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Anti-p53 Antibodies in Sera of Workers Occupationally Exposed to Vinyl Chloride

95-01-4

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Background: **The p53 tumor suppressor gene (also known as TP53) is often mutated in a wide variety of cancers, including angiosarcoma of the liver (ASL). Anti-p53 antibodies have been detected in the sera of patients with leukemia, childhood lymphoma, or cancers such as those of the breast, lung, colon, esophagus, and liver (hepatocellular carcinoma).** ***Purpose:*** **The objective of this study was to determine the prevalence and time of appearance of serum anti-p53 antibodies during the pathogenesis of ASL associated with occupational exposure to vinyl chloride.** ***Methods:*** **Enzyme-linked immunoassay (EIA) was used to detect anti-p53 antibodies in 148 serum samples from 92 individuals occupationally exposed (in France or in Kentucky) to vinyl chloride; 15 of these individuals (six from France and nine from Kentucky) had ASL. A subset of coded EIA-positive and EIA-negative sera was further analyzed for anti-p53 antibodies by immunoblotting and immunoprecipitation. Nucleotide sequence analysis of exons 5-8 of the p53 gene was conducted on ASL DNA from six patients. We tested sera from 31 men who had no occupational exposure to vinyl chloride; they made up the control group. Statistical analyses were done using the Kruskal–Wallis chi-squared approximation and the Wilcoxon two-sample test for normal approximation. All *P* values result from two-sided tests.** ***Results:*** **Fourteen serum samples (from nine individuals) were positive in the EIA. Five of the 15 individuals with ASL were positive for anti-p53 antibodies by EIA, immunoblotting, and immunoprecipitation: one individual at 11.3 and 10.8 years before diagnosis, another at 4 months before and shortly after diagnosis, and three when diagnosed or shortly thereafter. Four of the 77 vinyl chloride-exposed workers without diagnosed ASL were positive for anti-p53 antibodies; two of the four had symptoms related to vinyl chloride toxicity. Tumors from three of the six vinyl chloride-exposed workers from which sufficient DNA for analysis was obtained had A:T to T:A missense mutations of the p53 gene. Anti-p53 antibodies were detected in two of these individuals. Among the control group, two of 15 serum samples from 15 lung cancer patients and zero of 15 serum samples from control subjects without cancer had anti-p53 antibodies at substantially lower levels than the nine (10%) of 92 vinyl chloride-exposed workers who were positive for anti-p53 antibodies.** ***Conclusions and Implications:*** **Serum anti-p53 antibodies can predate clinical diagnosis of certain tumors, such as ASL, and may be useful in identifying individuals at high cancer risk, such as workers with occupational exposure to vinyl chloride. [J Natl Cancer Inst 1995;87:1400-7].**

Angiosarcoma of the liver (ASL) is the most common of the malignant mesenchymal tumors affecting human liver (*1*). Moreover, it is an extremely rare cancer in humans, except in instances of catastrophic exposure to carcinogens, the usual causal source (*2*). ASL has been associated with exposure to thorotrast (thorium dioxide), arsenic solutions, and more recently with occupational exposure to vinyl chloride [reviewed in (2)]. In 1974, three ASL deaths in Kentucky were traced to the workers' common exposure to vinyl chloride during their years of employment in the manufacture of polyvinyl chloride (*3*). Vinyl chloride-induced ASLs in rats were also reported in that year (*4*), and observations in humans were later confirmed in workers from European factories (*5*). Vinyl chloride-induced ASL evolves insidiously, frequently producing surface damage, i.e., Raynaud's syndrome (blood vessel obstruction in fingers, toes, or ears causing tissue damage at those sites), but few major symptoms until late in the disease (*3,6*). Protection against exposure to vinyl chloride in industrial environments was initiated in the 1970s, as were studies to monitor cancer incidence in previously exposed workers (*7*). Recent molecular epidemiology studies of vinyl chloride-exposed workers have found K-ras (*8*) and p53 (*9*) mutations in liver tissue of ASL patients and mutated p21 Ras protein (*10*) in the sera of workers with and without ASL.

The p53 gene (also known as TP53) is mutated in a wide variety of human cancers, including cancer of the liver [reviewed in (*11-15*)]. The predominant genetic alterations are missense mutations in four common regions of the most conserved domains of the protein, exons 5-8, resulting in altered protein conformation (*16,17*), loss of suppressor function (*18*), and immunochemically detectable accumulation of p53 in the nucleus of tumor cells [reviewed in (*11,19-21*)]. Reduced p53 tumor suppressor function can also be achieved by, for example, a missense mutation that does not alter overall protein conformation (*22,23*). The p53 mutational spectra may reflect mutagenic mechanisms leading to cancer and provide clues to their etiologic origin (*11,14*). Anti-p53 antibodies, initially detected in sera from breast cancer patients (*24*), have been detected in sera from a variety of cancer patients, including, but not limited to, those with childhood lymphoma (*25*), breast cancer (*26-28*), lung cancer (*29-32*), leukemia (*33*), colon cancer (*30*), hepatocellular carcinoma (HCC) (*34*), and esophageal cancer (Cawley HL,

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See "Notes" section following "References."

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Meltzer S, DeBenedetti VM, Trivers GE, Bennett WP, Harris CC: manuscript in preparation). Anti-p53 antibodies have also been detected in patients with chronic obstructive pulmonary disease who later developed lung cancer (*31*) or other malignancies (Trivers GE, Cawley HL, DeBenedetti VM, Caron G, Miller RD, Bennett WP, et al.: manuscript in preparation). These antibodies are associated with cellular p53 accumulation and p53 mutations in individual tumors (*26,29,32*). As a putative tumor-specific antigen, mutated p53 protein has been associated with a poor prognosis in patients with cancer, including those with breast, stomach, colon, and bladder cancers [reviewed in (*13*)]. Likewise, anti-p53 antibodies have also been suggested to indicate a poor prognosis in patients with lung (*35*) or breast (*27,36,37*) cancer. Most studies of serum anti-p53 antibodies, however, have used patients with relatively late stage cancer.

Here, we describe the detection of anti-p53 antibodies in sera from individuals occupationally exposed to vinyl chloride. Our objective was to determine the prevalence and time of appearance of these antibodies during the pathogenesis of ASL.

Subjects and Methods

Vinyl Chloride-Exposed Workers and Unexposed Control Subjects

Exposed workers. One hundred forty-eight serum samples were obtained from 92 workers recruited (via informed consent approval by the internal review boards of the University of Louisville, Kentucky, and the National Institutes of Health and Medical Research, Institut National de la Santé de la Recherche Médicale [INSERM], in Lyon, France) for two industry-initiated or industry-assisted projects in Kentucky (one factory) and France (INSERM, five factories) to study vinyl chloride-induced ASL and workers at risk for development of ASL due to prolonged, high exposure to vinyl chloride. Sera were obtained from 83 workers in France and from nine workers in Kentucky and were stored at *–70 °C from 6 months to 18 years before* being used for this study. Aliquots of the sera from the French workers were used previously to study p21 Ras protein (*10*). The workers in this study held a variety of positions providing high exposure to vinyl chloride for 12-34 years, including an extended period before 1974 (0-34 years) without protection from exposure, and a shorter period after 1974 (0-18 years) with protection (*3*). Medical and occupational histories of the workers were provided, including job classifications, smoking histories, information on alcohol consumption, clinical records, and years of exposure to vinyl chloride (high [before 1974]; low [after 1974]), along with the results of periodic, extensive physical examinations. Tumors, benign and malignant, were verified by histopathologic examination.

There were 92 factory donors (nine Kentuckians [aged 38-72 years; mean ± SD = 51.2 ± 11 years]; 83 French [aged 34-71 years; mean ± SD = 58 ± 6 years]). All nine Kentuckians had diagnosed ASL. Of the 83 French, 41 were asymptomatic, 26 were symptomatic for toxic effects of vinyl chloride exposure, eight had benign tumors, and eight had malignancies (six ASLs and two HCCs). Twenty-four workers (nine in Kentucky and 15 in France) donated multiple (two to eight) blood samples, some before and after diagnosis of ASL and/or surgery. Asymptomatic patients, those with symptoms, and those with angiomas or HCCs had similar years of vinyl chloride exposure in three exposure categories: high (before 1974, mean ± SD = 13.3 ± 0.9 years), low (after 1974, mean ± SD = 7.3 ± 0.5 years), and the total accumulated time (mean ± SD = 20.7 ± 1.2 years). ASL patients had approximately 50% more time (20 ± 5.7 years) in the higher exposure period, 50% less time (3.7 ± 4.2 years) in the lower exposure periods, and only a slightly greater (+15%) total time of exposure.

Unexposed control subjects. Serum samples as well as medical and smoking histories were obtained from 30 men (aged 41-81 years, mean ± SD = 64 ± 12 years; from an ongoing study of lung cancer, smoking, and anti-p53 antibodies), 15 of whom had lung cancer, 15 who did not have a history of any cancer, and none of whom had industrial exposure to vinyl chloride. One HCC patient from Kentucky without vinyl chloride exposure was included in the study as an additional control subject. Examination of a larger number of unexposed control subjects was considered unnecessary on the basis of published reports (*24-28*) that indicated that antibody-positive serum donors are found predominantly among cancer patients. In a recent study (*37*) that included 200 normal donors, only one antibody-positive serum (0.5%) was found in a patient who had no symptoms of cancer. Moreover, a survey of nine of the referenced reports (*24-26,28-31,34,37*) revealed a total of 167 (14%) positive donors among 1197 cancer patients, compared with three (0.4%) positive donors among 736 noncancer control donors, including 40 and 67 patients with organ-specific, noncancer clinical symptoms affecting the lung and liver, respectively.

Enzyme Immunoassay to Detect Anti-p53 Antibodies in Human Serum

We used modifications of enzyme immunoassay (EIA) methods previously described (*38*). Briefly, purified human p53 (*39,40*), unrelated protein as control (bovine serum albumin [BSA] fraction 5; Sigma Chemical Co., St. Louis, MO; selected in experimental comparison with mock protein and horse and human serum albumin), and all other reagents were added to wells of microtiter plates in 50-µL volumes. The plates were washed five times following each reaction (37 °C for 1 hour). We dried 2.0 ng/well of p53 and 2.0 ng/well of BSA in triplicate wells and stored them at –20 °C. Plates were washed, then blocked with 4% goat serum (GTS) (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) in 1× phosphate-buffered saline (PBS) containing 0.05% Tween-20 (GTS-T_{20}). The serum samples, stored at –70 °C, were thawed, diluted 1:100 in 1% GTS-T_{20}, and applied simultaneously to p53 and BSA in single, triplicate-well columns, 10 samples per test plate. Rabbit anti-p53 antiserum (1:40 000 dilution, CM-1, from Dr David Lane, Department of Biochemistry, Medical Sciences Institute, The University Dundee, Scotland, U.K.) in 1% GTS-T_{20} was added in a single column as positive control. Detection of bound antibody involved use of alkaline phosphatase-conjugated, goat anti-rabbit immunoglobulin G (IgG) or alkaline phosphatase-conjugated, goat anti-human IgG antiserum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). We added 0.1% paranitrophenyl phosphate (Sigma Chemical Co.) in 10 m*M* magnesium chloride and 10 m*M* diethanolamine (pH 9.6) to the wells, and the product paranitrophenol was measured at an absorbance of 405 nm (A_{405}) after 15 minutes, up to 6 hours, and after storage overnight at 4 °C.

Criteria for EIA-positive assays and samples. Each serum sample was assayed three times. A positive assay required a mean p53-to-BSA ratio of 1.5 or greater (without the deletion of the lowest wells in individual triplicate assays) and a difference between the two means equal to two standard deviations or greater. A positive sample required at least two positive assays. All samples were examined by EIA. Those samples with positive EIA results, along with a selected number of negative samples, were submitted for analysis by immunoblotting and precipitation.

Statistical evaluations of EIA. EIA results were converted to specific activities and compared in SAS (PROC NPAR1WAY; SAS Institute Inc., Cary, NC), using the Kruskal–Wallis chi-squared approximation and the Wilcoxon two-sample test for normal approximation. All *P* value results are from two-sided tests. Although the ratios were used for their readily observable magnitudes of differences desired for determining antibody positivity, we used specific activity (p53 A_{405} minus BSA A_{405}) for the statistical analyses of the results. We considered raw data to be more reliable for this purpose than a derived factor.

Immunoblotting

Procedures used were modifications of methods previously described (*41*). p53 (10 ng/lane) and BSA fraction 5 (10 ng/lane) as control antigen were fractionated in 12% polyacrylamide gels containing 10% sodium dodecyl sulfate. The proteins were transferred by electrophoresis to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) in buffer (1× PBS, 0.1% Tween 20, 0.02% NaN_3, and 3% BSA) for 1 hour, then incubated for 2 hours with 1:100 human sera and 1:10 000 rabbit anti-p53 (CM-1) as positive control. Antibodies were detected with 1:1000 anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) and 1:1000 anti-human IgG (Jackson ImmunoResearch Laboratories) as second antibody treatment (1 hour), followed by a 1-minute incubation in ECL Western detection reagents (Amersham Life Science Inc., Arlington Heights, IL). Molecular weight markers (49 500-205 000 daltons, prestained sodium dodecyl sulfate–polyacrylamide gel electrophoresis standards; Bio-Rad Laboratories) were run on each gel to identify the p53 bands. The nitrocellulose mem-

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branes were exposed to Hyperfilm (Amersham Life Science Inc.). All sera that were positive at a 1 : 100 dilution were reassayed at 1 : 100 or higher dilutions; sera negative at 1 : 100 were reassayed at 1 : 100 or lower dilutions (1 : 50; 1 : 25), where interpretation could become difficult because of increasing background staining, but frequently allowed the reproducible confirmation of an EIA result.

Purified p53 was prepared by modifications of procedures previously described (*39*). Recombinant baculoviruses (from Drs. D. O. Reilly and L. Miller, University of Georgia, Athens) transfected with the wild-type human p53 gene were used to infect SF9 insect cells (Invitrogen, San Diego, CA) grown in culture. The harvested cells were lysed, and the p53 protein was immunoaffinity purified on p53-specific monoclonal antibody (PAB421; Oncogene Science, Inc., Uniondale, NY) columns (A-Sepharose, Oncogene Science), using monoclonal antibody (Oncogene Science #1801) or rabbit anti-p53 antibody (CM-1) to define the elution profile (data not shown). The concentration of protein in column fractions was determined by protein assay (Bio-Rad Laboratories) and by silver staining (Bio-Rad Laboratories) after electrophoresis in polyacrylamide gels. The purified p53 protein produced one to three bands. Further characterization was also done by immunoblotting using additional p53-specific monoclonal antibodies. The choice of a control antigen was determined by experimentation. Using anti-p53 antibody-positive and antibody-negative human sera and rabbit anti-human p53 (CM-1) antiserum, we tested mock protein (immunoaffinity purified from SF9 cells infected with nontransfected baculoviruses), several different preparations of human serum albumin, horse serum albumin, and BSA (Sigma Chemical Co.), and proteins from p53-expressing ($p53^{++}$) and p53-non-expressing ($p53^{--}$) Calu 6 tumor cells (American Type Culture Collection, Rockville, MD: No. ATCC HTB 56, from a lung adenocarcinoma that does not express p53; transfected with p53 mut143ala in this laboratory by Dr. E. Felley-Bosco) (*42*). No control antigen was better than BSA fraction 5. Therefore, BSA fraction 5 was used as the negative control in the EIA.

Immunoprecipitation

In vitro translation of ^{35}S-labeled p53. Procedures were carried out according to directions provided with the Promega Kit (TnT SP6 Coupled Rabbit Reticulocyte Lysate System; Promega Corp., Madison, WI). DNA [plasmid: p-Select p53, 1.27 μg/μL, prepared by Dr. Felley-Bosco (*42*)], 20 μCi of ^{35}S-labeled cysteine (Du Pont NEN, Boston, MA), and the kit reagents were mixed in a total volume of 50 μL, then incubated at room temperature for 90 minutes. Plasmid DNA (2 μg per reaction) produced 200-300 ng p53 (by Coomassie blue staining) in 50 μL of the TnT system.

Immunoprecipitation procedures. A 10-μL volume of serum was diluted in 90 μL of 1% goat serum in GTS-T_{20}. We added 20 μL of this mixture to 80 μL (final dilution = 1 : 50) of the reaction solution, containing 4-16 μg (estimated by immunoblotting) of ^{35}S (Du Pont NEN)-labeled p53 protein. The positive control was 1 : 10 monoclonal antibody DO-1 (Oncogene Science). Protein A-conjugated agarose beads (Oncogene Science) were added, and the precipitated mixture was fractionated in a polyacrylamide gel along with Rainbow ^{14}C-methylated, molecular weight markers (14 300-200 000 daltons; Amersham Life Science) and exposed to film. The negative controls consisted of sera from cancer-free donors without vinyl chloride exposure. All assays for matched sera from the same individual were done in a blinded manner and not necessarily on the same day with the same ^{14}C-labeled p53 translation product.

DNA Sequence Analysis

Tumor and nontumor DNA from four ASLs (cases 26, 14, 8, and 3) and one HCC from the French workers had been previously examined for mutations in the p53 gene (*9*). DNA from an additional ASL (case 17) was obtained from a cell line generated with percutaneous biopsy tissue (Marion MJ, Boivin S, Martel-Planche G, Hollstein M, Montesano R: manuscript in preparation). Along with this case, DNA from the tumor of one ASL patient and DNA from the tumor of one HCC patient (both from Kentucky) were analyzed for p53 mutations during this study, making a total of six ASLs and two HCCs assayed for p53 mutations and antibodies. Exons 5-8 were amplified by polymerase chain reaction (PCR) using intronic primers and sequenced as previously described (*43*). Positive samples were confirmed by sequencing from a second independent PCR from tumor DNA.

Results

Of the 148 serum samples assayed by EIA at a 1 : 100 dilution, a total of 14 samples from nine workers had anti-p53 antibodies. Table 1 shows the antibody detection results for the nine positive workers and one negative worker as p53/BSA ratios. Mean ratios for EIA-positive samples ranged from 1.53 to 24.00, with those at higher levels giving titers (i.e., dilution factor to detect antibodies) of 400-1600 (worker 24, samples a and b, Fig. 1; worker 23, Fig. 2). Six of nine workers with anti-p53 antibodies provided multiple serum samples. Worker 24, who died of ASL, provided three serum samples at successive 6-month intervals (11.3 years to 10.3 years prior to diagnosis). The first two samples contained detectable anti-p53 antibodies (Table 1, Fig. 1); the third did not. Control sera included two positive for anti-p53 antibodies among 15 serum samples from lung cancer patients (data not shown) and none positive in 15 serum samples from control subjects without cancer diagnosis (Fig. 3); these prevalence rates were significantly lower than the five positive serum samples among 15 workers with diagnosed ASL ($P \leq .002$). All EIA-positive serum samples and an approximately equal number of randomly selected negative samples, combined with similarly selected samples from other studies, were coded and tested together in the immunoblotting and immunoprecipitation assays. Forty-two serum samples (27 from workers in France and 15 from Kentucky workers) were examined. Nine serum samples (from six workers), all EIA positive, were positive by immunoblotting. Ten serum samples (from seven workers) were positive by immunoprecipitation, including the six that were EIA positive. Seven workers were antibody positive in at least two of the three assays. Table 1 shows the close relationship between the increasingly higher EIA ratio and the detection of antibodies by immunoblotting and immunoprecipitation. Five of the 15 workers with ASL had anti-p53 antibodies that were detected by the three anti-p53 antibody detection assays. Two anti-p53-positive ASL workers had detectable antibodies prior to the diagnosis of ASL: worker 23, 4 months before and on the day of diagnosis (Fig. 2, A and B); worker 24, 11.3 and 10.8 years before the diagnosis (Fig. 1). Workers 17 and 26 were positive at the time of diagnosis, and worker 25 was positive 7.5 years after partial hepatectomy (Fig. 2, A and B). Anti-p53 antibodies were also detected by all three assays in sera from two of 43 asymptomatic workers (workers 21 and 22) (Table 1). However, two of 26 workers with nontumor clinical symptoms (workers 19 and 20) had anti-p53 antibodies that were detectable only by the EIA.

As previously reported (*9*), the two ASLs among the five tumors (four ASLs and one HCC) available from France for sequencing had A : T to T : A missense mutations: the ASLs from worker 3 (exon 7, codon 255: ATC to TTC) and worker 26, with the highest anti-p53 antibody titer (exon 7, codon 249: AGG to TGG). DNA from an ASL cell line (Marion MJ, Boivin S, Martel-Planche G, Hollstein M, Montesano R: manuscript in preparation; *see* "Subjects and Methods" section) from worker 17 (Table 1; Fig. 2, A and B) was examined and was also found to have an A : T to T : A mutation (exon 5, codon 179: CAT to CTT; the data have not been previously published). The only workers in Kentucky with sufficient DNA avail-

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Table 1. p53 antibodies in vinyl chloride workers with and without angiosarcoma of the liver (ASL) by descending enzyme-linked immunoassay (EIA)*

Worker No.	Age, y	Smoking status	(Years) exposed†	Occupation	Time‡ between assay and diagnosis	Clinical symptoms: survival; mutation	Mean EIA§ p53/BSA ratio	Result	Immunoblotting‖	Immunoprecipitation¶
26	59	Smoker	[26]16	Autoclave worker	At diagnosis	ASL#; p53MUT 249: AGG>TGG**	24.00	++	++	+
25	53	Never smoker	[28]19	Autoclave cleaner	7 y after resection††; 3 y, 4 mo, before death	ASL#	19.50	++	+	+
24										
Sample a	72	Unknown	[34]32	Chemical worker	11 y, 4 mo, before diagnosis	ASL#	4.06	+	+	+
Sample b					10 y, 10 mo, before diagnosis		2.69	+	+	+
Sample c					10 y, 4 mo, before diagnosis		0.70‡‡	–	–	±
23										
Sample a	53	Unknown	[28]27	Vat cleaner	4 mo before diagnosis	ASL#	3.90	+	+	+
Sample b					At diagnosis		2.60	+	+	+
22										
Sample a	68	Smoker	[28]23	Polymerization	4 y before assay	None	3.00	+	+	+
Sample b	74	Smoker		worker	3 y before assay	None	2.34	+	+	+
21										
Sample a	55	Never smoker	[36]23	Polymerization	6 y before assay	None	1.53	+	–	–
Sample b	57	Never smoker		worker	4 y before assay	None	2.02	+	+	+
20	56	Smoker	[28]14	Synthesis and polymerization worker	Not applicable	RS§§	1.95	+	–	–
19										
Sample a	60	Smoker	[25]14	Mechanic/maintenance	4 y before assay	RS§§	1.88	+	–	–
Sample b	61	Smoker		worker	3 y before assay	RS§§; hepatomegaly	1.72	+	–	–
Sample c	62	Smoker			2 y before assay	(recent liver	1.62¶¶	±	–	–
Sample d	64	Smoker			1 mo before assay	nodule)‖ ‖	1.46¶¶	±	–	–
17										
Sample a	57	Smoker	[23]5	Autoclave operator	3 y, 8 mo, before diagnosis	ASL# p53MUT**	1.04	–	–	–
Sample b	61	Smoker			At death	179:CAT>CTT	1.55	+	+	+
3										
Sample a	61	Smoker	[28]28	Autoclave cleaner	At surgery	ASL; PRT.HEPTY#,##	1.03	–	ND	ND
Sample b					21 d after surgery		0.87	–	ND	ND
Sample c	61	Smoker			36 d after surgery	255:ATC>TTC**	1.11	–	ND	ND
Sample d					63 d after surgery		1.00	–	ND	ND

*Table contains data from 10 vinyl chloride workers (six of 15 with ASL and four of 77 without an ASL diagnosis) examined for serum antibodies to p53 (*see* "Subjects and Methods" section).

†Values = [total years] and years of unprotected exposure to highest levels of vinyl chloride (before 1974, when protective measures were begun).

‡Time interval in years, months, and days between collection of serum sample and diagnosis of cancer.

§Mean of three triplicate-well EIAs. – = negative; + = positive; ++ = highly positive; ± = questionably negative.

‖Results of three immunoblottings. – = negative; + = positive; ++ = highly positive; ± = questionably negative; ND = not done.

¶Results of three immunoprecipitations of in vitro translated ^{35}S-labeled p53. Volumes of sera received from these patients were frequently insufficient to support required attempts to obtain conclusive results in assays with higher concentrations. ND = not done.

#Dead.

**Missense p53 mutation showing codon and amino acid changes in exons 7, 5, and 7, respectively, from patients 26, 17, and 3.

††Recurrence of ASL seen during aborted liver explant.

‡‡Detection indicates a high probability of the presence of p53 mutations; the loss of detectable anti-p53 antibody may be due to either antigen depletion or excess, causing antigen levels to recede below assay detection levels.

§§RS = Raynaud's syndrome.

‖ ‖Liver lesions were found subsequent to the positive assays for p53 antibodies.

¶¶Only one of three triplicate-well assays was positive.

##PRT.HEPTY = partial hepatectomy, performed before serum samples were taken, and no serum samples were available just before or just after surgery. Eight serum samples collected in 7 months were negative, the first three of which (21-63 days) are shown in the table.

able for analysis (one antibody-negative worker with ASL and the antibody-negative HCC control subject) were found to have wild-type p53 in the exons examined. Two of the three workers with mutations (workers 17 and 26) had anti-p53 antibodies. Worker 3 was negative for anti-p53 antibodies.

In addition to the 15 workers with ASL, 26 workers had clinical symptoms related to vinyl chloride toxicity. These symptoms included Raynaud's syndrome (*15*), hepatomegaly (*9*), and splenomegaly (2), occurring alone or in various combinations. Seven workers with benign angiomas and two with HCC were negative for detectable anti-p53 antibodies. None of the hepatic angiomas were available for sequence analysis. Worker 17 was diagnosed with an hepatic angioma 3.4 years before presenting with ASL. He provided two serum samples: one at 3.7 years before diagnosis and one at the time of the diagnosis, after admission to the

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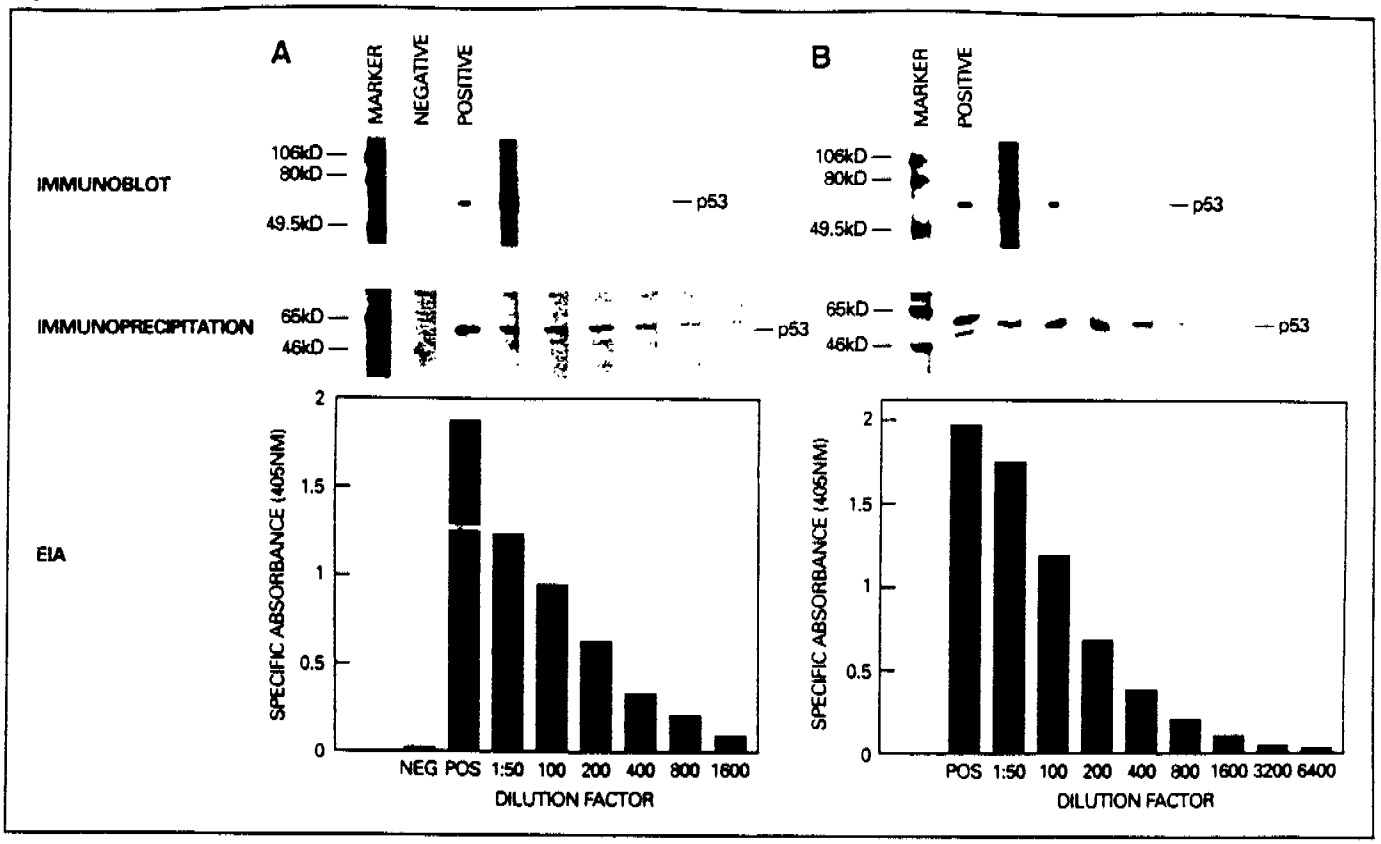

Fig. 1. Detection of serum anti-p53 antibody in vinyl chloride-exposed worker 24 by the enzyme immunoassay (EIA), immunoprecipitation, and immunoblotting (IMMUNOBLOT) assays (*see* "Subjects and Methods" section, sample analyses are vertically aligned for cross-reference). A) Detection in serum sample prepared 11 years, 4 months, before diagnosis of angiosarcoma of the liver. B) Detection in serum sample prepared 10 years, 10 months, before diagnosis of angiosarcoma of the liver. EIA and immunoprecipitation produced titers of 1600 and 800, respectively, from both serum samples. Immunoblotting achieved detection at 1 : 100 dilution only. A third serum sample prepared at 10 years, 4 months, before diagnosis (6 months later) was negative. Marker = molecular weight indicator (*see* "Subjects and Methods" section); negative = normal serum as negative antibody control; positive = rabbit anti-p53-specific antibody; kD = kilodaltons of molecular mass.

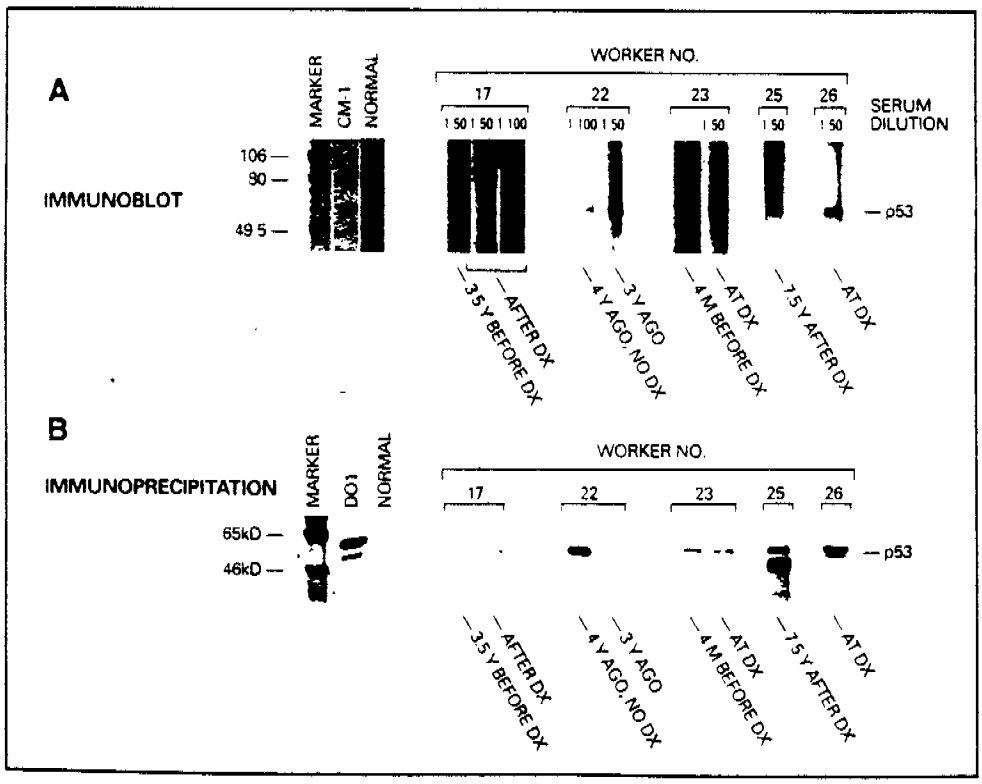

Fig. 2. Detection of serum anti-p53 antibody (*see* "Subjects and Methods" section) in vinyl chloride-exposed workers 17, 22, 23, 25, and 26 by the enzyme immunoassay, immunoblotting (IMMUNOBLOT), and immunoprecipitation assays (*see* "Subjects and Methods" section). Marker = molecular weight indicator (*see* "Subjects and Methods" section); CM-1 = rabbit anti-p53-specific antibody as positive control; normal = normal human serum as negative control; Y = years; DX = diagnosis, DO1 = p53-specific antibody-positive control; and M = months. A) Immunoblotting results show that worker 17 was negative before but positive at the time of diagnosis; worker 22, an asymptomatic worker who is still under observation, has had two positive serum samples 4 and 3 years before these assays were performed, requiring, respectively, a maximum dilution of 1 : 100 and 1 : 50 for detection; worker 23, an ASL patient now dead, had positive serum samples 4 months before and at the time of cancer diagnosis (this worker had an anti-p53 antibody titer of 400 by EIA, data not shown). B) Immunoprecipitation shows the same positive results for these workers and a more distinctly decreased titer in the second serum sample from asymptomatic worker 22.

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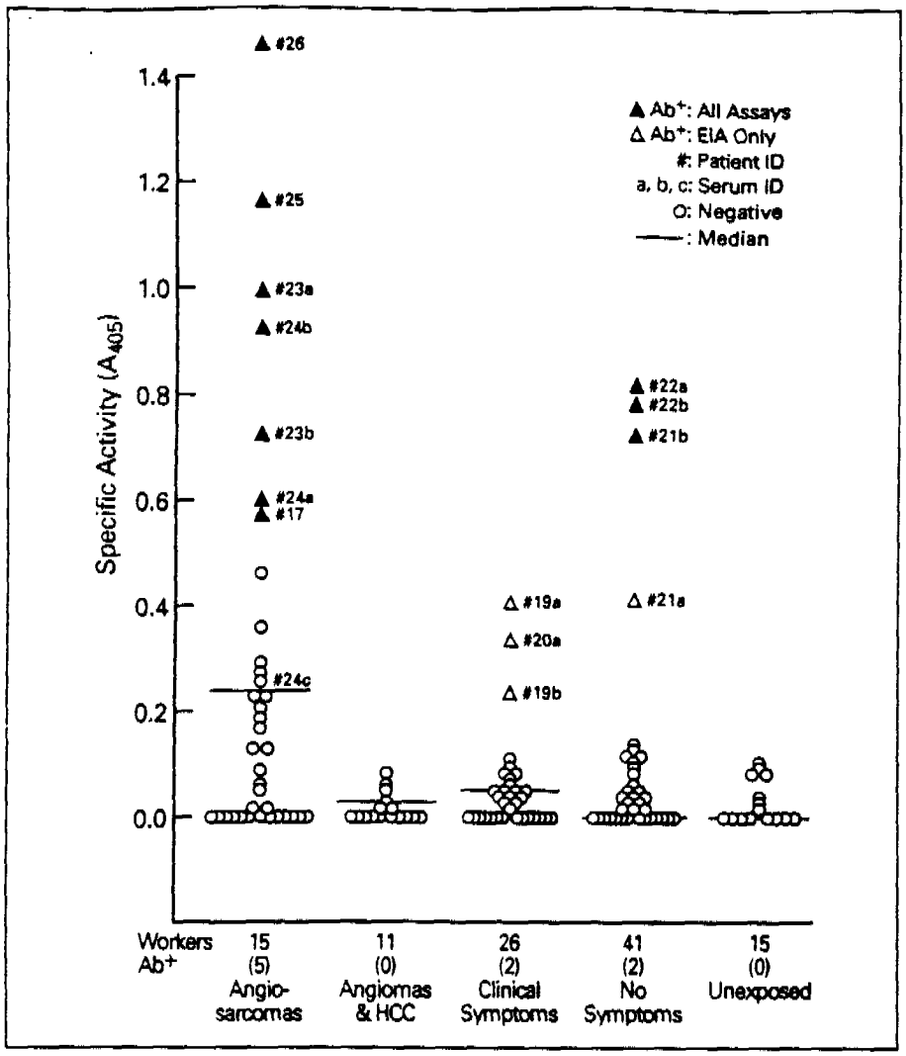

Fig. 3. Enzyme immunoassay (EIA) results shown as specific activities (mean p53 A_{405} minus mean bovine serum albumin A_{405}) of individual serum samples in groups of workers screened for anti-p53 antibody (*see* "Subjects and Methods" section) after 12-34 years of high industrial exposure to vinyl chloride: 31 serum samples from 15 workers with angiosarcoma of the liver (ASL), two of whom (patients 23 and 24) had detectable antibody before the diagnosis; 14 serum samples from 11 workers with other tumors (eight angiomas and three hepatocellular carcinomas [HCC]); 35 serum samples from 26 workers with clinical symptoms of vinyl chloride toxicity; 44 serum samples from 41 workers without symptoms; and 15 serum samples from 15 age-matched male control subjects without cancer and without industrial exposure to vinyl chloride. Results in workers with ASL were statistically significant when compared with those in workers with and without symptoms ($P \leq .001$ and $P \leq .0005$, respectively) and volunteers without vinyl chloride exposure ($P \leq .002$). Results in workers with and without symptoms were not significant when compared with those in unexposed volunteers ($P \geq .08$ and $P \geq .1$, respectively). However, the frequencies of positive vinyl chloride-exposed workers were statistically significant when compared with the results in anti-p53 antibody-positive normal and symptomatic cancer-free control subjects (three positive cases in 736) compiled from the published studies cited (*see* "Subjects and Methods" section) (i.e., versus five positive cases in 15 vinyl chloride-induced ASL: $P \leq .0001$; versus four positive cases in 77 non-ASL workers, $P \leq .001$).

hospital. Only the latter sample was positive, and the worker died shortly after admission to the hospital. Serum was unavailable immediately before and immediately after surgery (partial hepatectomy for ASL) for antibody-negative worker 3, and eight samples taken from 1.5 to 6 months after surgery were negative. (Results for the first three serum samples are shown in Table 1.) However, anti-p53 antibodies were detected by EIA in two workers (19 and 20) without clinically evident cancer (Table 1). These two workers had Raynaud's disease. Worker 19 also had hepatomegaly and later developed a liver nodule that was found subsequent to the report of finding the anti-p53 antibodies.

Discussion

The objectives of this study were to begin an elucidation of the molecular pathogenesis of ASL and to identify biomarkers for use in its early diagnosis. Workers with aggressive ASL survived about 6 months after diagnosis, and about 50% had metastases at the time of diagnosis. Long-term survival after partial hepatectomy has been observed for some ASL patients; however, because of the metastatic disease, few ASL patients survive more than 1-3 years after complete resection (*5,44*). Workers in this study often presented late, and some died the day of the ASL diagnosis.

In this study of 92 workers occupationally exposed to vinyl chloride, we have found serum anti-p53 antibodies in five (33%) of 15 workers with ASL and in four (5%) of 77 workers without clinically evident cancer. Two of the workers with clinical symptoms of vinyl chloride toxicity had antibodies detectable by one assay (EIA) only, a result similar to that reported in studies of breast cancer (*28*) and lung cancer (*31*) patients. These findings could reflect variations in assay sensitivities in different laboratories. However, we did not detect anti-p53 antibodies in three workers with HCC or in seven workers with liver angiomas. The incidence of anti-p53 antibody-positive ASL patients was somewhat higher than the 13%-25% incidence reported for breast cancer (*24,27*) and lung cancer (*29,32*) patients as well as for the incidence of most of the other types of cancer reported to date, including 20 (25%) of 80 HCC patients (*34*). The actual incidence could be lower because of the small number of ASL patients in this study. Two of three ASL patients with p53 mutations had p53 antibodies.

The most notable result was that, in two ASL patients, anti-p53 antibodies were detected from 4 months and 11 years, respectively, prior to the cancer diagnosis, and one patient had anti-p53 antibodies before and after surgery. The detection of anti-p53 antibodies this far in advance of diagnosable ASL is not clearly understood, but it could be the result of earlier antigenic presentation to the immune system, due to the more cytolytic nature of vinyl chloride toxicity. Long-term, high-level vinyl chloride exposure

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leads to extensive tissue damage and activation of multiple systemic disorders, including sclerotic syndrome, acro-osteolysis, Raynaud's-like symptoms, thrombocytopenia, and liver damage (*44*). In addition, vinyl chloride is mutagenic and carcinogenic, producing DNA strand breaks and gaps, chromosomal fragments, dicentrics, and rings (*5*). If this cellular damage occurs early in the carcinogenesis process, it provides a plausible description of conditions that might lead to early development of mutant cells (*45*) and presentation of an antigenic mutant p53 protein.

The process by which mutated p53 protein activates an immune response in humans is not clearly known at present. Mutated protein may complex with Hsp70 protein before presentation to the immune system in some breast cancer patients (*26*); missense mutations may not be associated with the epitope of the altered protein (*27,31*), and human anti-p53 antibodies may not be able to differentiate between mutated and wild-type protein (*29,30*); however, it does appear likely that mutations and accumulation of large amounts of the protein are the initiating source of its immunogenicity. The failure to detect anti-p53 antibodies in the third serum sample from previously positive worker 24 (Table 1, Fig. 3) is attributable only to the poorly understood, multifactorial immune mechanisms and host responses functioning in p53-related carcinogenesis.

The spectrum of p53 mutations can provide clues to the exogenous and endogenous mutagenic mechanisms leading to cancer (*11,14*). The finding of one additional missense mutation of the p53 gene raises the total to three of six vinyl chloride-induced tumors with mutations and extends the results of this and a previous study of ASL (*9*). These mutations appear to be consistent with the premutagenic ethenoadenosine DNA adducts induced by vinyl chloride (*46*) and might be explained by the recurring or increasing lesions caused by continuing high exposure to vinyl chloride.

To our knowledge, this is the first study of anti-p53 antibodies in patients with and without cancer who have had time-measured industrial exposure to a known chemical carcinogen, vinyl chloride. In other studies, however, anti-p53 antibodies were detected in one patient with chronic obstructive pulmonary disease and one with a benign tracheal tumor 4 months and 15 months, respectively, before a diagnosis of lung cancer (*31,47*) and in four of 36 women with a positive family history of breast cancer (*28*). In addition, we (unpublished results) have detected anti-p53 antibodies in four patients with chronic obstructive pulmonary diseases before diagnoses of cancer of the lung (6 and 7 months), breast (5 months), and prostate (11 months). These results are consistent with the finding that p53 mutation and nuclear protein accumulation can occur in preinvasive lesions during carcinogenesis in human esophagus (*48*). Cancers in which alterations in p53 have been shown to occur as early events in carcinogenesis include cancers of the lung, skin, breast, liver, and esophagus [reviewed in (*14*)]. The current data on the relationship between p53 mutations and anti-p53 antibodies indicate that studies to detect anti-p53 antibodies in populations at high risk for cancers that typically exhibit early changes in the p53 gene might be informative (Fig. 3), particularly with respect to the usefulness of antibodies as surrogates for early p53 mutations and as potential aids in the early detection of some cancers.

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Notes

Supported in part by Public Health Service grant P20ES06832-01 from the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services.

Manuscript received February 3, 1995; revised June 23, 1995; accepted July 5, 1995.

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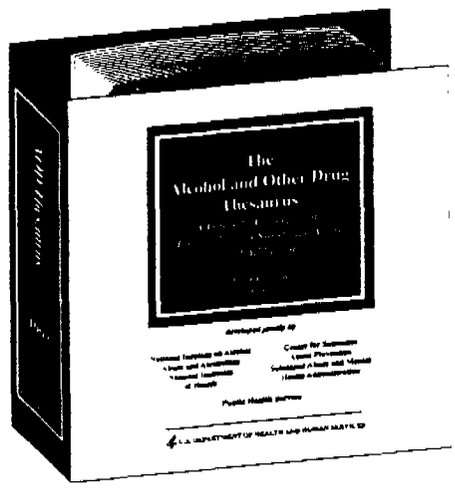

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