proliferation. The wild-type *p53* gene is a tumor suppressor gene and has been mapped to chromosome 17p, a region often reduced to homozygosity in common cancers. It is the most frequently altered gene in human cancers (Jones *et al.*, 1991). In analysis for mutations of *p53* in HCC, in patients from China (Hsu *et al.*, 1991) and from Africa (Bressac *et al.*, 1991), 11 of 13 mutations have resulted in an arginine to serine substitution in codon 249 (AGG) of *p53*. Additionally, 12 of 13 point mutations found in these patients were G → T transversions. Aflatoxin–N^7-G is the most likely cause of mutation. The specific mutant *p53* acts as a dominant oncogene and may interact further with a hepatitis B protein to provide a growth advantage in hepatomas. Other types of mutations, including frameshift and deletion, also may enhance clonal expansions. It appears that *p53* mutations in colon cancer, leukemias, and sarcomas are not induced by carcinogen–DNA adducts (Jones *et al.*, 1991); therefore, patterns of base changes in *p53* induced by aflatoxins may be considered footprints of their activities on DNA.

Vinyl Chloride (Angiosarcoma): Synthetic Environmental Toxin

The original association of vinyl chloride (VC) with angiosarcoma of the liver (ASL) in humans was made at a Louisville plastics and synthetic rubber plant in 1973. Since that initial discovery, the University of Louisville and B. F. Goodrich Company have been involved in a 17-year cooperative prospective medical surveillance study involving 600–1200 active and 150–200 retired employees of the Louisville plant. The biologic data include annual historical, physical, radiologic, physiologic, pathologic, and biochemical data obtained on each employee. The environmental data include rank-ordered exposure estimates to 22 toxic chemicals and yearly individual job and area monitoring for specific vinyl monomers.

The prospective human study of VC-associated ASL illustrates the following points. First, the initial discovery of ASL, a very rare liver tumor, was not linked specifically to VC. Polyvinyl chloride (PVC) and acrylonitrile (AN), as well as other chemicals, were also initially suspect. Medical examinations did not identify the causal agent(s) and, in only a few cases, the existence of liver disease. Basic biochemical screening tests identified one or more abnormalities in 30–35% of the work force. Federally required specific liver tests found abnormalities in 10–20% of the work force. Definitive investigation confirmed only 10% of the work force as having persistent or significant liver dysfunction; 0.4% (four) had pre- or malignant disease.

Individual rank-ordered retrospective or prospective work histories for 22 major work-related chemicals (Table 20.5) were used to identify which of these chemicals' cumulative exposure ranked months (CERMs) correlated with liver disease and angiosarcoma (Figure 20.3A,B). Only four chemicals were associated with ASL cases: VC, hexane, dimethyl maleate (DMM), and

TABLE 20.5 Selected Chemicals for Exposure Indices

Chemical code	Chemical name
01	Acrylic acid
02	Acrylamides—acrylamide, methyl, *n*-octyl
03	Acrylonitrile
04	Acetylene
05	Acrylates—ethyl, methyl, methyl-meth, 2-ethyl hexyl, *N*-butyl
06	Bisphenol A
07	Butadiene
08	Caprylyl chloride
09	Chlorinated solvents—carbon tetrachloride, chloroform, trichloroethylene
10	Chloroethyl vinyl ether
11	Diethyl maleate
12	Mercuric chloride
13	Methanol
14	Phenol
15	Toluene
16	Vinyl chloride
17	Vinylidene chloride
18	Vinyl acetate
19	PVC dust
20	Catalysts
21	Styrene
22	Hexane

catalysts. All other plastics-related chemicals and all the synthetic rubber chemicals showed no relationship. The catalyst group was used for VC products only and hexane was the major solvent for the VC catalyst, therefore both were always present when VC was used. DMM was a specific catalyst for a specialized PVC product and was used only periodically. This chemical is used by toxicologists to deplete GSH in animals in order to potentiate the toxicologic effect of the agent under study. Among the ASL cases, individuals with DMM exposure have shorter latency periods (Tamburro *et al.*, 1984).

VC also causes characteristic histologic liver injury (Tamburro, 1984). These histologic characteristics correlate very well with total (CERMs) relative VC exposure job rank, as shown in Figure 20.4. Study of biochemical and metabolic liver markers in detecting chemical injury, using CERMs and liver histology for specific lesions, revealed that ICG clearance provided the best combination of sensitivity and specificity (Figure 20.5A); GGT provided the highest sensitivity but also had the lowest specificity (highest false positivity; Figure 20.5B); and AP had the highest specificity (Figure 20.5C). Finally, individual job and area monitoring of VC and AN were shown to be

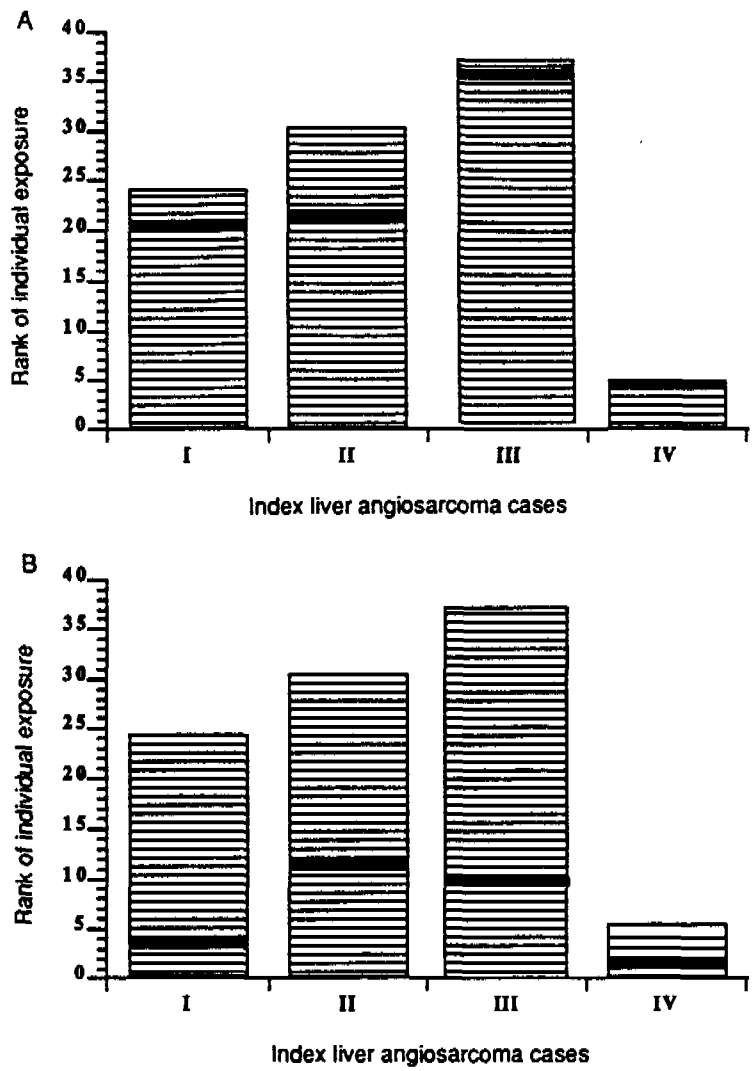

FIGURE 20.3 Relative vinyl chloride (A) and acrylonitrile (B) exposure rankings of index cases of hepatic angiosarcoma relative to their controls (individuals who worked the same years and number of years as index cases). ■, Angiosarcomas; □, matched controls.

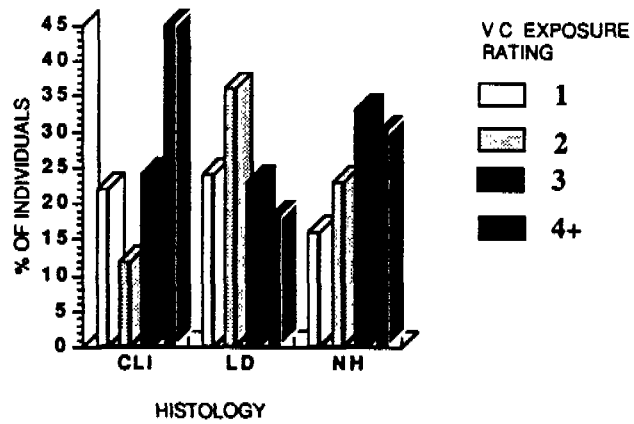

FIGURE 20.4 Correlation of hepatic injury and chemical exposure illustrated by the significantly larger percentage of markers whose liver histology showed evidence of chemical liver injury (CLI) that had vinyl chloride (VC) exposure ranking of 4 or greater. LD, Liver disease, nonchemical; NH, normal histology.

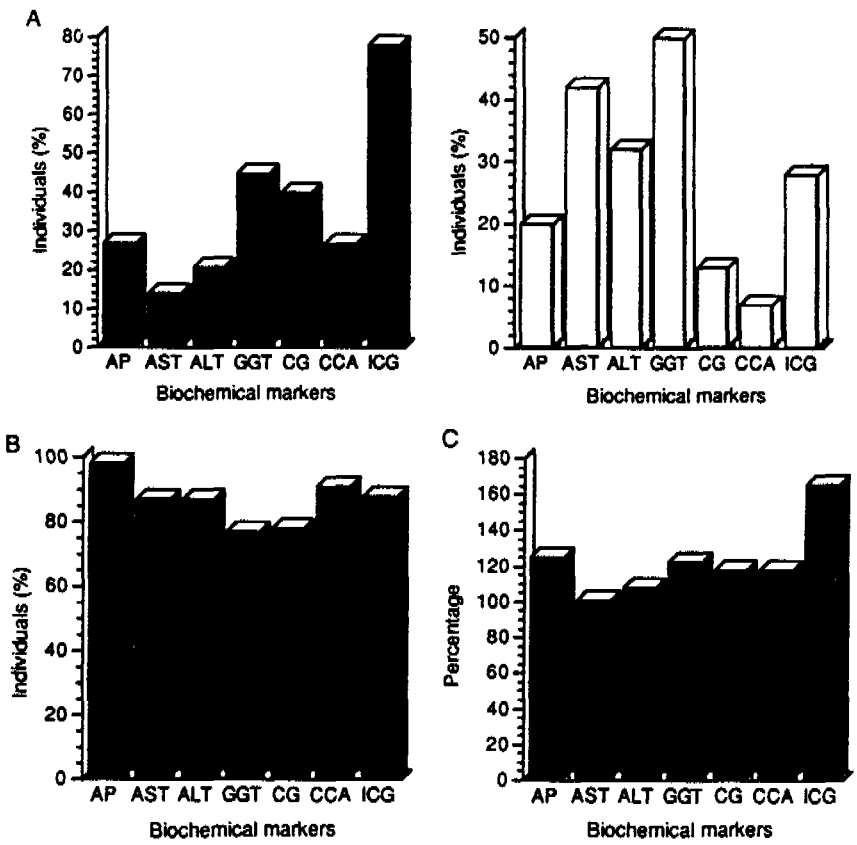

FIGURE 20.5 (A) Sensitivity, (B) specificity, and (C) sum (sensitivity and specificity) for hepatic biochemical biomarkers in chemical (■) and nonchemical (□) liver injury.

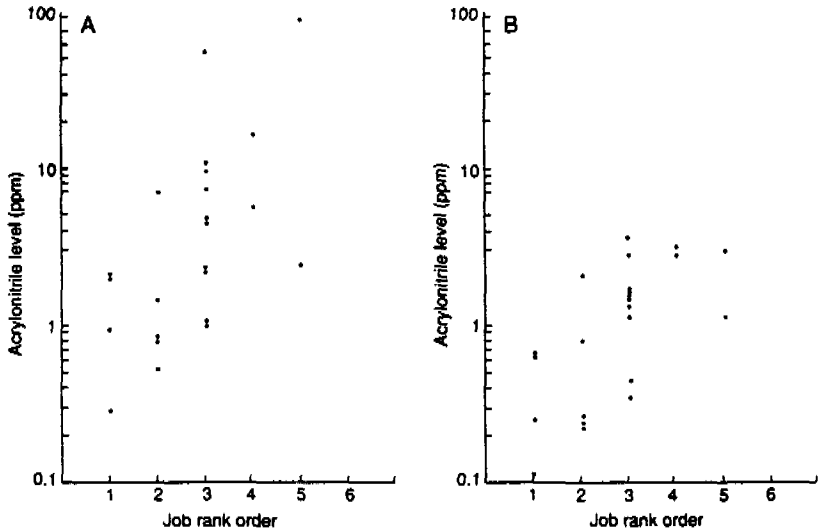

FIGURE 20.6 Correlation of job-specific acrylonitrile environmental exposure with job exposure ranking, at maximum level (A) and at mean/average level (B).

highly correlated to CERMs, to verify the relative exposure estimates of the CERMs, and to provide a ppm value for the CERMs (Figure 20.6).

This study identified and verified the cellular toxicity and carcinogenicity level of VC and established its biologic threshold level for humans. These data now can be used to estimate human risk to past and future exposure accurately (Tamburro, 1984). Finally, similar analysis of the other chemicals, via the relational database system, provided strong evidence that no association or relationship existed between VC exposure and other malignancies in the cohort. A 1982 report summarized the initial multidisciplinary research developments on techniques and methods for the detection and prevention of carcinogenesis in this cohort of industrial workers (Tamburro *et al.*, 1982).

As illustrated in the ASL case, because of the multiple metabolic and synthetic roles of the liver, no single marker, biologic or analytical, is sufficient for molecular epidemiologic purposes. The essential requirements for effective epidemiologic study in the occupational surveillance of hepatic injuries are listed in Table 20.6. Guidelines for detection of hepatotoxicity due to chemical exposure were outlined by Davidson *et al.* (1979).

A comprehensive review of the VC epidemiologic studies (Doll, 1988) confirms that the association between VC and cancer is confined to ASL. However, concern remains regarding individual human variation and prior VC animal exposure studies showing other cancers (Maltoni *et al.*, 1981).

TABLE 20.6 Essential Elements for Prospective Surveillance of Occupational Environments

1. Medical history
2. Physical examination
3. Basic biochemical screening tests
4. Specific biochemical markers of liver (target organ)
5. Medical protocol for evaluation of positive finding
6. Defined investigation (radiologic, physiologic, pathologic) for hepatic evaluation
7. Biologic storage bank (blood, tissue)
8. Individual work history with rank-ordered cumulative exposure to key chemicals in work environment
9. Individual job and area analytical monitoring of key chemicals (agents) in the work environment
10. *Computerized relational database for storage of all data*

Therefore, a dosimetry method based on molecular markers of VC metabolites is needed to evaluate the current regulatory exposure limit of 1 ppm. Potential biomarkers for the metabolites shown in the scheme in Figure 20.7 are under current development (Tamburro *et al.*, 1982; IARC, 1986; Joseph *et al.*, 1990).

Among the DNA adducts detected after exposure of experimental animals to VC, the major product, N^7-(2-oxoethyl)guanine (OEG), is derived from guanine N^7 alkylation by chloroethylene oxide (CEO). The detection limit was 10 pmol OEG/μmol unmodified guanine (Fedtke *et al.*, 1990). Rat tissue DNA was depurinated using mild acid hydrolysis. The hydrolysates

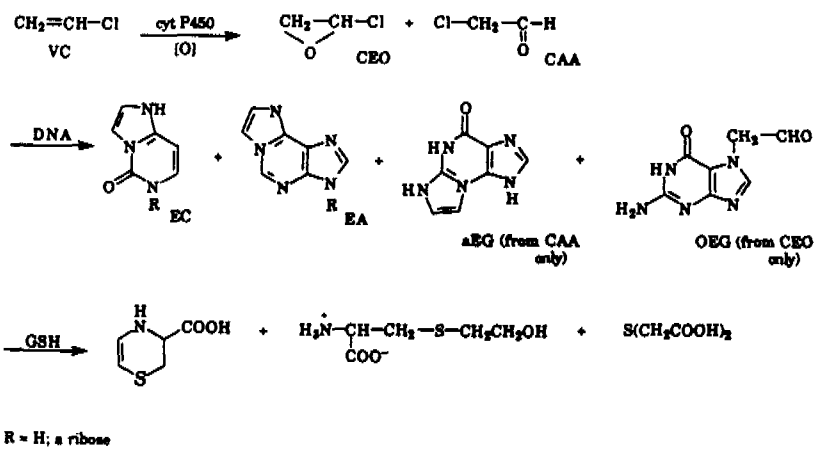

FIGURE 20.7 Potential biomarkers for vinyl chloride metabolites.

d-phase strong cation exchange column
on at 225 nm with a 340-nm emission
to give more reproducible results than
p with tritiated sodium borohydride or
h *O*-methylhydroxylamine for GC–MS

clic etheno derivatives whose formation
LA (E for etheno; G, C, and A are DNA
determined in mild acid DNA hydroly-
phy fraction was electrophore-labeled
The dipentafluorobenzyl derivative was
ndard $^{13}C_4$-EG using GC–MS with nega-
suring the m/z ion ratio of 354/358. The
μmol guanine (Fedtke *et al.*, 1990), an
LC method with fluorescence detection.
G was found to be approximately 1:100
r VC exposure (600 ppm by inhalation,
ratio in the liver increased to 1:14 1 week
at the half-life of OEG was 62 hr, but that
owing a greater persistence of the ethen-
these two major VC adducts is required
n.

nucleosides EC and EA can be achieved
unoassay. The former procedure reported
preceded by separating the adducts as
ersed-phase HPLC. Then the molecules
and the mixture was treated with a nucle-
theno[5′-^{32}P]monophosphates, collected
uid scintillation counting, yielding detec-
7/μg DNA. Monoclonal antibodies that
sine or ethenocytidine at approximately
an alternative detection method (Young
oassay for their presence in exposed rat
al., 1990). The concentrations measured
idine and 0.13 pmol EA/μmol deoxy-
xposed to 500 ppm VC (7 hr per day for
r of magnitude lower than the EG value
described earlier.

NA adducts from animal tissues can be
ajor DNA adduct formed in livers of rats
hereas the etheno derivatives (but not
ats chronically exposed to VC. In addi-
osure to VC-type chemicals, each adduct
genotoxicity. The predominant adduct

OEG, derived from the putative metabolite CEO, has a short half-life and lacks miscoding properties (IARC, 1986). It probably contributes only indirectly to the mutagenic effects of VC via depurination and mispairing opposite the apurinic sites. In contrast, the three minor etheno adducts have been reported to be efficient in causing mispairing during DNA replication (Jacobsen *et al.*, 1989; Singer *et al.*, 1987), although conflicting data point to low miscoding efficiency of EA and EC (Bartsch and Singer, 1985). All three cyclic adducts can be attributed to the other putative metabolite chloroacetaldehyde (CAA), thereby suggesting CAA to be responsible for VC genotoxicity. However, bacterial mutagenesis assays showed CEO to be much more potent than CAA (Perrard, 1985). Also, under comparable conditions when CEO was found to produce skin tumors in mice, CAA produced no increase in benign or malignant tumors (Zajdela *et al.*, 1980). Thus, the detoxification of CEO and CAA must be considered in assessing individual risk to VC exposure.

Strengths and Limitations

Enzymes

Microsomal P450

The induction enzymes, such as cytochrome P450, can be measured directly from liver samples obtained by needle or surgical biopsies (McPherson *et al.*, 1982). Such measurements have limitations based on differences in regional distribution of P450 and other enzymes, and on the different forms of the groups of enzymes, the levels of which may be reduced or induced by the xenobiotics themselves. In addition, these methods are limited by overlap (Table 20.2), variable xenobiotic induction, and background induction caused by high natural diet exposure, air pollutants, and life-style factors (Watkins, 1990). Knowledge of the "usual" background level (steady state) of induction is required also to identify any changes attributable to the suspect xenobiotic(s). Indirect measurement by ABT is the alternative to using tissue for these enzyme determinations.

Aminopyrene Breath Test

ABT itself has several limitations. It cannot distinguish among the various levels of liver disease (Hepner and Vesell, 1975). The overlap between individuals with adaptive or mild liver dysfunction makes the test less useful for those in most need of such evaluation, that is, individuals with subclinical disease. ABT has been found to be more reliable in predicting short-term changes, clinical improvement, and the histologic severity of chemical liver disease (e.g., alcohol related) than the more conventional liver tests. The predictive value of ABT for steatonecrosis, pericentral fibrosis, and cirrhosis (in-

active) is less than the standard predicted value of a BLT. At the moment, there is no evidence that one breath test has anything to offer over another. Such "surrogate" methods (ABT) with high sensitivity are desirable. However, without concurrent high specificity and high disease occurrence, such surrogate markers can be potentially more psychologically or socioeconomically harmful because of high false-positive and false-negative rates.

Glutathione *S*-Transferase

The clinical usefulness of GST as a potential biomarker is uncertain. For example, although glutathione *S*-transferase is involved in catalyzing the detoxification of diol-epoxide carcinogenic metabolites of polycyclic aromatic hydrocarbons (Vander Jagt *et al.*, 1985), these transferases have distinct but overlapping substrate specificities bordering on the complexities of the cytochrome P450s.

Metabolic and Physiologic Tests

ICG and other clearance tests mainly reflect hepatocellular injury or physiologic dysfunction. They provide only indirect evidence of xenobiotic injury. They are effective markers when the agent(s) and its exposure level are known and when other toxic associations can be excluded by epidemiologic or statistical analysis. Their major strength is their selectiveness for the liver.

Proteins

The major limitation of tests of antigen or antibody induced by xenobiotics is their sensitivity and specificity for the chemical agent. Exposure to the chemical agents acting as antigens may not be followed by antibody induction due to inadequate antigen production or structure derangement caused by its hepatic metabolism. Hepatic biomarkers of this type can be enhanced by PCR amplification for better detection.

Adducts

Monoclonal antibodies for specific chemical agents or their metabolites provide the most promising biomarker methods. A major limitation, at present, is that many adduct markers are not hepatically selective. Adducts with GSH, albumin, and hemoglobin reflect highly sensitive methods of identifying hepatic exposure over various periods of time. Monoclonal antibodies to various hepatic metabolites allow identification of different routes of metabolism (albumin and hemoglobin adducts) and the effectiveness of detoxification (GSH adducts). DNA adducts are best used to identify high-risk effects of exposure and provide a potential method of assessing the reparative capability of individual DNA. This methodology can be applied indirectly (circulating tissue: white blood cells) and directly (hepatic tissue: via biopsy).

The data suggesting strong xenobiotic associations or even causations in "group" data are insufficient for use on an individual basis. Such adduct markers still require confirmation of their specificity and sensitivity in individuals whose exposure and hepatic disease has been well characterized. The adduct surrogate (e.g., hemoglobin adduct for a hepatotoxic xenobiotic also must be shown to reflect the target organ (the liver) under surveillance correctly (i.e., selectivity).

Genetic Markers

Polymorphism

Use of gene mutation in the clinical setting of hepatic disease may be limited because (1) gene mutation may be present only in the end stages of the carcinogenic processes, (2) gene mutation may require multiple "hits" before becoming established, and (3) gene mutation may be seen only in liver tissue and not in the more accessible body tissues.

Oncogene Markers

In human cancer, the *ras* oncogene was found in 90% of adenocarcinoma in the pancreas, 50% in the colon, 30% in the lung, 50% in the thyroid, and in 30% of myeloid leukemia (Bos, 1989). The NIH3T3 transfection–transformation assay may not be sensitive enough to select *ras* activation in all the liver tumor DNA. Until a more sensitive assay is used, interpretation of the detection percentages of activated *ras* gene as a biomarker cannot be made with confidence. However, one should note the potential of the *ras* oncogene as a specific disease marker for the causative agent. Increasing evidence suggests that mutational spectra are highly correlated with each chemical carcinogen and reflect the predicted base substitution, that is, G·C → T·A transversion for benzo[a]pyrene, which forms predominantly the N^2-BPDE–deoxyguanosine adduct, and A:T → T:A resulting from N^6-deoxyadenosine bonded to the diol-epoxide of 7,12-dimethylbenzanthracene (Singer and Grunberger, 1983). It is plausible that molecular analysis of mutationally activated *ras* genes (a feat readily achievable with PCR) will reflect promutagenic DNA adduct formation and the mutagenic activities elicited by specific environmental carcinogens. With more complete molecular information, such structure–function correlations between *ras* DNA adducts and *ras* activities may be made, even in the presence of confounding factors such as spontaneous mutations producing G·C → T·A transversions.

The *p53* tumor suppressor gene appears to be more suited as a hepatic biomarker. The *p53* gene is mutated in diverse types of human cancers (Hollstein *et al.*, 1991); germ line mutations in *p53* predispose to cancers of the breast, soft tissues, and brain. Mutant *p53* has been found in hepatocellular carcinoma in connection with aflatoxin and hepatitis B. However, it is not

certain which of these two agents has caused the *p53* mutations in the China and southern Africa studies. Analysis of liver tumors from regions in which either aflatoxin or the hepatitis B virus is the predominant agent will be instructive. At present, mutant *p53* is associated with driving selective clonal growth, a critical step in the neoplastic process. Its detection in liver and other tissues means a risky prognosis.

Clinical Field Application of Biomarkers

Further application of these methods in the early detection of hepatic injury in multiple exposure environments, such as the workplace, is needed. Their application for determining hepatic cancer risk, however, will have strong socioeconomic and ethical impacts. Their field application is vital in showing their ability to: (1) identify high-risk individuals, (2) identify an individual's specific hepatic metabolism for xenobiotics (e.g., degree of oxidation/detoxification, DNA adduct/repair), (3) confirm the safety of work environments containing potential carcinogens (e.g., acrylonitrile, TCDD, PCB), (4) show levels of individual exposure not associated with hepatic functional or structural changes beyond the adaptive response (Level 1), and (5) differentiate the cause of hepatic injury in multiple agent involvement (e.g., acrylonitrile and vinyl chloride).

Due to the multiple and complex functions of the liver, molecular epidemiologic investigations require joint disciplinary research between the basic molecular biologist and the clinical hepatologist. By nature, human investigation must be conducted in well-characterized environments, in a prospective surveillance-type system containing and applying the essential elements set forth in Table 20.6. This control is especially relevant to hepatotoxin exposure and hepatogenetic markers. More than any other hepatic biomarkers, the genetic markers raise serious ethical and social questions. In industrially developed countries, the incidence of hepatic cancer is low, whereas in underdeveloped countries it is high. Having DNA damage or a cancer-susceptibility gene does not necessarily lead to cancer, although it may identify an individual as high risk. Determining whether such individual information outweighs the benefits requires continuous reassessment. In the low HCC-incidence populations, high risk identification is associated with increased anxiety, discrimination, depression, decreased job security, or uninsurability. In high HCC-incidence populations, such information often impairs personal economic growth or opportunity.

Research Needs

In the hepatic organ system, no single molecular marker will provide adequate information about exposure, effect, or susceptibility (risk) nor will it

answer all clinically relevant needs. Research in hepatic molecular markers is needed in four distinct but interdependent areas.

The first need deals with exposure identification of causative agent(s) when liver disease is found in a new occupational or environmental setting. Because of the variety of chemicals customarily present in such situations, it is often neither practical nor feasible to use specific markers such as MAbs to assess exposures. Under these circumstances, screening approaches are needed to identify the environmental chemicals or their hepatic metabolites. Mature analytical techniques for analysis of body fluids, such as GC-MS, are well developed for stable and volatile compounds such as dioxin (TCDD) and polychlorinated biphenyls (PCB). However, gaseous or gas-like compounds such as formaldehyde, methyl chloride, vinyl chloride, acrylonitrile, or butadiene escape easily from the aqueous samples to be so determined. Better techniques are needed for detection of their hepatic metabolites or conjugates in body fluids or tissue. In addition, more technological development is needed for nonvolatile agents or by-products. Intensive research is on-going to develop mass spectrometry for trace analysis of highly polar and nonvolatile compounds of complex mixtures; none, however, has been directed to liver-specific assays. Under development are derivatizations of adducts or adduct hydrolysates to increase volatility followed by GC-MS; liquid chromatography-mass spectrometry (LC-MS); tandem mass spectrometry (TMS or MS-MS) with desorption ionization (DI) via fast atom bombardment (FAB) or laser microprobe (LAM); LC-MS-MS; and so forth. Particularly promising for liver tissue analysis is the TMS technique. Here, the key innovation is the DI technique to produce ions from nonvolatile surfaces for mass analysis. Ions are formed from sputtered molecules after irradiating samples with a high-energy particle beam (FAB) or a focused laser beam (LAM). TMS is a nonchromatographic method for direct-mixture analysis that can yield molecular weight and structural information. A TMS experiment is performed as the name implies: two mass spectrometers (MS-1 and MS-2) are connected together so that MS-1 separates a particular ion Ma^+ (molecular weight information), formed by direct ionization of the sample, and the fragment ions formed by dissociation of Ma^+ are mass-analyzed by MS-2 (structure identification). Thus, TMS performs both the separation and the analysis step with sensitivity of detection reaching to subpicogram levels. The technique has been applied to biomolecules such as vitamin B_{12}, chlorophyll, and bradykinin (Burlingame *et al.*, 1984). Burlingame and co-workers reported assignment of specific residues in human hemoglobin modified by styrene oxide using TMS (Kaur *et al.*, 1989).

In the second area, for the more defined exposures under which routine screening of biological samples is required, MAbs to metabolites of commodity or known high-risk chemicals such as vinyl chloride, acrylonitrile, and the environmental toxin aflatoxin are very much wanted. However, the problem of false positives must be addressed more keenly, since cross-

analysis of a type-I collagen gene and its association with alcoholic cirrhosis in man. *Mol. Asp. Med.* 10, 159–168.

Wong, J. L., Ma, F. F., and Zhang, Y. (1990). Antibodies to acrylonitrile–glutathione conjugate. *Antibody Immunoconj. Radiopharmaceu.* 3, 194.

Wu, T. G., and Wong, J. L. (1991). Adsorptive stripping voltammetric speciation of nickel(II)-histidine in aqueous ammonia. *Anal. Chim. Acta* 246, 301–308.

Young, T. L., and Santella, R. M. (1988). Development of techniques to monitor for exposure to vinyl chloride: Monoclonal antibodies to ethenoadenosine and ethenocytidine. *Carcinogenesis* 9, 589–592.

Zajdela, F., Croisy, A., Barbin, A., Malaveille, C., Tomatis, L., and Bartsch, H. (1980). Carcinogenicity of chloroethylene oxide, an ultimate reactive metabolite of vinyl chloride and bis(chloromethyl)ether after subcutaneous administration and in initiation-promotion experiments in mice. *Cancer Res.* 40, 352–356.