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UNIVERSITY of LOUISVILLE

October 27, 1994

Hasmukh Shaw, M.D.
Chemical Manufacturers Association
2501 M. Street, N.W.
Washington, D.C. 20037

RE: Study of Brain Cancer in Vinyl Chloride Exposed Workers

Dear Dr. Shaw:

As per Mr. Michael Sweeney's request, I am submitting our time and cost estimate for the rapid completion of our study of brain cancer occurrence in vinyl chloride exposed workers. Enclosed you will find a summary of the budgetary assistance needed for us to complete the updating and publication of this brain cancer and vinyl chloride exposure study. The need to update is due in part to the recent reorganization of the B.F. Goodrich Company. Although all the original workers are still physically present in the original B.F. Goodrich Company Bells Lane plant, that site is now split into three different corporate structures: B.F. Goodrich, Geon, and Zeon Kentucky. As I indicated to Mr. Sweeney during their recent visit, we are now reestablishing a working network with all three companies that will provide for scientific information to be made available. This updating, based on part-time effort, was expected to proceed to completion over the next two years and one-half years.

We are prepared to proceed at a more rapid rate to complete the brain study with a 20-year prospective follow-up. This update would also provide a 25-year combined retrospective evaluation of the relationship of vinyl chloride to brain cancer. Each brain cancer case will be paired with all available matched controls and be analyzed by rank ordered analysis (reference Greenburg and Tamburro, JOM 1981). By this approach, each case will become an individual causal study. Each additional case will add further confirmation to the absence or presence of an identifiable relationship between any of 22 or more chemicals studied. In this manner we expect to revalidate our first observation, that no causal relationship exists between vinyl chloride exposure and brain cancer.

The assistance we require, in the form of a grant to the University, would allow us to analyze workers exposed to vinyl chloride both by rank order and environmental levels. The study hypotheses to be confirmed are based on our initial observation. That:

1. Vinyl chloride exposure is not associated, clinically or histologically with brain cancer development, based on 20-years of prospective and 25-years of retrospective study.

2. There is an environmental vinyl chloride level or biological threshold (minimum environmental level) at which no association with any brain disease has been found.

3. The biological threshold for disease development for humans exposed to vinyl chloride is 50ppm (parts per million) or less TWA (time weighted average) exposure for less than 3 years.

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We feel, scientifically, that the complete updated analysis will reconfirm these initial observations.

Our time estimate regarding updating, verifying, analyzing, and preparing the report for submission to a recognized national peer reviewed journal would be completion in 7-9 months. Enclosed is a summary of the work and services required to update the B.F. Goodrich study database in this time frame.

If I can provide any further clarification or information, please do not hesitate to give me a call. I look forward to hearing your response at your earliest convenience.

Sincerely,

Carlo H. Tamburro, M.D., M.P.H.
Professor of Medicine
Director of Liver Research Center & Lab
Professor of Pharmacology & Toxicology
Chief, Division of Occupational Toxicology

CHT:sam
Enclosures

BUDGET FOR CMA

PERSONNEL NAME	TIME ON PROJECT	ROLE ON PROJECT	SUMMARY COST
Carlo H. Tamburro, MD, MPH	10%	Principle Investigator	13,000.
H. Philip Fortwengler, MS	75%	Occupational Data Manager	33,066.
Lark Reynolds, BS	75%	Data Coordinator	15,190.
Mary Heck, MT	25%	Histological Procurement	7,420.
Stephen W. Looney, PhD	20%	Biostatistician	18,130.
SUBTOTAL			86,806.
EMPLOYEE CONSULTATIVE COSTS[1]			2,140.
SUPPLIES, EQUIPMENT[2]			1,900.
TRAVEL[3]			200.
SUBTOTAL			4,240.
TOTAL (DIRECT COSTS)			91,046.
INDIRECT COSTS (25% S&W)			21,702.
GRAND TOTAL			112,748.

1. Consultative cost for past and present key employee needed to work with U of L staff in work and environmental record data collection and verification, also cost for external independent biostatistical and epidemiology review.

2. Includes special computer updated software to increase speed and efficiency of large data set analysis, use of university main frame (computer time), telephone, mailing and support services cost.

3. Costs that will be encountered to check all regional hospitals for tissue verification of tumor diagnosis, especially the 100 not having complete cause of death data.

PROPOSAL AND OUTLINE
LABOR AND SUPPLIES NEEDED
FOR RAPID UPDATING OF
THE 20 YEAR PROSPECTIVE/45 YEAR RETROSPECTIVE
BRAIN CANCER STUDY ON VINYL CHLORIDE EXPOSURE

INFORMATION NEEDED:

1. **Contiguous Work Histories.** Updating of the contiguous industrial work histories will need to be completed and verified on all employees from the original B.F. Goodrich Company and the two offspring companies, Zeon and Geon. Some employees have been continually employed, others are new hires, retired, or have died since the last update. A reasonable estimate is 600-700 employees per year from 1979-1993 need updating or verification.

2. **Job Title Listings by Industry, Plant, Building, and Year.** Review and updating of all past (before 1980) and new job titles (after 1980). New plant construction, renovations, and manufacturing location changes, which have occurred in recent years necessitate this step. In addition, there have been physical plant areas segregated into offspring companies. These job title listings will be used to produce a table of updated exposure ranking for all employees for all of the 1980's and 1990's.

3. **Exposure Rankings.** Exposure ranking updates are required on the cohort as described above. Updated ratings (ranking) will be given to all new job titles and old job titles used in the old and new sections of the plant(s) from 1979-1994. Participation of experienced employees will be needed and used for rating processes.

4. **Chemical History.** Updated documentation/verification of the continuous use of the 22 chemicals originally identified is needed. The area and personal monitoring data, where available, for these chemicals under evaluation will also be updated. This will be used for quantitative confirmation of rank order. The years and the buildings in which any of the 22 chemicals under study were used will also be reconfirmed.

5. **Death Confirmation Data.** Death certificate data is available on 484 vinyl monomer workers. Forty-six have incomplete data or need updating for cause of deaths. Retired, and separated employees data needs to be completed and reverified.

6. **Cancer Records.** The histology, where and when ever available, will be examined for all brain cancer cases. This will be done for verification and confirmation of the histological tissue type. There will also be a follow-up conducted by phone or mail for retired former employees living outside the greater Louisville area if contact information is available.

LABOR EXPENDITURES REQUIRED:

Where needed, employees from B.F. Goodrich, Zeon, and Geon, paid by and working for the U of L staff, will be used to assist in gathering all pertinent work records and documents. The U of L staff will supervise the acquisition of all employee work records and the transfer of data via computer into the occupational interrelational data bank.

RECORDS GENERATED:

1. **Employee-Year Records.** An estimated 600-700 employees per year for 15 years yields approximately 9,750 employee-years of work histories.

2. **Maximum Estimated Cancer Cases.** Brain cancer cases (all types) from 1945-1993 is estimated (based on all Kentucky cancer deaths 200/100,000) at no greater than 5-7/1,000. This estimate includes non-death cancer cases. At this point, we have identified 136 cancer cases (all types) and 10 brain cancer cases in the 484 deaths.

3. **Analysis.** Analysis regarding exposure and cancer by site and tissue type will be analyzed by nonparametic statistics. These methods are used and validated in the publication: Greenburg RA, Tamburro CH, Exposure indices for epidemiological surveillance of carcinogenic agents in an industrial chemical environment. *Journal of Occupational Medicine* 1981; 23(5):353-358.

Exposure Indices for Epidemiological Surveillance of Carcinogenic Agents in an Industrial Chemical Environment

Richard A. Greenberg, Ph.D., M.P.H., and Carlo H. Tamburro, M.D.

A prospective system for establishing chemical exposure indices was developed and implemented for 22 chemicals used at a Louisville chemical plant. Validation of the indices was done statistically using industry-related cancer (liver angiosarcoma) and worker-matched controls. A rank ordered system for exposures was used to identify a relationship between the occurrence of disease and the presence of a suspect chemical used in the industrial environment.

A major difficulty in the epidemiology of occupational carcinogenesis is obtaining accurate exposure data, especially if the data must be procured after cancer develops. While epidemiological investigations of outbreaks of disease, infectious or chronic, are always retrospective, the long latent period between exposure to a causative factor and the occurrence of cancer complicates this problem. Routine continuous recording of exposure to possible carcinogens is an ideal goal. Unfortunately, this is not practical for most chemicals in a modern industrial setting. What is eminently practical, however, is a system utilizing rank ordering of exposures for highly suspect chemicals.

The B. F. Goodrich Louisville Chemical plant developed such a system in 1974 in response to the discovery of cases of hepatic angiosarcoma.[1-5] This system was the basis of the initial reports. It was extensively modified during a prospective medical screening program established by the University of Louisville under contract NO1-CN-55212 with the cancer control program of the National Cancer Institute.[6,7] The authors are unaware of other similar existing data sets. Occupational studies are usually based on group, rather than individual, exposures. An excellent review of the literature is given by Gamble et al.[8] A recent discussion of a computerized system is given by Kerr.[9]

From the University of Louisville, Dept. of Community Health (Dr. Greenberg) and Dept. of Medicine (Dr. Tamburro), P.O. Box 35260, Louisville, KY 40232.

The system for establishing exposure indices on an ongoing basis was developed through employee work histories and rank ordered job exposure categories. All jobs are classified uniquely by both area location and work description by means of area-description (A-D) codes which identify employment occurring in a particular building or area, independent of job; in a particular job, independent of building or area; or by both area and description.

The exposure index combines two components, i.e., work history and job exposure category, by utilizing the A-D code. The chemical exposure rating is an ordered, six-category ranking assigned to each A-D number for each calendar year, as follows:

Rating	Level of Exposure
0	Absent from Environment (on leave, furlough, layoff, etc.)
1	Lowest Exposure (includes exposure up to somewhere near one hour per day)
2	Minimal Exposure to Low Levels (chemical in building — not handled; low vapor pressure and dust level; individual probably works on different floor)
3	Moderate Exposure (works around the chemical, but exposure is minimal; individual is frequently exposed to little spills or leaks and infrequently — less than once per month — to large spills or leaks)
4	Works in Area Subject to High Occupational Exposures (normally exposure is minimal, but large spills or leaks occur once per month or more)
5	Works in Areas Where Level is High (exposure levels in area are frequently high; might consider that some risk is involved if the chemical is very toxic)
6	Intimate Contact — Skin or High Inhalation (includes individuals with daily and direct contact with the chemicals, such as poly cleaner in the old days and those who handled slurry)

Reprinted from Journal of Occupational Medicine
May, 1981, Volume 23, No. 5
pp. 353-358

Table 1. — Detailed Work and Exposure History.

	1A Work History			1B Exposure Rank for Each Chemical				
Year	A Building	D Job	No. Months	Vinyl Chloride	2*	3*	.....	22*
1944	000	576	6	2	1	1	.....	4
1945	000	576	5	2	1	1	.....	4
1945	111	194	7	5	1	1	.....	1
1946	111	194	12	6	4	1	.....	1
1947	111	194	12	6	4	3	.....	1
1948	111	194	8	5	4	3	.....	1
1948	121	192	4	4	3	1	.....	1
1949	121	192	4	4	3	1	.....	1
1949	112	253	8	2	6	3	.....	2
1949	112	253	8					
1949	112	253	8					
1949	112	253	8					
1957	121	235	12	4	3	1	.....	1
1957	121	235	12					
1957	121	235	12					
1957	121	235	12					
1972	117	573	5	1	2	1	.....	1
1972	000	574	7	2	1	1	.....	4
1973	000	574	12	2	1	1	.....	4
1974	000	574	8	2	1	1	.....	4
1974	Terminated							

*Other chemical

Twenty-two chemicals from two distinct manufacturing processes, one related to synthetic rubber and the other to plastics, were selected for rating. The ratings were assigned by panels of chemical exposure judges. The full six-point scale was not utilized for all chemicals. Although it would have been ideal to have a quantitative, continuous scale of actual exposure to parts per million, this was not available for all B. F. Goodrich employees for all chemicals (nor is it likely to be available in any other industry). In order to overcome the subjectivity inherent in this exposure rating system, knowledgeable people from each area of the plant were selected to serve as that area's chemcal exposure judges. The primary criterion for their selection was experience in the given area. As a rule, production foremen and technical people (i.e., chemical engineers) were in the majority among each area's panel of judges. The area production foremen were especially invaluable as they are experienced in and knowledgeable of all jobs performed in their areas. Most valuable, however, were those individuals who had been at the plant since its inception, or shortly thereafter. The chemical exposure judges met as a group and agreement was by consensus.

Table 1A gives the work history for a hypothetical individual. Note that each job is identified for each year indicating both the A-D number and the number of months worked during the year. This individual, therefore, began work in 1944 and worked for six months on A-D number 000576. He worked an additional five months on this number during 1945. He completed the last seven months in 1945 working at A-D number 111194. Table 1B identifies the exposure rating assigned to each of the 22 monitored chemicals for each A-D number for each year. For instance, the first row indicates the exposure ratings assigned in 1944 to A-D number 000576. In 1945 these remained unchanged. The third row indicates the exposure ratings for A-D number 111194 during 1945. The information in Tables 1A and 1B is combined to give cumulative exposure rank months (CERM) and average exposure ranks (AER). In this example, this individual in 1944 would have had 12 CERM for vinyl chloride in 1944, having worked six months at an exposure rank of 2 during the year. During 1945, this individual would have had CERM to vinyl chloride of (5 x 2) plus (7 x 5) or 45. He would have worked a total of 12 months and would have had an AER (45 ÷ 12) equal to 3.75. The assumption is made that the CERM can be used as a rank order statistic. The assumption is empirically validated later in this paper. The CERM cannot be considered as representing interval data and no such use of it is intended. The results given here are based on revised work histories and A-D codes developed during the NCI contract period. These same codes are now applied to the individual monitoring of all employees.

The data are accurately obtained in a prospective manner. Work histories are obtained on an ongoing basis. Each time an employee changes jobs, the change is identified by a payroll change card indicating the last A-D number, the new A-D number and the date of change. In addition, the rating of exposures by A-D number is now done on an annual basis using auxiliary information available about the A-D number including individual and area monitoring pertinent to the particular A-D number. Thus far this is available for only three chemicals and only since 1974. The rating of exposures is now obtained from chemical exposure judges consisting of representatives of both labor and management. This process takes about two weeks annually.

In order to determine whether such a system could work retrospectively, work histories were abstracted by A-D numbers from payroll records for all employees who worked on or after January 1, 1974, from records dating back to the opening of the plant in 1942. Payroll records

Table 2. — Standard Normal Deviates Comparing Exposure Ranks of Angiosarcoma Cases with the Average Exposure of Matched* Controls (by Selected Chemicals).

Chemical	Group 1 (23 Controls)	2 (29 Controls)	3 (36 Controls)	4 (4 Controls)
1	−1.43	2.32	−0.66	−2.20
2	−2.13	−0.93	−2.00	−0.53
3	−3.54	−2.16	−3.16	−1.50
4	−3.31	−1.79	−0.06	−1.76
5	3.97	0.84	5.08	1.11
6	−2.01	−2.58	−1.59	—
7	−2.33	−2.59	−1.93	−1.50
8	0.29	−0.47	1.56	—
9	−3.92	−0.27	6.44	−2.28
10	−0.76	3.64	0.17	−0.71
11	11.27	7.59	0.93	5.08
12	−1.69	−1.35	0.45	−1.00
13	−1.78	−1.41	5.95	0.00
14	−2.41	−2.91	−2.35	−0.85
15	−1.87	−1.63	0.40	−1.21
16	5.13	3.12	5.77	2.55
17	−0.77	−3.43	5.53	−1.50
18	1.55	−1.45	5.68	1.07
10	2.56	−4.36	4.64	−0.83
20	9.11	2.65	3.72	2.60
21	−3.87	−2.39	−2.91	−1.31
22	6.55	2.34	2.36	2.85

*Matched by age, sex, race, year of employment and survival as a B. F. Goodrich employee to January 1, 1974

did not have the A-D numbers that were subsequently developed; these older records referred to many jobs which no longer existed, and to some which were performed in buildings long since torn down. However, a staff of knowledgeable employees matched the jobs listed on these payroll records with current A-D numbers. Final determination for controversial work records was made, wherever possible, by an individual employee's review. The chemical exposure judges, who were assigning ordered rating exposures to A-D numbers for each year, faced these same problems. They used, in addition to their memory, whatever records of chemical processes and procedures that were available. (The company maintains a file on all products ever produced and all processes ever used at the plant.)

This system of determining work exposure data was *validated empirically* in the following manner: The incidence of hepatic angiosarcoma at the B. F. Goodrich plant since January 1, 1974, presented in four subjects. Each of the four subjects was matched by exact year of birth, by sex and race (white or non-white), by exact year of employment, and by continuing employment at the B. F. Goodrich plant through January 1, 1974. All matches are included in the subsequent analysis. Each of the angiosarcoma subjects and the corresponding matched group were compared on CERM separately for each calendar year. The results were then ranked within each calendar year and the ranks were summed over the calendar years. If the work histories and the ordered rating exposures were no better than random assignments, one would expect a uniform distribution of ranks within each year and independence from year to year. Otherwise, rank order exposures should demonstrate higher exposure to vinyl chloride in those individuals with hepatic angiosarcoma.

Table 2, which gives standard normal deviates, was calculated from the observed sum of ranks, the expected sum of ranks, and the theoretical standard error (conditional on the observed pattern of tied ranks) of the expected sum of ranks. Sums were over years. The results show a very clear pattern of high exposure to chemical number 16, vinyl chloride. This is to be expected a priori if the work history data and the exposure rank data are valid. A second finding of importance is that all four angio-

Table 3. — Comparison of Frequency of Observed and Expected Excess Exposure to Selected Chemicals.

Number of Angiosarcomas Showing Excess Exposure	Expected* Proportion	Observed Frequency	Expected Frequency	Contribution to Chi Square
0	1/16	6	1.25	18.05
1	4/16	6	5.00	0.20
2	6/16	2	7.50	4.03
3	4/16	1	5.00	3.20
4	1/16	5	1.25	11.25
Total		20	20.00	$36.73 = X^2$, $p < 0.001$

*Calculated from the binomial distribution with $n=4$ and $p=1/2$

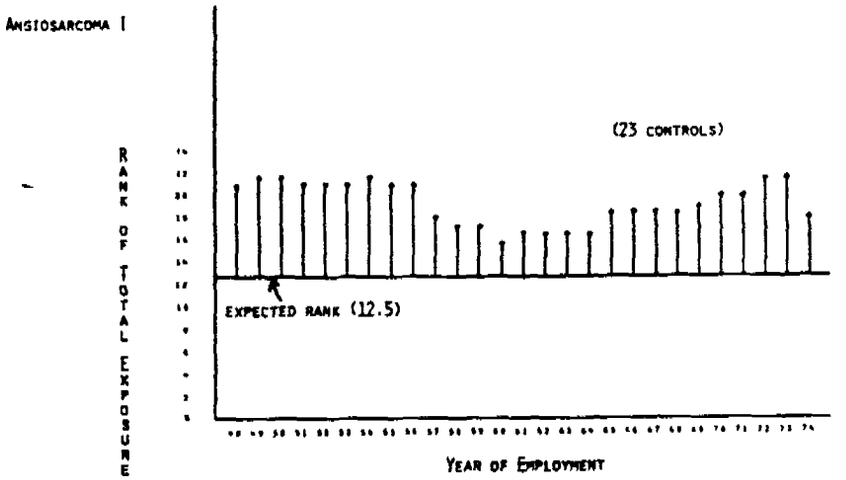

Fig. 1 — Observed and expected exposure rank to vinyl chloride for each full year of employment matched by age, sex, race, year of employment and survival as a B. F. Goodrich employee to January 1, 1974.

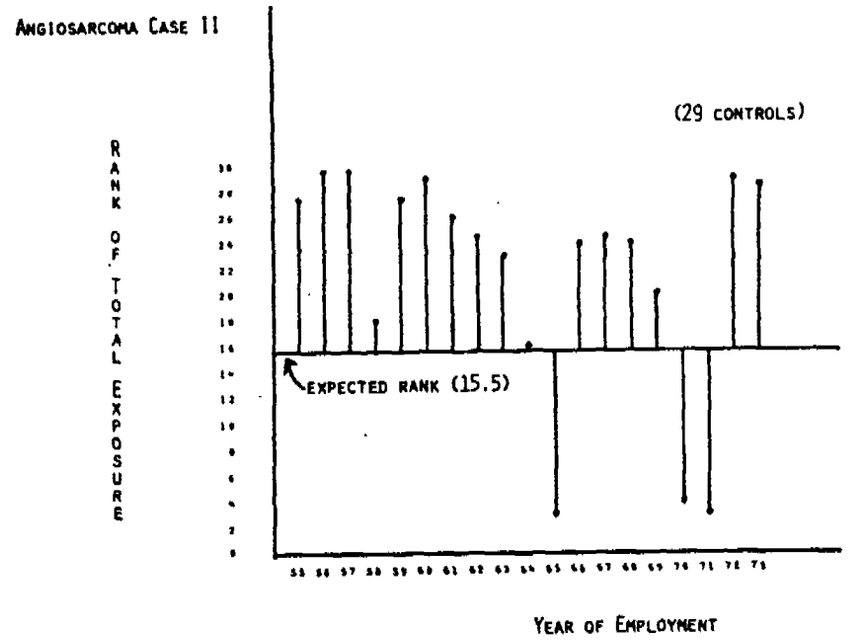

Fig 2. — Observed and expected exposure rank to vinyl chloride for each full year of employment matched by age, sex, race, year of employment and survival as a B. F. Goodrich employee to January 1, 1974.

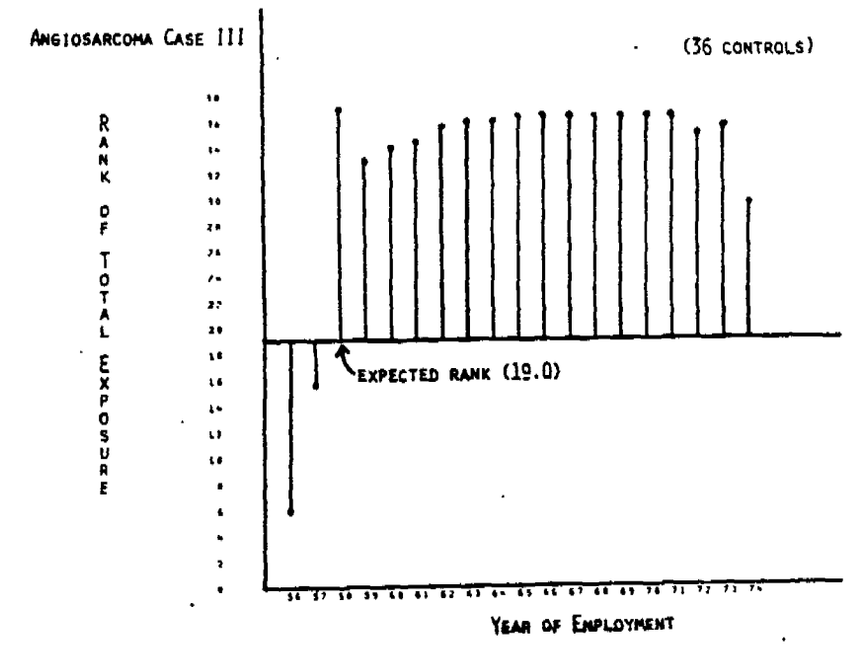

Fig 3. — Observed and expected exposure rank to vinyl chloride for each full year of employment matched by age, sex, race, year of employment and survival as a B. F. Goodrich employee to January 1, 1974.

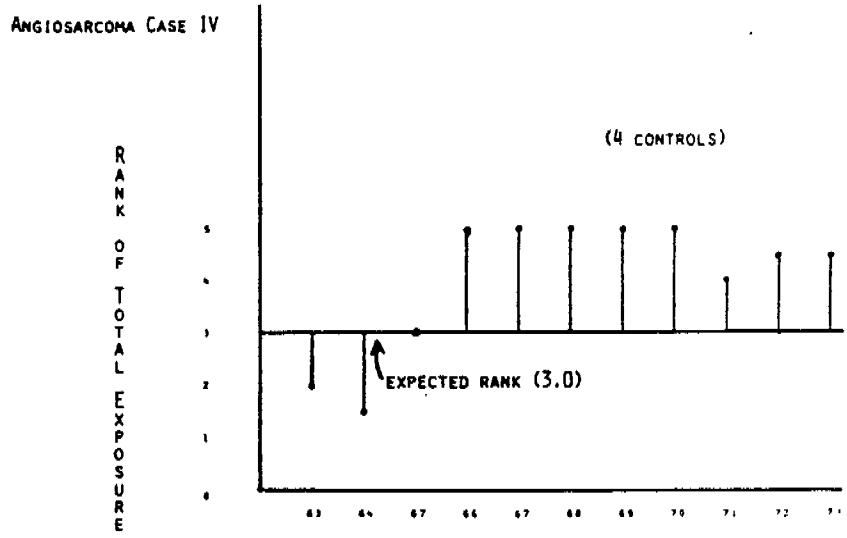

Fig 4. — Observed and expected exposure rank to vinyl chloride for each full year of employment matched by age, sex, race, year of employment and survival as a B. F. Goodrich employee to January 1, 1974.

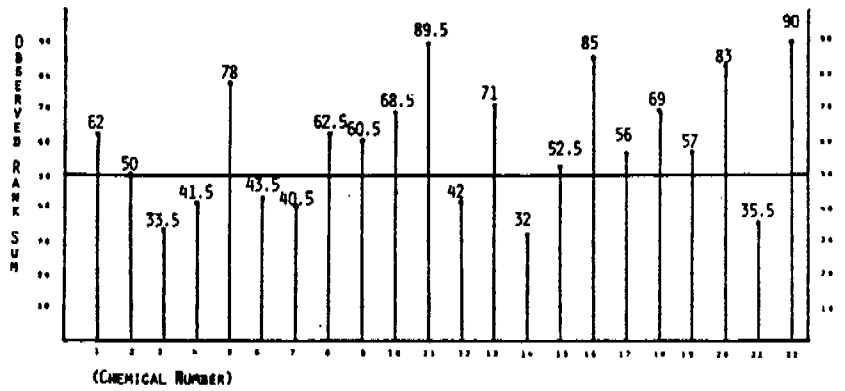

Fig 5. — Observed and expected cumulative exposure rank sums for four angiosarcoma cases compared with matched sets of controls. (Matched by age, sex, race, year of employment and survival as a B. F. Goodrich Chemical Company employee to January 1, 1974. The numbers of controls for the four angiosarcoma patients were 4, 23, 29 and 36. The maximum possible rank sum is 96 and the minimum is 4. Expected rank sum is 50. A rank sum of 80 or more would occur by chance 2.6% of the time. A rank sum of 20 or less would occur 2.6% of the time.)

sarcoma subjects also showed significantly high exposures to chemicals number 20 and 22. Chemical number 20 is a group of catalysts used only with vinyl chloride and chemical number 22 is hexane, used as a solvent for the group of vinyl chloride catalysts. In addition, the data also strongly suggest a high exposure to chemical number 11, diethyl maleate, also a specialized catalyst used only for a specialized process with vinyl chloride.

A clear pattern of exposure is apparent in Table 2. All four angiosarcoma subjects showed excess exposure to 5 of 20 chemicals (numbers 5, 11, 16, 20, 22). By chance, this would be expected to occur for only 1.25 chemicals. All four angiosarcoma subjects also had less than expected exposure to six additional chemicals (numbers 2, 3, 4, 7, 14, 21). The expectation again is only 1.25. When the binomial distribution is inspected (i.e., the distribution of the number of successes in four trials with the probability of success at each trial being one-half), the results are statistically significant ($\chi_4^2 = 36.7$: $p < 0.001$). These are summarized in Table 3. Once more the null hypothesis of random assignment of exposures is rejected. For chemicals number 6 and 8, the exposure was tied for all members of group 4 and they were not included in the analysis. Figs 1 to 4 show the observed to the expected rank exposure for each employee with angiosarcoma by each full year of exposure. The pattern is one of consistent high exposure as compared to the matched controls. It is evident that the system does reflect the autocorrelation in jobs over time as well as the exposure to vinyl chloride among the subjects with angiosarcoma.

To remove the assumption of independence from year to year (as would be required in new field studies), the CERM were summed over years for each angiosarcoma subject and the associated controls. These then provided a single ranking for each matched group. The observed independent ranks of the four angiosarcoma subjects were then summed and compared to the expected rank sums in 5. The exact distribution of the rank sums was obtained by direct enumeration. The angiosarcoma subjects again show significantly high exposure to chemicals number 16 (vinyl chloride), number 20 (catalysts), number 22 (hexane used as a catalyst solvent), and number 11 (diethyl maleate). Fig 6 displays the vinyl chloride rank of the angiosarcoma subject in each matched group; this is the chemical through which empirical validation of the procedure is achieved.

Prior knowledge of the etiology of angiosarcoma was used by the authors to validate these exposure indices. It is questionable what the situation would have been if such prior knowledge had not existed. This study would have shown that employees who subsequently developed angiosarcoma had had high exposure as compared to

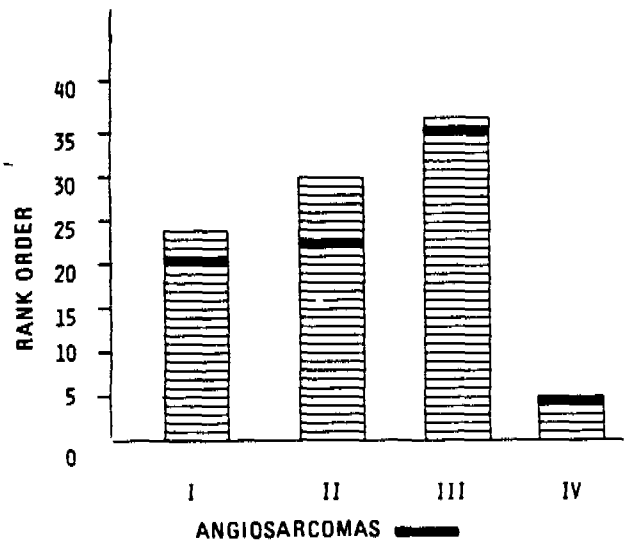

Fig 6. — Vinyl chloride exposure rank of matched individuals with angiosarcoma. (Matched by year of birth, sex, race, year of employment and survival as a B. F. Goodrich Chemical Company employee to January 1, 1974.)

matched controls for a set of four highly correlated chemicals selected from a larger set of 22 studied. These 22 had been selected because of potential toxicity or carcinogenicity by the following criteria: known hepatotoxin, suspected carcinogen, degree of toxicity, degree of contact, and location relative to vinyl monomer polymerization areas. The chemicals would certainly be suspect, but because of correlated exposures, a determination of an exact cause-effect relationship would not be possible. It is certain that further studies would be initiated to interpret the observed events.

The total cost incurred in setting up this system retrospectively (i.e., the cost of going back into records covering plant operation from the beginning up to the present), including the standardization of 321 job classifications ($450), the application of A-D codes to work histories for 1400 employees ($4,760), the assignment of exposure rating to each job classification for each of 35 years for each of 22 chemicals ($880), the extraction of medical history data ($6,000), and computerization ($7,920) was $20,010, or $16 to $17 per employee. The prospective cost, i.e., the cost of work that is recorded regularly forward in time (not including work and exposure history from past times) averaged $3,281 per year, or $2 to $3 per employee, with a breakdown as follows: job classification (1 to 3 new jobs per year), $60; work history (240 new employees per year), $136; exposure indices (20 new chemicals per year), $160; medical data $1,425; and computer costs $1,500. Costs were determined on the basis of actual man-hours worked using industrial hourly wages of the individuals who performed the work.

These data demonstrate empirically that a system of rank ordered individual exposure indices (CERM) for highly suspect chemicals can be implemented in an industrial environment and can identify a known causative relationship between exposure and the development of disease. This system can provide a means of monitoring industrial environments of any size at minimum costs. It can also allow for the introduction of sophisticated monitoring methods and prospective surveillance of an environment, taking into account co-factors which influence biological outcome.

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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

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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-

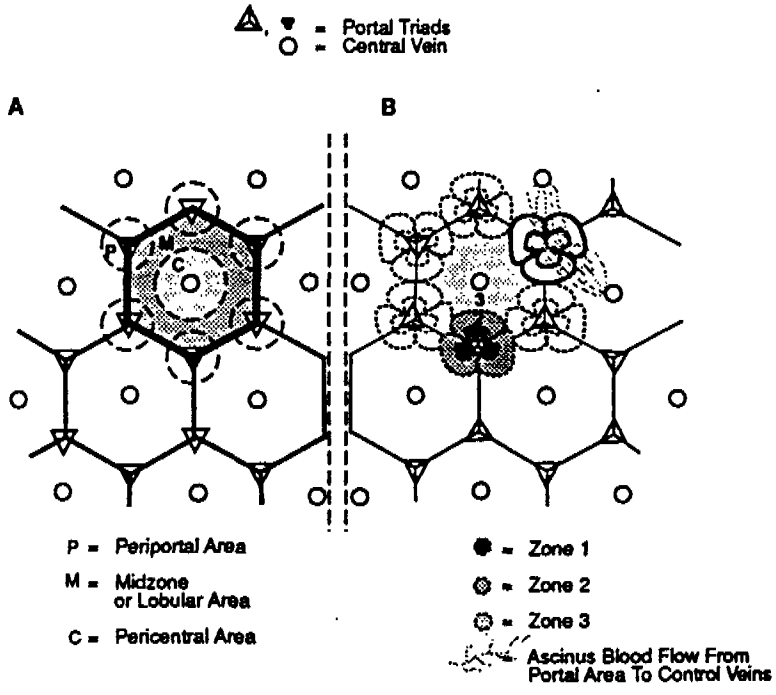


FIGURE 20.1 Normal architecture of the liver illustrating the structural concept of hepatic lobule (A) and the functional concept of hepatic ascinus (B).

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).

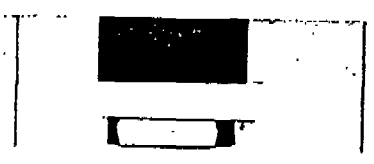
terials by its phagocytic activity via the reticular endothelial system (RES), which also encompasses parts of the immune system. The metabolic role of the liver makes it vulnerable to toxic injury from exposure to a variety of metabolic and xenobiotic insults. Hepatic biomarkers can identify this injury 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·C → A·T or G·C → T·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

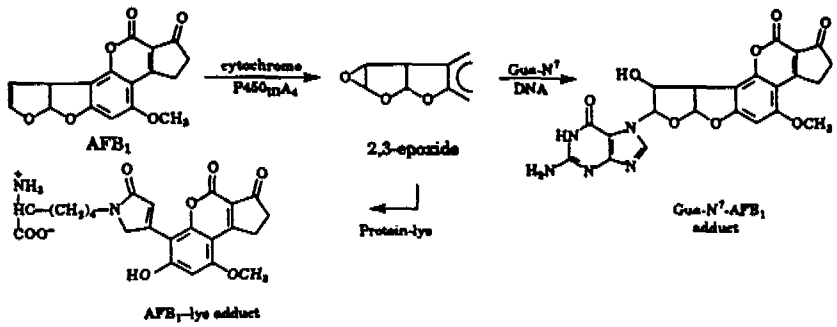


FIGURE 20.2 AFB_1 epoxide covalently bonding to DNA, which might lead to hepatocellular transformation.

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

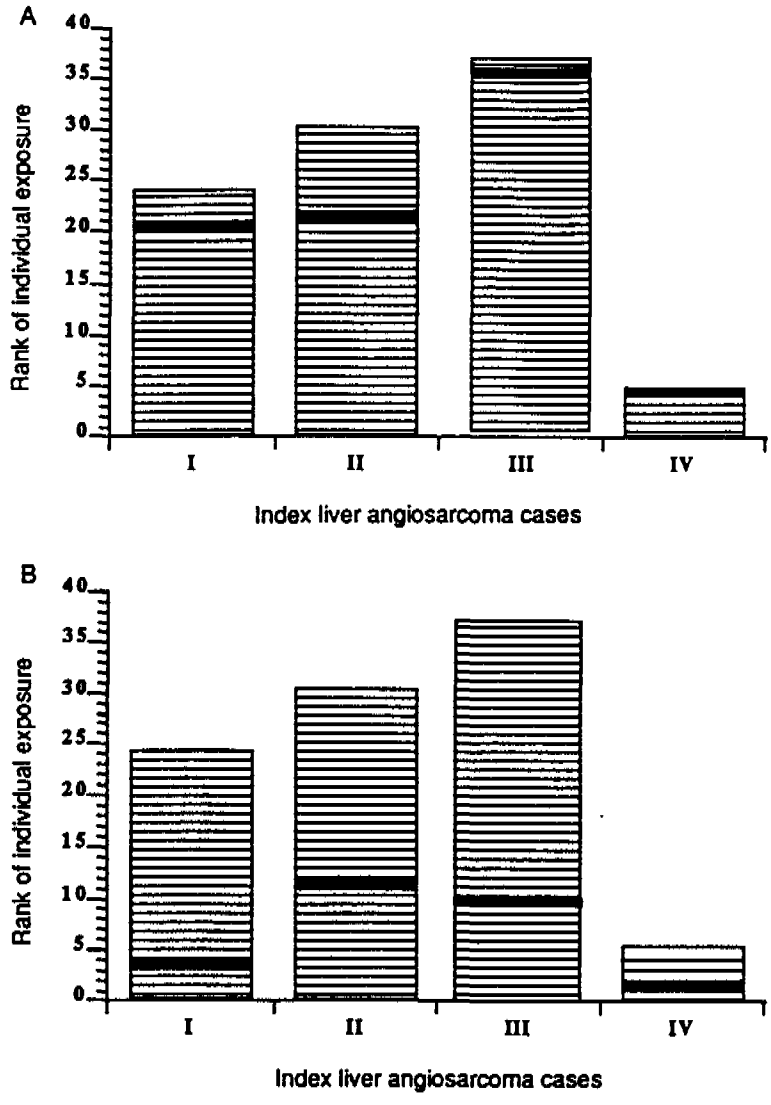


FIGURE 20.3 Relative vinyl chloride (A) and acrylonitrile (B) exposure rankings of index cases of hepatic angiosarcoma relative to their controls (individuals who worked the same years and number of years as index cases). ▬, Angiosarcomas; ▭, matched controls.

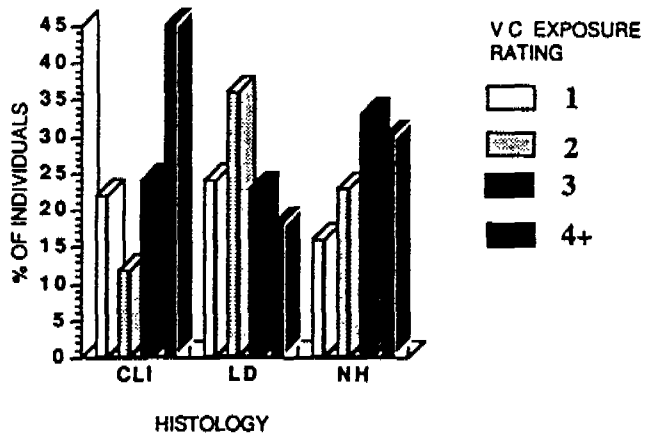


FIGURE 20.4 Correlation of hepatic injury and chemical exposure illustrated by the significantly larger percentage of markers whose liver histology showed evidence of chemical liver injury (CLI) that had vinyl chloride (VC) exposure ranking of 4 or greater. LD, Liver disease, nonchemical; NH, normal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

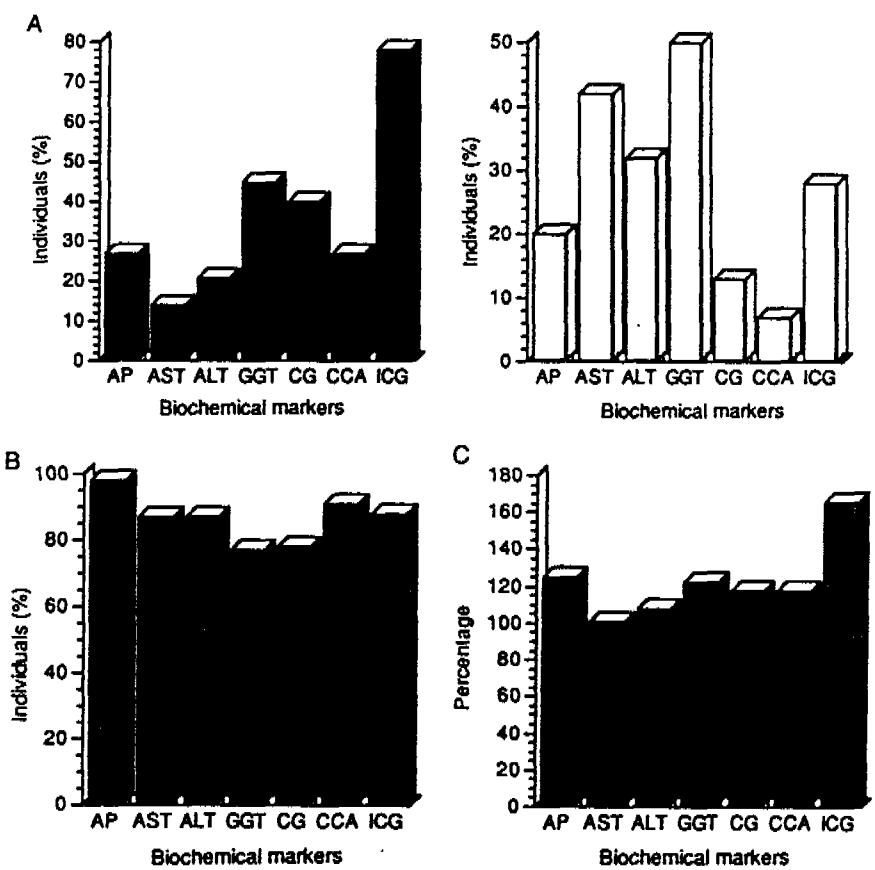


FIGURE 20.5 (A) Sensitivity, (B) specificity, and (C) sum (sensitivity and specificity) for hepatic biochemical biomarkers in chemical (■) and nonchemical (□) liver injury.

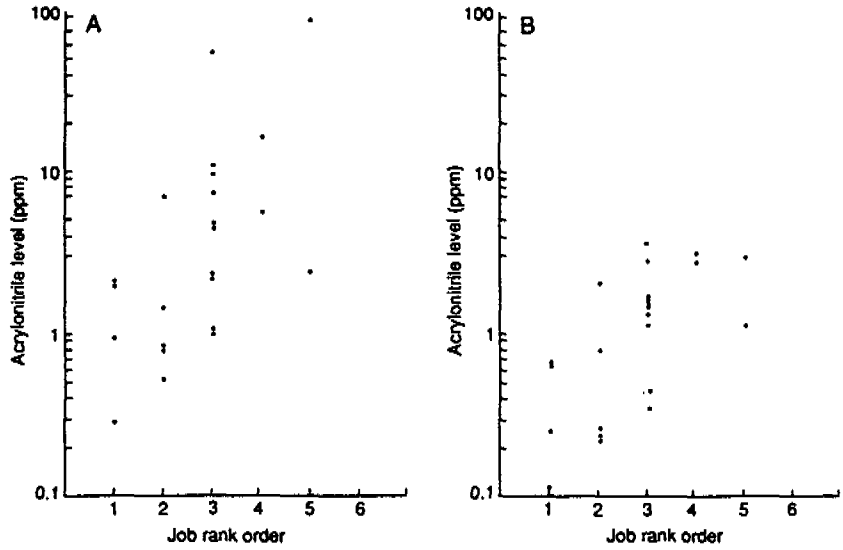


FIGURE 20.6 Correlation of job-specific acrylonitrile environmental exposure with job exposure ranking, at maximum level (A) and at mean/average level (B).

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).

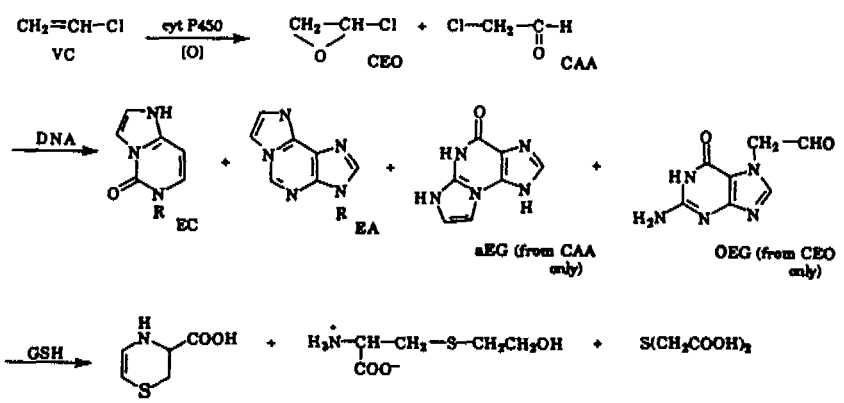
A comprehensive review of the VC epidemiologic studies (Doll, 1988) confirms that the association between VC and cancer is confined to ASL. However, concern remains regarding individual human variation and prior VC animal exposure studies showing other cancers (Maltoni *et al.*, 1981).

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



R = H; a ribose

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
EA (E for etheno; G, C, and A are DNA
determined in mild acid DNA hydroly-
iphy 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-
C/μg DNA. Monoclonal antibodies that
ine or ethenocytidine at approximately
an alternative detection method (Young
noassay 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
whereas 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-

reactivity of MAbs that is not identified during laboratory development may become significant in its field applications.

To date, liver histology (biopsy) remains the standard of environmental liver injury. However, specific hepatic tissue biomarker assays are needed for hepatocytes, biliary ductal cells, reticuloendothelial cells, and macrophages to help identify organ tissue target sites. Along this line, liver specific tissue antigen biomarkers could help identify specific liver function impairment (effect) without the need for liver tissue. Further, hepatic biomarkers with high specificity are more important socioeconomically than those with high sensitivity. These markers are needed to avoid the high false-positive findings that often outweigh the true positive benefits.

Especially needed for chronic exposure are quantitative markers of hepatic reserve, both anatomical and functional. Special attention needs to be directed to methods that can quantitate hepatic collagen content, decreased protein substrate, or synthesis. Quantitative biomarkers of hepatic collagen (effect of injury) or substrate content or synthesis (effect of function impairment) would have very high clinical diagnostic value.

Susceptibility research, the third area of hepatic biomarkers of risk, should be directed at two major categories. Methods that will identify putative metabolic pathways of exposure in individuals (individual susceptibility), for example, MAbs and MS, are needed. Another area of susceptibility involves tests to detect gene injury. In liver tissue, susceptibility of groups or populations will depend greatly on the history of exposure (dose, duration, and recentness) and the evidence of hepatic injury. Tests directed at gene injury or activation, for example, analysis of *p53* and the *ras* gene using PCR and RLFP techniques, offer promise for hepatic biomarker development. This development should start with verification of the effect of each gene presently implicated in hepatic tumors (e.g., hepatocellular carcinoma and HBV-aflatoxin or angiosarcoma and vinyl chloride). Retrospective and prospective applications should be in a defined population, for example, first-generation Asian immigrants to the United States and vinyl monomer workers. Both are valid populations in which to apply the outcomes of susceptibility research to demonstrate its benefits or limitations.

Finally, the fourth area, the most time consuming and the most vital, is research in the further development and maintenance of well-characterized prospective surveillance programs of at-risk populations. These studies are absolutely essential to validate the application and interpretation of these evolving techniques. Long-term cooperative nonadversarial efforts between industry, its workforces, and the scientific community already have shown that such programs can be financially, ethically, and scientifically feasible. These programs need to be enhanced by incorporating the standardization of hepatic nomenclature and criteria for hepatic function, injury, and disease (Leevy *et al.*, 1976) into environmental surveillance. Issues of background marker frequency, determination of relative risk assessment, and disease fre-

quency (M, P, and R, respectively) in the workplace and in noncommercial exposed environments cannot be addressed properly without such population or group studies.

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Specific Antisera to Unique Antigenic Determinants in Human Hemoglobin-Acrylonitrile Adducts[1]

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Key Words: antisera, immunoassay, hemoglobin, acrylonitrile, adducts

Running Title: polyclonal antibodies to assay human hemoglobin-acrylonitrile adducts as biomarkers

Footnotes:

[1] Supported in part by a grant from the National Institute of Environmental Health Sciences ES 05353 and by a postdoctoral fellowship from Graduate Programs and Research of the Univ. of Louisville.

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ABSTRACT

Specific immunogenic hemoglobin adducts were used to develop an immunoassay for dosimetry of exposure to potential human carcinogens. Pathological human hemoglobin (Hb) conjugates of acrylonitrile (AN) were isolated as Hb-AN1 and Hb-AN2. Structural modification of Hb was probed by comparing the kinetics of S-alkylation of blood thiols by the hydrophobic AN and hydrophilic chloroacetaldehyde hydrate, a vinyl chloride metabolite. This and ^{14}C counting for up to 3 AN per Hb and gel analysis of the adducts appear to be compatible with increasing levels of cyanoethylation in the distal heme pocket region to yield two antigenic adducts. The antisera raised in Balb/c mice to Hb-AN1 and Hb-AN2, which should recognize various Hb epitopes, showed in ELISA a binding specificity for the adducts 2.7 to 3.3 times over Hb before immunopurification. After the antisera were passed through a Hb-Sepharose 4B column to retain antibodies to normal Hb, the polyclonal antibody filtrate showed 16.5 times specificity towards Hb-AN in blood with a sensitivity of 5 ng. The lack of cross-reactivity with confounders such as Hb-chloroacetaldehyde and Hb-glycolaldehyde was demonstrated. Immunoassay of human blood exposed to AN in vitro gave a dose-response, illustrating the clinical applicability of specific immunoassay for the detection of hemoglobin adducts. To our knowledge, this represents the first example of an unique approach to the development of immunoassays of Hb adducts as biomarkers of human exposure to chemical carcinogens.

INTRODUCTION

Acrylonitrile (AN) represents an example of an important industrial chemical of high U.S. production [2.65 billion lb in 1991 (1)] whose carcinogenic risk to humans remains in serious dispute. AN is used mainly as a starting material for synthetic fibers, resins and rubber, and is released to the environment as fugitive emissions and in wastewater during its production and use. It has been classified by the International Agency for Research on Cancer to be a Group 2A agent that is probably carcinogenic to humans (2). A risk level of E-4 (1 in 10,000) was estimated for respiratory cancer in workers exposed to an air concentration of 1 μg of AN per cubic meter (0.47 ppb) (3). However, two recent epidemiological studies of workers exposed to AN, one a cohort of 2,671 men who had worked at American Cyanamid (4), and the other consisted of 6,803 workers in the Netherlands (5), have found no indications that AN has a carcinogenic effect. If specific biomarkers had been available for measuring AN exposure in these populations, the human studies would have provided a more preferred and accurate assessment of risk than the default assumption based on animal carcinogenesis data of AN (6).

Considerable progress has been made in the dosimetry of chemical carcinogens from adducts of human hemoglobin A (Hb) (7). Regarding the Hb-AN adducts, gas chromatography-mass spectrometry analysis of N-(2-cyanoethyl)-valine, an adduct formed by addition of AN to the N-terminal valine of Hb, has been reported (8). The adduct levels among 41 chemical workers were 0.02-66 nmol/g Hb or 1.29-4260 ppm. However, none of this developed methodology is readily applicable to large-scale epidemiological screening or routine monitoring. A specific and sensitive immunoassay to determine human exposure to AN and other chemicals would be ideal for human health assessment. AN is known to be metabolized by direct addition to the double bond, a major pathway, and epoxidation (9). We have been interested in developing immunoanalysis of the direct conjugation products of AN. In our preliminary study of the glutathione-AN adduct (10), a synthetic immunogen was prepared by coupling glutathione-AN to hemoglobin as a carrier protein, which was

used to immunize Balb/c mice to raise hybridoma. After excluding those which exhibited anti-carrier or cross-reactivity, two monoclonal antibodies were found to show specificity to the glutathione-AN adduct, but only at moderate sensitivity. We have since focused on developing antibodies to intact Hb-AN. These adducts were implicated by the effects of AN on rat erythrocytes and the slow clearance of ^{14}C-AN from red cells (half-life 825 h) (11). The Hb-AN adducts apparently involve modifying the distal heme pocket region to yield new antigenic sites in Hb. Herein we describe the kinetics, level of alkylation, and electrophoresis of Hb-AN adducts, the specificity of two Hb-AN antisera, and immunoassay of the pathological Hb-AN conjugates in human blood exposed to AN in vitro. The antisera are the first of its kind which provide a firm basis for the development of immunoassays of hemoglobin-carcinogen adducts to serve as biomarkers of human exposure where dosimetry has relied mainly on chromatography and mass spectrometry.

MATERIALS AND METHODS

Chemicals and Animals. Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), including AN which contained 35-45 p.p.m. of hydroquinone monomethyl ether as a polymerization inhibitor. The radiotracer, [2,3-^{14}C]-AN at 0.3 mCi/ml in ethanol:water 1:1, 5.3 mCi/mmol, and purity verified by nuclear magnetic resonance, was obtained from Sigma Chemical Co. (St. Louis, MO), as were other biochemicals including human hemoglobin A (Hb), rabbit anti-Hb antiserum, human serum albumin (HSA), and glutathione (GSH). Another rabbit antiserum, anti-HbF, was purchased from CalBiochem (La Jolla, CA). Balb/c mice, male, 6-8 weeks old, were purchased from Charles River Breeding Laboratories (Raleigh, NC).

Hemoglobin-Acrylonitrile Adducts in Human Blood. To 2 ml of human blood collected in an EDTA-containing tube (Vacutainer, Becton Dickinson, Rutherford, NJ) was added 40 µl of AN. The reaction was carried out in a shaker-water bath at 37^0 C for 15 to 180 min. Aliquots of treated blood, 0.2 ml each, were removed and worked up to collect Hb according to a standard procedure (12): centrifuged to pellet RBC, washed in 0.9% NaCl, lysed in water, added Sephadex G25 for 10

min, and centrifuged at 12,000 x g to obtain Hb in the supernatant. The Hb concentration was determined at OD 307 nm. The Hb-AN adducts were separated by PAGE as described below to compare with Hb from a control experiment with no AN added. Five human blood samples were used to show reproducibility of the AN exposure study. Also, Hb isolated from fresh human blood was treated with AN in the same manner, yielding the same adducts as shown by PAGE.

Kinetics of Alkylation of Blood Thiols and Adducts Obtained. Rate studies were carried out in a deaerated solution of 0.5 M Tris-HCl (pH 7.4) : methanol 9 : 1 in a shaker-water bath at 37°C in triplicates. Three blood thiols: hemoglobin (Hb) and serum albumin (HSA), both 0.6 mM, and glutathione (GSH) 5 mM, were alkylated with either acrylonitrile (AN) or chloroacetaldehyde hydrate (CAA), which was used at a concentration of 150 mM for reaction with Hb and 60 mM for HSA or GSH. The Hb reaction was monitored for up to 3 h by removing 50 µl aliquots to determine the free SH with 4,4'-dipyridinedisulfide (4-PDS) according to the method of Morell et al. (13). Briefly, 4-thiopyridone liberated was determined at 324 nm and Hb concentration at 307 nm. The GSH and HSA reactions were followed for up to 1 h by titrating the free SH with Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and monitoring the liberated chromophore, 2-nitro-5-mercaptobenzoic acid at 412 nm (14). The optical density values were graphed as log C_t/C_o vs. time, where C_t is the SH concentration at time t and C_o the initial concentration. Statistical analysis with linear regression was carried out by using standard plotting software. At the completion of alkylation of Hb and HSA, the reaction mixtures were centrifuged in a Centricon microconcentrator (Amicon, MW cut-off 10,000) to isolate Hb-AN, Hb-CAA, and HSA-AN. In addition, glycolaldehyde (GA) and D-glucose were allowed to react with Hb under the same conditions used for CAA to prepare the Hb-GA and Hb-glucose adducts.

AN Level by Radiotracer in Hb-AN. To 0.5 ml of deaerated Tris-HCl (pH 7.4) - methanol 9:1 was added Hb (19.4 mg, 0.6 mM) and the solution was kept under nitrogen. Triplicate solutions were prepared, two for counting controls, and the third to react with AN: 1.5 µl of [2,3-^{14}C]-AN (4.6 µg) and 5 µl of AN (4.03 mg) for 150 mM with 878 fold radiolabel dilution. These solutions were capped under nitrogen, incubated at 37°C for 0.5 or 3 h, and 50 µl (30 nmol protein) was

transfered into a vial containing 1.2 ml of 0.14 M of phosphate buffer (PB, pH 7.4) and 0.8 ml of 25% trichloroacetic acid (TCA) to precipitate Hb. To remove any nonspecifically bound AN, Hb was centrifuged, washed, dissolved in phosphate buffer, precipitated by adding TCA, and the process repeated from 3 - 6 times until the supernatant gave only background counts. The dark-colored Hb preparation was decolorized by adding 30% H_2O_2. To convert cpm to dpm, one control vial was used to determine the protein background cpm by duplicating the above work-up. To the second control vial was added 1.5 µl of U-^{14}C-D-glucose (0.02 µCi/µl, ICN Biochemicals, Inc., Costa Mesa, CA), and 50 µl (6,660 dpm theoretical) of this solution was added to 2 ml of PB, followed by 8 ml of a complete counting cocktail, which yielded a counting efficiency of 16.4% for Hb.

Electrophoresis of Protein Adducts. Electrophoresis was carried out on vertical polyacrylamide gel: 10% T resolving gel in Tris-HCl buffer (pH 9.15) and 4% T stacking gel in Tris-HCl buffer (pH 7.4) in 5 mM Tris-glycine running buffer (pH 8.3) for 6 h at a constant current of 15 mA through the stack and 20 mA through the resolving gel.. Each sample well was loaded with about 25 µg of protein containing 0.002% bromophenol blue tracking dye. For preparative gel separation, the visible Hb-AN bands after electrophoresis were cut out and the protein recovered by electroelution. For separation of the α and β chains of Hb and Hb adducts, SDS-PAGE with 12.5% T resolving gel was applied. The Hb adducts were dissolved in 0.02 M Tris-phosphoric acid buffer, pH 6.8, containing 8 M urea, 1 % SDS, 0.02 % 2-mercaptoethanol, and 0.008 % bromophenol blue tracking dye, and electrophoresed in 0.1 M Tris-phosphoric acid, pH 6.8, containing 0.1 % SDS by applying 50 V overnight.

Immunization of Balb/c Mice and Antisera. The mice were treated as follows. Week 1: intraperitoneal (i.p.) injection of 100 µg of an immunogen (Hb-AN1 or Hb-AN2 from preparative gel) in complete Freund's adjuvant (FA). Week 3: 100 µg of immunogen in incomplete FA (i.p.). Week 5: 75 µg of immunogen in incomplete FA (i.p.). Normally at week 6 but sometimes 16.5-20, the immunized mice were bled and assayed by ELISA for antibody activity. The antisera were purified by a standard procedure followed by immunoadsorption on a Hb-Sepharose 4B affinity column prepared according to manufacturer's instructions. Briefly, 0.5 g of CNBr-activated Sepharose 4B gel coupled

with 10 mg of Hb at 4^0C for 20 h was used for 0.6 ml of Hb-AN antiserum. The filtrate enriched in anti-HbAN antibodies was assayed for protein content by calibrated absorbance at 280 nm.

ELISA. The ELISA methodology used was adapted after Coleman et al. (15) and Santella et al. (16). Briefly, Nunc-immuno maxisorp plate was coated at 4°C overnight with 0.1 ml of 0 (blank control), 1 or 10 μg of antigen per ml of 0.1M sodium carbonate. The plate was washed according to a standard procedure, 0.15 ml of 10% calf serum was added at 25° for 1 h to block nonspecific binding, and the wash procedure was repeated. Sera collected from immunized mice were serially diluted to 50, 250, 1250, 6250, 31250 and 156250 fold in PBS-T containing 0.05% calf serum, and 0.1 ml of these dilutions was added to the microwells, and incubated at 37°C for 2 h. After washing, goat anti-mouse (or goat anti-rabbit for rabbit antiserum) IgG-alkaline phosphatase (diluted 1:4000, 0.1 ml) was added and the plates incubated for 2 h at 37°C. The wash was repeated, and 0.1 ml of p-nitrophenyl phosphate in diethanolamine buffer at pH 9.8 (1 mg/ml) was added, left at 25°C for up to 1 h, and the color development was stopped by adding 50 μl of 3N NaOH. The absorbance was read at 410 nm by a microplate reader in triplicate wells for statistical analysis. For competitive ELISA, wells were coated with 0.1 ml of an antigen as above. The antiserum was diluted 1 : 1250, 50 μl of which was mixed with 50 μl of an inhibitor solution which was serially diluted from 1000 to 0.1 μg/ml, and the mixture was added to the wells. Subsequent steps were as those for direct ELISA.

RESULTS

The structural modification of Hb by AN was investigated as follows. It is already known that AN formed adducts to N-terminal valine of Hb in humans (8). Moreover, among the modified amino acids isolated from hemoglobin of mice treated with ^{14}C-AN, the predominant radioactivity peaks were found to correspond to products formed by addition of AN to cysteine and to histidine (17). Although there are 6 SH groups in the human Hb tetramer, cysteines α104 and β112 are "masked" and only β93 has a "free" sulfhydryl group that reacts with non-mercurial thiol reagents (18). Hence, Cys β93 can be singled out for monitoring the major Hb alkylation. On the other hand, there are 38

histidines in Hb, 10 in α- and 9 in β-chain. Locating these cyanoethylated residues in Hb-AN would require a lengthy sequence analysis which may not elucidate its antigenic determinants. Therefore, we chose to compare the rates of S-alkylation of hemoglobin, glutathione, and human serum albumin by AN and CAA in order to probe the structural change in Hb-AN. At time = 0, the mole ratio of free SH of Hb was titrated to be 1.89, or 2.30 when determined after treatment with 50 mM of 2-mercaptoethanol for 2 h at 25°C. All the rate studies yielded first order plots and the rate constants are compared in Table 1. Since polyacrylamide gel electrophoresis has been used to detect hemoglobin hybrids of α and β variants (19), we have worked out a PAGE technique to separate Hb from Hb-AN. Figure 1 depicts the electrophoretic behavior of the Hb adducts. The Hb-AN mixtures, derived either from human blood exposed to AN for 3 h or from a synthetic reaction with Hb, gave rise to two adduct bands (1A). The first one, identified as Hb-AN1, was detected in reactions from 10-120 min, but the second band, Hb-AN2, appeared only in the 120 min reaction (1B). The latter was dominant in the Hb-AN 4 h mixture, but no additional band could be discerned from prolonged exposure. Thus, Hb-AN1 and Hb-AN2 represent two identifiable levels of increasing cyanoethylation of Hb. Under denaturing SDS-PAGE conditions in 8M urea, both Hb and its adducts gave the same α- and β-chain bands (1C). The number of AN molecules conjugated to one Hb was determined by using [2,3-^{14}C]-AN. After exhaustive removal of non-covalently bound AN, the products gave a ratio of 0.7 and 3.1 AN per Hb after 0.5 h and 3 h, respectively. It appears likely that Hb-AN1 may have about 1 AN per Hb tetramer whereas Hb-AN2 may have 2-3 AN.

The specificity and sensitivity of the antisera raised to the two levels of Hb-AN adducts were determined by performing ELISA. The means of triplicate ELISA data, coefficient of variation < 7%, are used for the following plots. In Fig. 2 are shown the binding curves for various antisera in serial dilutions against 0.1 μg of coated antigen on the microwell: anti-HbAN1 vs. Hb and Hb-AN1 (2A), anti-HbAN2 vs. Hb and Hb-AN2 (2B), anti-Hb vs. HSA, Hb, Hb-AN1, and Hb-AN2 (2C), and anti-HbF vs. fetal Hb-F, Hb, Hb-AN1, and Hb-AN2 (2D). The cross-reactivity of anti-HbAN1 (2E) and of anti-HbAN2 (2F) with the AN adducts of Hb and HSA are also shown. Fig.3 depicts the competitive ELISA results: anti-HbAN1 antiserum in 1:1250 dilution against 1 μg of Hb-AN1 coated

antigen in the presence of increasing amounts of Hb-AN1, Hb-AN2, Hb-chloroacetaldehyde, and Hb as inhibitors (3A), a similar competitive inhibition plot for anti-HbAN2 vs.1 μg of Hb-AN2 coated antigen (3B), a similar competitive inhibition plot for anti-Hb vs. 0.1 μg of Hb plated (3C), and a similar plot for anti-HbAN1 vs. 0.1 μg of Hb-AN1, with Hb-AN1, Hb-glucose, Hb-glycolaldehyde, and Hb as inhibitors (3D). Fig.4 compares the detection of Hb-AN1 over Hb by the Hb-AN1 antisera before and after immunopurification on Hb-Sepharose 4B. Two antisera, collected 16.5-20 weeks after immunization, were purified: one from mouse A which was re-immunized 2 weeks before bleeding (4A) and the other from mouse B was not so treated (4B). The immunopurified antiserum from mouse A was used in 1:1250 dilution in a blood immunoassay where Hb-AN adducts were serially diluted as shown in Fig.5A to find the limiting signal-to-noise level for detecting Hb-AN over Hb. In Fig.5B, serial dilutions of the antiserum showed dose response to blood which was exposed to AN from 0 to 180 min.

DISCUSSION

Antigenic structure of Hb-AN. In this study, we have raised polyclonal antibodies which can distinguish between hemoglobin, Mr 64,500, and its adducts with 1-3 AN conjugated. The structural modification due to size must be minimal since the cyanoethyl group is smaller than even glycine. We surmise that AN has modified Hb antigenicity by presenting an unique determinant in the pathological hemoglobin adduct. There is no information on the antigenicity of any carcinogen-hemoglobin adduct, but human hemoglobin has been shown to possess 10 continuous antigenic sites, five each on the α and the β chain (20). Although none was found for the heme pocket region of β91-105, it was suggested that there should be an antigenic site at β93-98 by extrapolating the conformational location of a myoglobin antigenic site. It is likely that the quaternary tetrameric structure of Hb may have obstructed access to this site. Indeed, the SH group of Cys β93 in deoxy-Hb is known to be shielded by the β-C-terminal salt bridge between Asp β94 and His β146, but NMR shows that this is broken upon modification with thiol reagents (21). Thus, the Hb-AN adducts may lead to an

accessible antigenic site due to Cys β93 S-cyanoethylation. Furthermore, a comparison of sulfhydryl titration with ^{14}C counting reveals the extent of N-alkylation: 0.3 SH alkylated vs. 0.7 ^{14}C-AN conjugated in 0.5 h in forming Hb-AN1, and 1.4 SH vs. 3.1 AN in 3 h in forming Hb-AN2. Since only a weak 4% binding, relative to the β-chain as 100%, was shown by the N-terminus 1-15 in the study of β-chain antigenic sites (22), the terminal Val-AN adduct should not cause substantial change in the antigenicity of Hb. On the other hand, the strongest binding affinity of the β-chain antigenic sites was mapped to the C-terminus peptide 131-146 with 55% of binding (22). In this connection, tandem mass spectrometric analysis of tryptic peptides derived from the in vitro reaction of Hb with styrene-7,8-oxide showed prominent alkylation at the externally accessible His β143 in addition to Cys β93 (23). In light of these observations, we speculate that His β143 N^{τ}-cyanoethylation may make significant contribution to the development of unique antigenic determinants in Hb-AN1 and 2. This is illustrated in Fig.6 with the structure of the distal heme pocket region near the β-C-terminus (24), which includes Cys 93 and His 143, for comparing the fate of Hb alkylations by AN and CAA.

Our premise is that the rate of S-alkylation is indicative of the stereoelectronic environment of the nucleophilic reaction. We have derived structural information from the first order kinetics of blood thiol alkylation shown in Table 1 as follows. A mean value of 2.2 free SH groups per Hb tetramer, including two Cys β93, has been reported (24), which is similar to our titration of Hb-SH. Using glutathione as the standard thiol, the reaction of AN with Cys β93 in Hb was slower by about 20 times, but the corresponding reaction rates with CAA as the substrate were practically the same. The difference between AN and CAA in S-alkylation may be attributed to their different physical and chemical properties. Referring to Fig. 6, reaction of the hydrophobic AN with Cys β93 would perturb both Tyr β145 and the Asp β94 salt bridge, hence a slower reaction relative to glutathione. With the hydrophilic and bisalkylating CAA, it may crosslink the β93 SH group with the side chain of Ser β89 or Lys at β95 or β144 with less strain. In this context, the fat-transport protein HSA, with the only free SH group at Cys 34, reacted with AN about 41 times faster than the corresponding Hb-AN reaction, but only 5 times faster when CAA was the alkylating agent. The faster rate of HSA cyanoethylation could be explained by the binding of AN to a hydrophobic pocket in the transport

protein wherein ready access to nucleophilic attack by the SH group occurred. Concurrent with S-cyanoethylation is that Hb also undergoes His N^{τ}-conjugation, possibly at the nearby β143 as shown in Fig.6 analogous to styrene oxide, yielding a less protonated imidazole ring due to the inductive effect of the cyanoethyl group. Thus, increasing levels of cyanoethylation of Cys β93 and His β143 may increasingly disrupt the ionic and hydrogen bonds in the distal heme pocket region of Hb, thereby creating unique antigenic determinants in Hb-AN. For substantiation, gel analysis of the Hb adducts was performed as shown in Fig.1. Two bands were obtained for the AN conjugates, identical ones being formed from AN reacting with either purified Hb or with Hb in the red blood cell (1A). Both bands were faster moving than that of Hb from the negative to positive electrode in non-denaturing PAGE. They represent consecutive levels of modification of Hb according to the time study shown in 1B. The formation of a faster moving component in non-denaturing electrophoresis implies the consumption of a positively charged group, presumably a quarternary nitrogen, hence freeing a carboxylate anion to facilitate migration from the negative to the positive electrode. The combined effects of Cys β93 S- and His β143 N^{τ}- cyanoethylation, progressing with time from Hb-AN1 (about 1 AN) to Hb-AN2 (about 2-3 AN), are compatible with such migration pattern. That no cross-linking of chains or chain cleavage of the Hb α and β chains has taken place can be seen by detecting the same α and β bands by SDS-PAGE for both the adducts and parent Hb in a denaturing gel analysis (1C).

Antisera Binding of Hb Antigens. Our hypothesis that increasing levels of cyanoethylation at Cys β93 and at His β143 generate the two isolable antigenic adducts, Hb-AN1 and Hb-AN2, is supported by the antisera binding studies. The antisera to Hb-AN1 and Hb-AN2, which are polyclonal in nature and hence should recognize various Hb epitopes, did not bind to Hb as well as to the AN adducts (Fig.2A and 2B). At 1250 fold dilution of anti-HbAN1 antiserum and 0.1 μg of plated antigen, the ELISA absorbance for Hb-AN1 (0.74) was 2.7 times that for Hb (0.27). A similar pattern was given by anti-HbAN2 antiserum with 3.3 times the specificity between Hb-AN2 and Hb (0.63 vs. 0.19) under the same assay conditions. The control mouse sera gave only baseline values for the two AN adducts showing that the Hb-AN antisera were specifically raised to the immunogens inoculated. On

the other hand, the commercial anti-Hb rabbit antiserum could recognize Hb, Hb-AN1, and Hb-AN2 with the same absorbance, which was about 2 times that of the unrelated protein HSA (Fig.2C), indicating common Hb epitopes among the three Hb antigens. Another rabbit antiserum to human fetal hemoglobin, anti-HbF, was able to discern some differences among these Hb antigens (Fig.2D). At 1250 fold dilution of anti-HbF, there seems to be increasing binding in the order of Hb ($\alpha_2\beta_2$ tetramer) < Hb-AN2 < Hb-AN1 < Hb-F ($\alpha_2\gamma_2$). It seems that the AN-modified β chain in Hb-AN may at certain point resemble the γ chain in Hb-F, e.g., His β143 when cyanoethylated may become uncharged like Ser γ143, inspite of 39 different amino acids between the β and γ chain (24). The similarity between Hb-AN1 and Hb-AN2 is indicated by cross-reacting the antisera. The binding specificity of Hb-AN1 and Hb-AN2 displayed no substantive difference when assayed with either anti-HbAN1 (Fig.2E) or anti-HbAN2 (Fig.2F), although each antiserum did recognize the corresponding antigen slightly better in all serum dilutions. In respect to binding unrelated proteins, both antisera showed little affinity for either HSA or its AN conjugate, HSA-AN.

The cross-reactivities of the Hb-AN antisera with other hemoglobin adducts are shown in Fig.3. The two adducts of particular concern are Hb-CAA and Hb-GA, which are derived from chloroacetaldehyde hydrate (CAA) and glycolaldehyde (GA), respectively. Both CAA and GA are metabolites of vinyl chloride (26) and have multiple environmental origins as well. Mice exposed to vinyl chloride resulted in alkylation of cysteine and histidine of hemoglobin to yield the 2-oxoethyl derivatives (27). It is therefore gratifying that competitive ELISA with anti-HbAN1 (Fig.3A) and anti-HbAN2 (Fig.3B) found no specific inhibition by Hb-CAA, which behaved like Hb, showing only low level non-specific inhibition. In contrast, the Hb-AN adducts were highly inhibiting to their own antibodies. The Hb-AN1 antiserum was inhibited by both adducts equally well, but the Hb-AN2 antiserum was more sensitive to Hb-AN2 than to Hb-AN1, consistent with Hb-AN2 having a higher level of similar antigenic determinants than Hb-AN1. In the presence of anti-Hb, all the adducts of AN and CAA were just as competitive which were better than Hb itself as inhibitor (Fig.3C), indicating the prevalence of the normal Hb epitopes in these adducts. When either Hb-GA or Hb-glucose was used to inhibit the binding of Hb-AN1 by anti-HbAN1 (Fig.3D), neither was found to be

inhibiting. This is not surprising since both of these adducts are formed by a mechanism which is different from that for Hb-AN, involving Schiff base formation, followed by Amadori rearrangement to carbonyl, and possibly a second addition by a neighboring nucleophile (28).

This lack of cross-reactivity with Hb and other Hb adducts suggests that an unique antigenic determinant may have been formed in Hb-AN, provoking specific antibodies. Accordingly, immunopurification of the mouse antisera was illustrated with anti-HbAN1 using an affinity column of Sepharose 4B covalently linked to Hb. As shown in Fig.4, the binding specificity of the two antisera reflects the immunization history of their sources. The protein concentrations of antiserum A before and after the column treatment were 49.4 and 8.4 mg/ml, respectively. In ELISA, the Hb-AN : Hb specificity ratio increased dramatically from 1.02 (before Hb-column) to 16.5 (after) at 20 μg of antiserum A per well. For antiserum B, the protein concentrations were 64.0 mg/ml before and 4.9 mg/ml after the Hb-column. Here the specificity ratio increased only moderately from 1.0 (before) to 6.2 (after) even at 121.5 μg of antiserum B per well. Since mouse B was not re-immunized 2 weeks before being bled as did mouse A, the immune system memory effect would favor producing more of anti-Hb in the long run against the more prevalent Hb epitopes, hence less anti-HbAN1 antibodies were present in antiserum B. This explanation is also consistent with the low initial specificity ratio of ~ 1 for these late antisera, collected in 16.5-20 weeks, instead of a ratio of ~3 for the antisera collected 6 weeks post-immunization. The immunopurified anti-HbAN1 antiserum A displayed useful specificity and sensitivity in immunoassay of human blood as shown in Fig.5. At 1250 times dilution of the antiserum and serially-diluted Hb antigens, the Hb isolated from human blood given AN for 1.5 h was clearly discernible from that of the control blood up to 5 ng of antigen plated (Fig.5A). The positive control in this assay, a 4 h reaction product of Hb with AN, showed slightly better sensitivity which could be differentiated from the negative control Hb at 2 ng plated. This antiserum sensitivity exceeds the lowest level of Hb-AN adduct of 0.02 nmol/g Hb (1.29 ppm) detected in occupationally exposed workers by gas chromatography-mass spectrometry (8). A dose response for blood exposed to AN for 180, 120, 60, 15, and 0 min (control) was obtained at various dilutions of antiserum A (Fig.5B). Thus, the immunopurified antiserum is capable of determining the levels of Hb-AN

biomarkers in human blood, making it feasible to scale-up to rabbit antisera as well as to screen for monoclonal antibodies for validation studies. The present investigation has shown the specific immunogenicity of the Hb-AN adducts, characterized their likely antigenic modifications, and verified the sensitivity and specificity of their antibodies. Furthermore, they have illustrated the clinical applicability of specific immunoassay for the detection of carcinogen-hemoglobin adducts for the study of human dosimetry and environmental risk. To our knowledge, this represents the first example of an unique approach to the development of immunoassays for the detection of carcinogen-hemoglobin adducts in exposed humans.

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Table I. Pseudo lst order rate constants of S-alkylation of thiols at pH 7.4 and 37°C by linear regression of SH group remaining.

Thiols	CH_2=CH–CN (AN) $k/10^{-4}s^{-1}$ (r^2)		Cl–CH_2–$CH(OH)_2$ (CAA) $k/10^{-4}s^{-1}$ (r^2)	
Hb[a]	1.0	(0.98)	17.8	(0.97)
GSH[b]	19.8	(0.98)	18.1	(0.99)
HSA[c]	41.4	(0.98)	89.8	(0.97)

[a] [Hb] = 0.6 mM, [AN or CAA] = 150 mM, reaction time 120 min

[b] [GSH] = 5 mM, [AN or CAA] = 60 mM, reaction time 12 min

[c] [HSA] = 0.6 mM, [AN or CAA] = 60 mM, reaction time 6 min

Legends of Figures

Fig. 1 Comparison of non-denatured PAGE and SDS-PAGE of hemoglobin-acrylonitrile adducts. (A) non-denatured PAGE with 10% T resolving gel. **Lane 1** Hb isolated from RBC, **Lanes 2 and 3** replicates of Hb isolated from blood pre-treated with AN, 3h, 37°, containing Hb-AN adducts, **Lane 4** Hb standard, **Lane 5** Hb treated with AN, 3 h, 37° contained Hb-AN1 (band ahead of Hb) and Hb-AN2 (fastest-moving band). (B) PAGE of the time course of Hb-AN adduct formation with 10% T resolving gel. **Lane 1** BSA standard, **Lane** 2 Hb standard, **Lanes 3,4, and 5** Hb-AN reaction at 37° for 10, 30, and 120 min, respectively. (C) SDS-PAGE with 12.5% T resolving gel. **Lane 1** HbAN1 isolated from preparative PAGE, **Lane 2** Hb-AN2 isolated from preparative PAGE, **Lane 3** Hb pretreated with CH_3CHO as control, **Lane 4** Hb standard.

Fig. 2 Antisera binding of Hb and related antigens: anti-HbAN1 (A), anti-HbAN2 (B), anti-Hb (C), anti-HbF (D), cross-reactivity of anti-HbAN1 (E) and anti-HbAN2 (F).

Fig. 3 Competitive inhibitions of anti-HbAN1 (A), anti-HbAN2 (B), anti-Hb (C), and anti-HbAN1 (D) antisera in binding to Hb and adducts.

Fig. 4 Immunopurification of anti-HbAN1 antisera on Hb-Sepharose 4B column. (A) Balb/c mouse A was bled 20 weeks after 1st immunization, re-immunized with HbAN1 in incomplete FA 2 weeks before and tail injection 4 days before blood collection . (B) Balb/c mouse B was bled 16.5 weeks after 1st immunization, tail injection 4 days before blood collection (B).

Fig. 5 Immunoassay of Hb-AN in human blood with immunopurified Hb-AN1 antiserum. (A) Serial dilutions of antigen. (B) Serial dilutions of antiserum against blood exposed to AN for various lengths of time.

Fig. 6 Nucleophilic additions of AN and CAA in the distal heme pocket region near the β-C-terminus of human hemoglobin.

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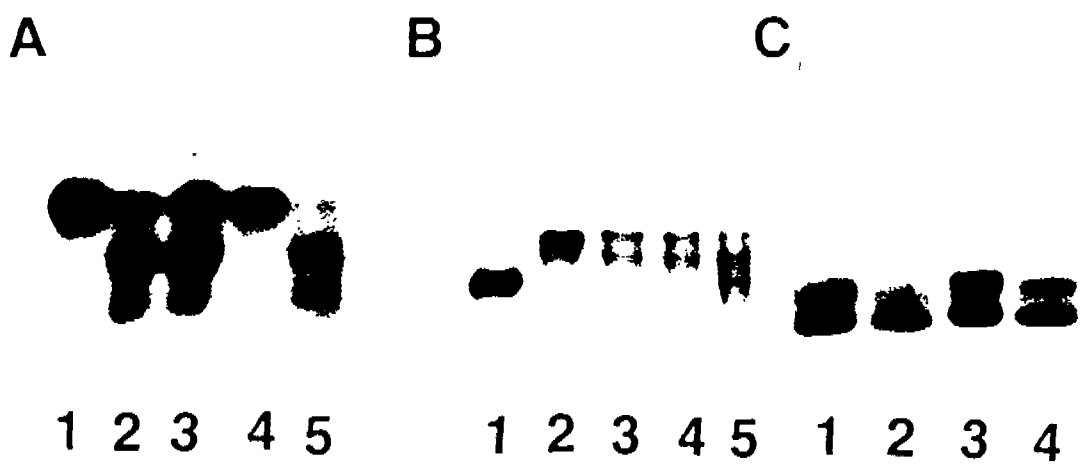

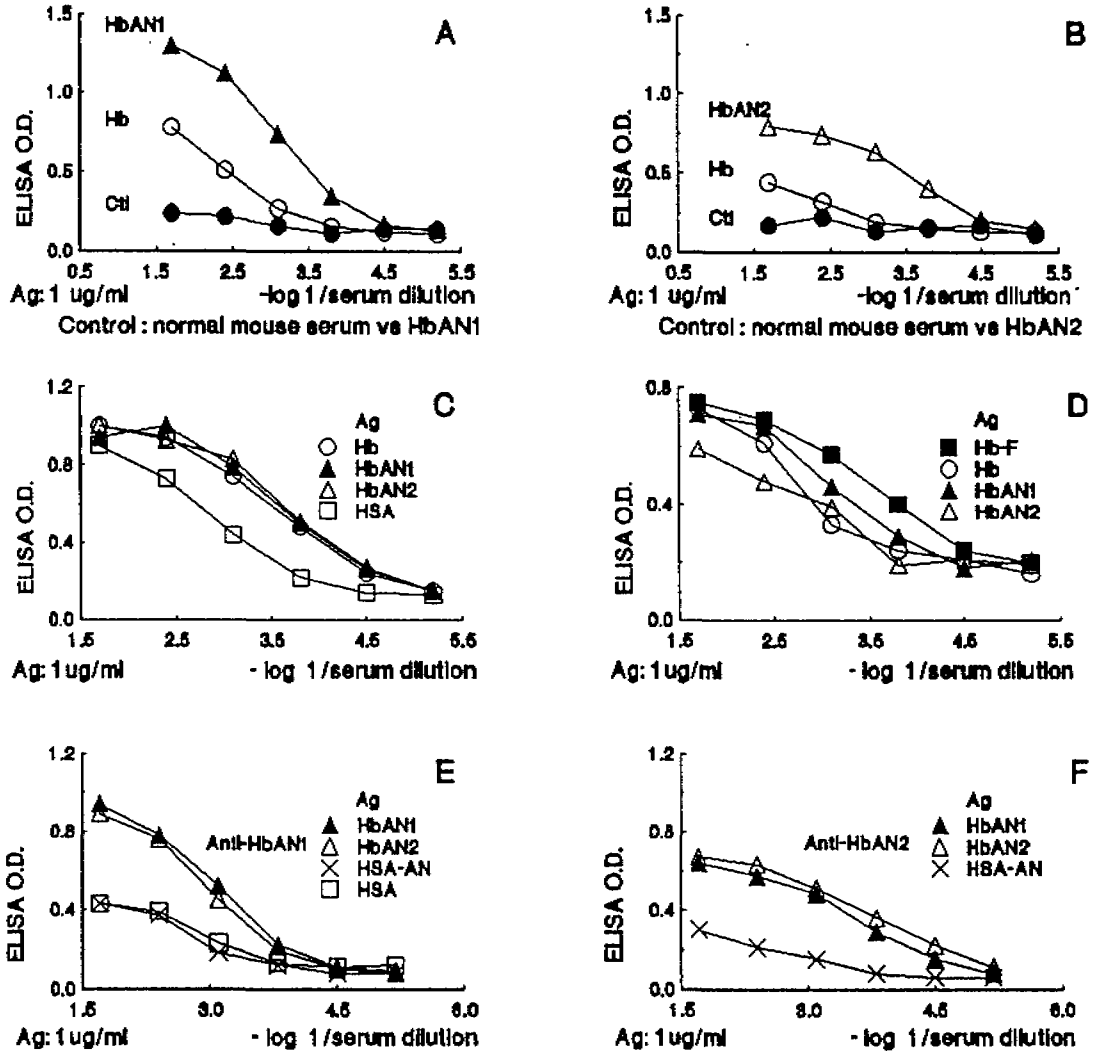

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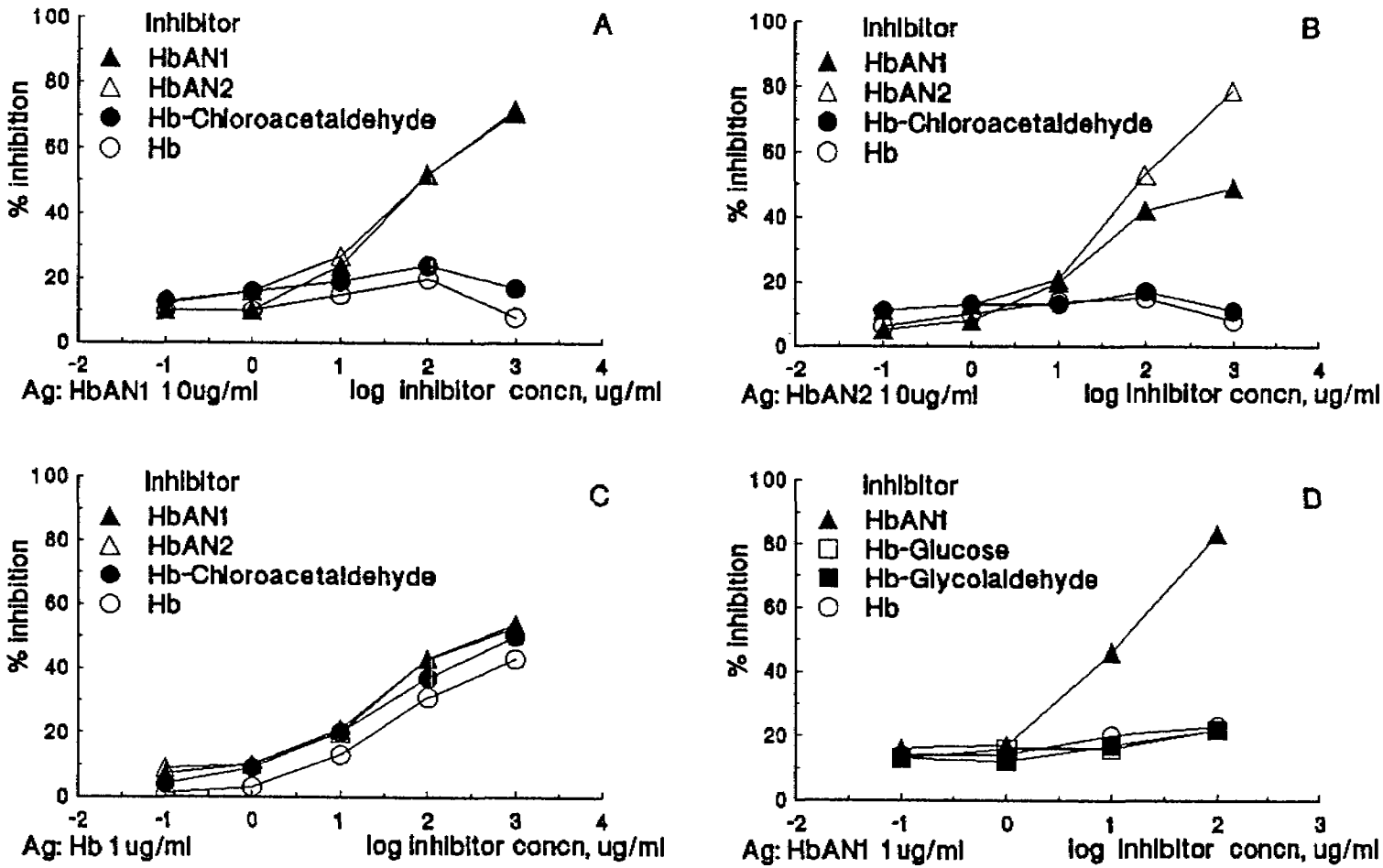

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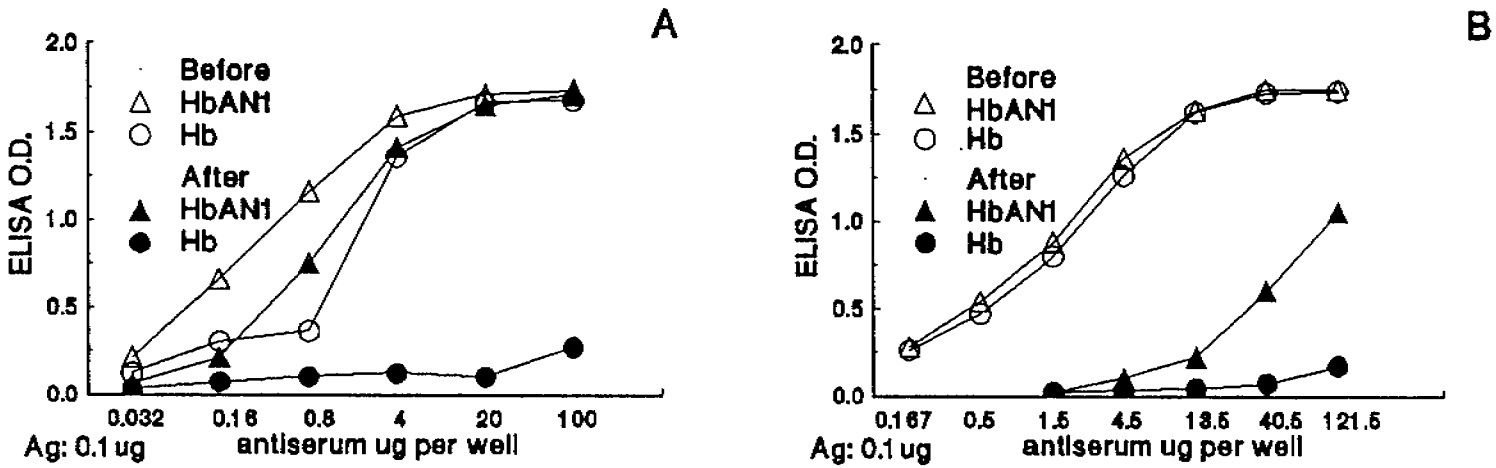

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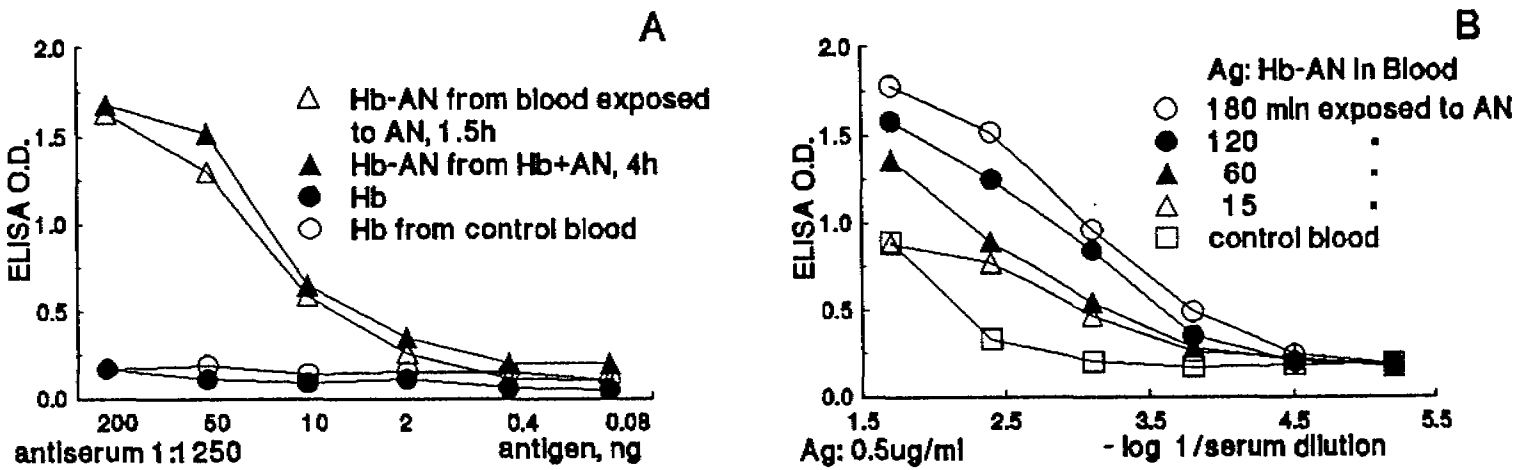

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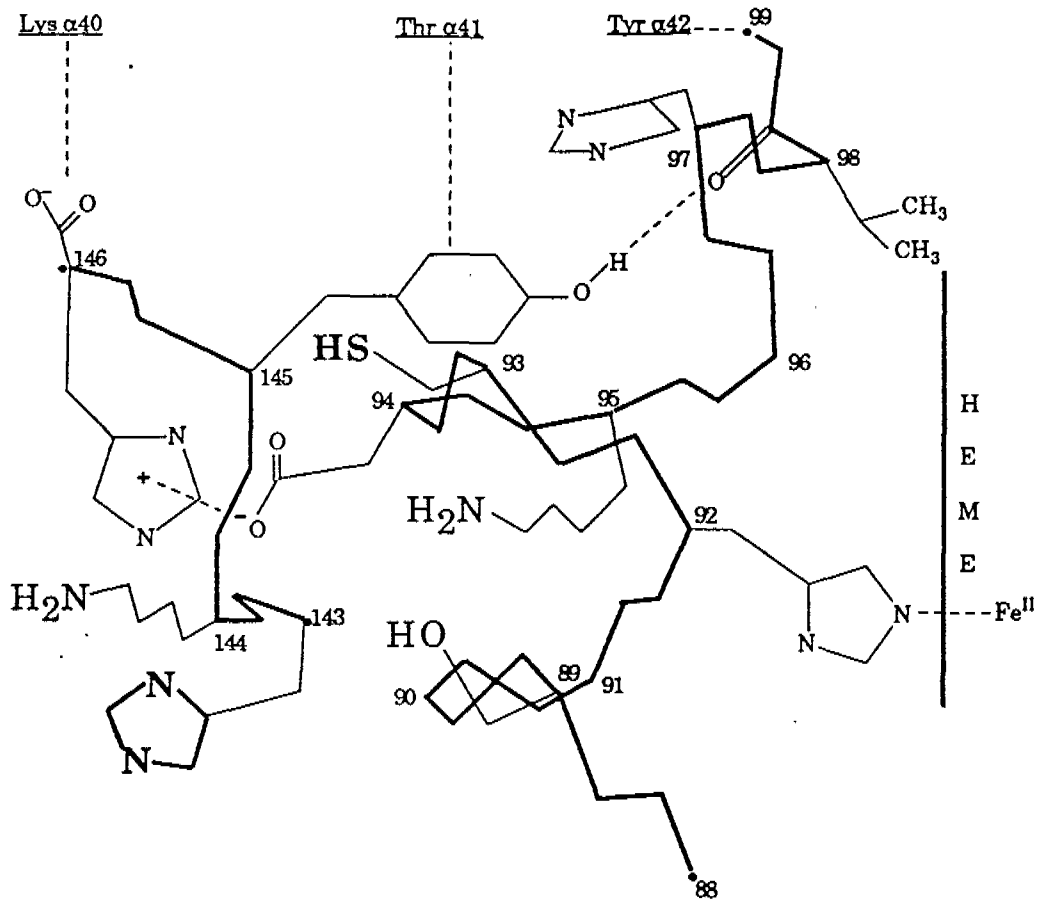

AN $CH_2{=}CH{-}CN$ hydrophobic

93$-SH \longrightarrow$ **93**$-S-CH_2CH_2CN$

143$-N^r \longrightarrow$ **143**$-N^r-CH_2CH_2CN$

CAA $Cl-CH_2-CH(OH)_2$ hydrophilic

93$-SH \longrightarrow$ **93**$-S-CH_2-\underset{\displaystyle X}{\overset{\displaystyle OH}{CH}}-X$

143$-N^r \longrightarrow$ **143**$-N^r-CH_2\underset{\displaystyle X}{CH}-OH$

X = **89**– O; **95**–, **143**–NH

Fig. 6 Nucleophilic additions of AN and CAA in the distal heme pocket region near the β-C-terminus of human hemoglobin

Academic Press, Inc.
1250 Sixth Avenue, San Diego, California 92101-4311

United Kingdom Edition published by
Academic Press Limited
24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging-in-Publication Data

Molecular epidemiology : principles and practices / edited by Paul A. Schulte, Frederica Perera.
p. cm.
ISBN 0-12-632345-3
1. Molecular epidemiology. 2. Biochemical markers. I. Schulte, Paul A. II. Perera, Frederica P.
[DNLM: 1. Biological Markers. 2. Epidemiologic Factors. 3. Molecular Biology. QH 506 M71925]
RA652.5.M65 1993
614.4–dc20
DNLM/DLC
for Library of Congress 92-49193
CIP

PRINTED IN THE UNITED STATES OF AMERICA
93 94 95 96 97 MP 9 8 7 6 5 4 3 2 1

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