Time-Resolved Immunofluorometric Assay of p53 Protein

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A common feature of human tumor tissue is mutant p53 protein accumulation. Here we evaluate a new "sandwich" immunoassay for p53 protein incorporating modifications to a previously reported method, including the use of microtiter plates coated directly with the anti-p53 monoclonal antibody DO-1, a detergent- and mouse serum-containing sample diluent, and a labeled secondary antibody diluent containing goat serum. The use of CM-1 antiserum to probe the immunocaptured p53 and the detection of bound complexes by a labeled secondary antibody allows coupling to a time-resolved fluorescence detection system. The new assay yielded p53 concentrations comparable with those by the previous assay for breast tumor cytosols (n = 198), nondiseased breast tissues (n = 70), and five transformed cell lines, but showed differences in p53 values measured in sera from patients without cancer (n = 78). These serum differences were found to reflect nonspecific interferences affecting the original method, which implies that the new immunoassay has improved specificity for serum p53 quantification.

Indexing Terms: *enzyme-linked immunosorbent assay/tumor markers/breast cancer*

Mutations of the chromosome 17p13-localized *p53* tumor suppressor gene are the most frequent genetic alterations in human cancers, occurring in tumors of the colon (*1*), breast (*2*), lung (*3*), ovary (*4*), and virtually every other tissue (*5–7*). It is currently believed that abrogation of *p53* function by mutation disables critical regulatory pathways, induced by DNA damage, that lead either to cell cycle arrest at the G1/S phase boundary (*8*) or to apoptosis (*9*). Loss of *p53* function is therefore expected to favor the propagation of mutations throughout the genome, some of which may promote tumor cell evolution. The detection of *p53* gene abnormalities, which occur predominantly as missense point mutations in conserved regions coupled with loss of heterozygosity (*10*), has been shown to be of clinical significance, given that the presence of a mutated *p53* gene in tumor tissues is often associated with aggressive tumor phenotypes (*11–13*).

Correlating very closely with *p53* genetic changes is the accumulation of a conformationally altered mutant p53 protein, usually in the nucleus of transformed and tumor-derived cells (*14*). Normal cells, in contrast, typically have undetectable concentrations of wild-type p53 protein. Because of the relative ease of the immunochemical detection of p53 protein compared with the approaches for targeting changes at the genetic level, immunohistochemical techniques to visualize p53 protein in situ, with either monoclonal antibodies against mutant p53 protein or polyclonal antisera, have become the standard tools used in the majority of studies reporting p53 protein overexpression (*15*). Using such methods, investigators have demonstrated that the overexpression of p53 protein in a patient's tumor tissue may predict reduced disease-free survival and (or) overall survival for several tumor types (*16–18*). However, quantitative immunological methods for p53 protein measurement in cellular extracts offer an alternative approach for quantifying p53 protein in tumor tissues and in cultured cell lines.

Despite the potential advantages of immunological assay systems (*19*), the availability of a large number of p53-specific monoclonal and polyclonal antibodies, and the recognition that more precise quantitative estimations of p53 accumulation in tumor tissues are needed than are at present provided by immunohistochemical methods (*20*), few immunoassays for quantifying soluble p53 protein have been described (*21–23*); nonetheless, two p53 ELISA kits are commercially available (Oncogene Science, Uniondale, NY). A p53 immunoassay developed in our laboratory (*23*) has been used to quantify p53 protein in tissue extracts prepared from breast (*23–25*) and ovarian (*18*) tumors. In this assay design, a mouse monoclonal anti-p53 antibody was immobilized on microtiter plates precoated with anti-mouse immunoglobulin. The monoclonal antibody-bound p53 was then probed with polyclonal rabbit antisera raised against recombinant p53. The subsequent addition of enzyme-labeled anti-rabbit immunoglobulin allowed coupling of the immunocaptured p53 protein with a sensitive time-resolved fluorescence detection system (*26*).

Although this assay was successful when applied to tumor tissue extracts, preliminary studies with serum specimens indicated nonspecific interference by serum components. In the present study, we have developed and evaluated a new assay that incorporates one of a new generation of anti-p53 antibodies as the initial capture immunoreagent; we also added blocking agents (animal serum and detergent) to sample and

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labeled antibody diluents. The result is a p53 assay suitable for any sample type, including serum.

Materials and Methods

Production of Monoclonal Antibodies

The DO-1 hybridoma cell line (*22*), provided by David P. Lane (University of Dundee), was cultured in Dulbecco's modified Eagle's medium supplemented with 100 mL/L fetal bovine serum, 100 mg/L sodium pyruvate, and 50 g/L gentamycin (all from Gibco BRL, Gaithersburg, MD). Approximately 5×10^6 log-phase growing cells were collected, washed with phosphate-buffered saline (PBS), and injected intraperitoneally as a 0.5-mL suspension in PBS into adult female BALB/c mice that had been primed 7 days earlier by injection with pristane.[5] Ascites fluid removed after 1 to 3 weeks was subjected to Protein A affinity chromatography with Econo-Pac Protein A columns (Bio-Rad Labs., Richmond, CA), according to the manufacturer's instructions, after which the eluates were desalted by dialysis against 0.1 mol/L $NaHCO_3$. The concentration of DO-1 antibody was determined spectrophotometrically at 280 nm.

Production of Recombinant p53 Protein and Preparation of Calibrators

Sf9 insect cells were cultured in Grace's insect medium with supplements (Invitrogen, San Diego, CA) and infected with recombinant baculovirus for both viral amplification and protein expression according to routine practices (*27*). Low-titer stock of a baculovirus vector containing the full-length wild-type human *p53* gene (gift of T. Soussi, INSERM, Institut de Génétique Moléculaire, France) was used to infect log-phase Sf9 cells; 5 days later, the supernate was harvested and used to reinfect Sf9 cells at a multiplicity of infection between 5 and 10. Infected cells were collected after 48 h, washed 3 times in PBS, and lysed in a lysis buffer (10 mmol/L Tris, pH 8.0, 1 mmol/L EDTA, 150 mmol/L NaCl, 10 mL/L NP-40, 10 g/L sodium deoxycholate, 1 g/L sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/L pepstatin, and 10 mg/L each of leupeptin and aprotinin) on ice for 30 min. The soluble extract was collected after ultracentrifugation at 100 000*g* for 30 min at 4 °C and aliquoted for storage at −80 °C. The presence of p53 protein was confirmed by Western blot analysis, i.e., electrophoresis of the Sf9 lysate on a precast 8–16% Tris-glycine minigel (Novex, San Diego, CA), transfer to a nitrocellulose membrane, probing with polyclonal CM-1 antiserum (*21*) (Novocastra Labs., Newcastle upon Tyne, UK) diluted 1000-fold in buffer A [50 mmol/L Tris, pH 7.80, 60 g/L bovine serum albumin, and 0.5 g/L NaN_3], and detection with the ECL Western blotting kit (Amersham, Bucks, UK).

Sf9 lysate containing p53 protein was assayed at several dilutions with our new immunofluorometric procedure (see below) to determine the concentration of p53 relative to arbitrary calibrators prepared from a breast tumor lysate containing a very high concentration of p53 protein (*24*). Assay of lysates prepared from uninfected Sf9 cells demonstrated undetectable results for p53 protein. On the basis of assay results of several dilutions made in buffer A of the Sf9 cell lysate, we estimated that the concentration of p53 protein in the undiluted Sf9 lysate exceeded 40 000 arbitrary units per liter (units/L), or 130 000 units/g of total extract protein. Dilutions of the p53-quantified Sf9 lysate in buffer A yielded calibrator solutions of 0, 2, 10, 50, 200, and 1000 units/L, which were aliquoted and stored at −80 °C until use. Comparison of these calibrators with p53 calibrators purchased from Oncogene Science showed the following relationship: 1 μg/L of p53 protein (Oncogene Science) ≈13 units/L (our arbitrary calibrator).

Optimization of Assay Conditions

Variations of the previously described immunofluorometric assay for p53 protein (*23*, *24*), hereafter referred to as the "original assay," were first evaluated by assaying in duplicate 50-μL volumes of p53 calibrators prepared by dilution of a breast tumor extract. In common with all of the configurations described in this report were the use of 96-well white polystyrene microtiter plates (Dynatech Labs., Chantilly, VA) and a programmable plate washer (Adil Instruments, Strasbourg, France). The wash solution consisted of 5 mmol/L Tris, pH 7.80, containing 150 mmol/L NaCl and 0.5 g/L Tween 20 (Sigma, St. Louis, MO). All assay configurations also shared a final enzymatic activity measurement step. This included addition of a stock solution of 0.01 mol/L diflunisal phosphate in 0.1 mol/L NaOH, diluted 10-fold in 0.1 mol/L Tris, pH 9.10, containing 0.15 mol/L NaCl, 1 mmol/L $MgCl_2$, and 0.5 g/L NaN_3 as a substrate for alkaline phosphatase, which was conjugated to one of the immunoreagents. In all cases, incubation of the wells with 100 μL of this enzyme substrate for 10 min at room temperature (~25 °C) on a shaker platform was followed by the addition, without washing the plates, of 100 μL of a developing solution of 1 mol/L Tris, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA; after a 1-min incubation with shaking, fluorescence at 615 nm was measured in a time-resolved mode on a Cyberfluor-615 Immunoanalyzer (Cyberfluor, Toronto, Canada). Calibration curve-fitting and interpolation to determine unknown analyte concentrations were performed by the instrument software. The advantages of lanthanide chelates as fluorescence labels and of time-resolved fluorometry have been discussed previously (*26*, *28*).

To assess differences between direct vs indirect methods of coating the microtiter plates with anti-p53 antibodies, we used plates coated overnight at 4 °C either directly or indirectly with antibody solution, 100 μL per well. For direct coating we used monoclonal antibodies PAb240, recognizing the p53 protein in a

[5] Nonstandard abbreviations: GaMIg, goat anti-mouse immunoglobulin; GaRIg-ALP, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin; PBS, phosphate-buffered saline; TSH, thyrotropin; and LH, lutropin.

mutant conformation (*29*); panspecific PAb421, recognizing both mutant and wild-type p53 (*30*); DO-1, also recognizing both forms; or a monoclonal antibody against human thyrotropin (TSH) (clone 5404; Medix Biochemica, Kauniainen, Finland) as a negative control. In other configurations, wells were coated indirectly by first coating with 100 μL of goat anti-mouse immunoglobulin (GaMIg; Jackson ImmunoResearch, West Grove, PA). All antibodies for plate coating were diluted in Tris buffer, 50 mmol/L, pH 7.40, containing 0.5 g/L NaN_3, and used at concentrations of ~4 mg/L (100 μL per well); antibodies against p53 had been previously purified from murine ascites fluid as described above for DO-1. The hybridoma cells producing PAb240, PAb421, and DO-1 were provided by David Lane (University of Dundee). Microtiter wells indirectly coated with GaMIg were subsequently incubated with either PAb240, PAb421, or DO-1 cell culture supernates at 50-fold dilutions in buffer A containing 0.5 mol/L KCl. We have used as our original assay method the configuration consisting of plates coated first with GaMIg to capture subsequently added PAb240. In control wells we added the anti-TSH antibody (2 mg/L) diluted in the same buffer. In the directly coated plates, the p53 calibrators were added along with assay buffer (50 μL each). In the GaMIg-coated plates, the p53 calibrators were added at the same time as the anti-p53 antibody (also 50 μL each). Thereafter, all assays were identical: incubation with shaking at 37 °C for 3 h; washing of the wells four times; incubation with 100 μL of polyclonal CM-1 antiserum diluted 5000-fold in buffer A for 1 h at 25 °C; washing four times; incubation with 100 μL of 0.12 mg/L goat anti-rabbit IgG conjugated to alkaline phosphatase (GaRIg-ALP) (Jackson ImmunoResearch); washing six times; and finally the detection procedure as described above.

Other experiments were carried out to determine the optimal concentration of DO-1 and duration of incubation for microtiter plate coating; the effect of diluting samples twofold in the wells with buffer A supplemented with 0.5 mol/L KCl, 10 mL/L mouse serum, and 5 mL/L Tween-20 detergent; the optimal sample incubation duration and temperature; the optimal duration, temperature, and antibody concentrations (CM-1 and GaRIg-ALP) of subsequent incubation steps; and the effect of adding various amounts of goat serum to the GaRIg-ALP diluent.

The assay of all calibrators, controls, cell lysates, and clinical specimens (see below) were performed in duplicate unless otherwise stated.

Assay Evaluation

The use of all materials of human origin to evaluate the new immunoassay had been approved by the Committee for Research on Human Subjects at the University of Toronto. Clear, turbid, or hemolyzed serum samples were supplemented with lysates from Sf9 cells expressing recombinant p53 and lysates from COLO 320HSR cells [obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium supplemented with 100 mL/L fetal bovine serum], cells known to overexpress mutant p53 (*23*, *31*). The COLO 320HSR cell extract was prepared by lysing ~10^7 cells for 30 min on ice with 300 μL of a buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mL/L NP-40, 10 mg/L phenylmethylsulfonyl fluoride, and 1 mg/L each of leupeptin and aprotinin, followed by centrifugation for 30 min at 14 000*g* at 4 °C to collect the supernate. Each of the serum samples, and buffer A as a control, were used to dilute Sf9 lysate 400-fold and COLO 320HSR lysate 10-fold. Recovery of p53 protein in each serum was expressed as the ratio of the p53 protein concentration measured in the serum (to which either cell lysate had been added) to the p53 protein concentration measured in buffer A to which an equivalent amount of the corresponding lysate had been added. Three serum specimens were also supplemented with COLO 320HSR lysate and assayed for p53 protein immediately and after a 1-h incubation at 37 °C. Sera and buffer A containing no added lysate were also assayed similarly. Lysate prepared from 10^7 COLO 320HSR cells was serially diluted twofold in buffer A to yield 16 p53-containing solutions. This dilution series was used to assess the linearity of the new assay. Within-assay precision was determined from the assay of three breast tumor extracts with p53 protein concentrations of ~0.15, 0.75, and 4.5 μg/L.

Immunoassay Applications

We compared the new assay with the original assay for the analysis of soluble extracts from breast tumors, nondiseased breast tissue, and transformed cell lines and for sera collected routinely from hospitalized patients.

Breast tumor cytosolic extracts prepared for routine steroid hormone receptor analysis, as described previously (*24*), were provided by a regional service laboratory at Sunnybrook Health Sciences Centre in Toronto, Ontario. Consecutive specimens (n = 198) were assayed for estrogen receptors and progesterone receptors by enzyme immunoassay kits (Abbott Labs., North Chicago, IL) and stored at −80 °C until assayed for p53 by both methods in parallel. All results were expressed relative to the total protein content of the extracts, measured by the Lowry method (*32*). For both steroid hormone receptors, 10 fmol/mg was used as the cutoff for positive receptor status, as in previous work by our group (*24*). Cytosols were rerun in dilution if the initial p53 protein concentration exceeded 75 μg/L.

Bilateral breast tissue specimens were also obtained from 35 women who underwent cosmetic breast reduction surgery. Representative portions of each of these tissues (n = 70), snap-frozen immediately after surgery, were pulverized and extracted as described previously (*18*), before assaying for p53 protein. Concentrations of p53 were expressed as micrograms per gram of total protein in the extract, the latter being determined by a commercially available kit based on the bicinchoninic acid method (Pierce, Rockford, IL).

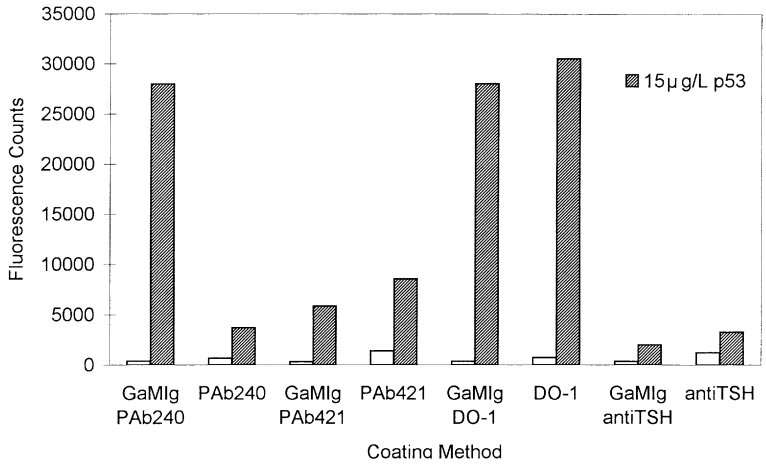

Fig. 1. p53-associated fluorescence from immunoassay designs involving direct or indirect coating with anti-p53 or irrelevant antibodies.

Unshaded bars indicate background fluorescence.

Extracts from cultured cell lines were prepared as described above for COLO 320HSR. The COLO 320HSR, T-47D, DU-145, and MCF-7 cell lines were obtained from the ATCC; the pRNS-1 cell line was a gift from Donna Peehl (Stanford University School of Medicine). Culture media for the COLO 320HSR and T-47D cell lines consisted of RPMI-1640 supplemented with 100 mL/L fetal bovine serum and, in the case of T-47D, with 10 mg/L insulin. The MCF-7 and DU-145 cell lines were cultured in Eagle's minimal essential medium with nonessential amino acids supplemented with 1 mmol/L sodium pyruvate, 1 mg/L insulin, and 100 mL/L fetal bovine serum. For culturing the pRNS-1 cells, we used keratinocyte serum-free medium supplemented with 5 μg/L epidermal growth factor and 50 mg/L bovine pituitary extract. All culture reagents were from the same supplier (Gibco BRL). Both immunoassays were used to quantify p53 protein in the cellular extracts relative to the total protein concentration, measured by Pierce protein assay kit. Extracts with p53 values >75 μg/L were repeated in dilution.

Randomly selected serum specimens (n = 78) collected for routine bloodwork on hospitalized patients were provided by the Department of Pathology and Laboratory Medicine at Mount Sinai Hospital in Toronto, Ontario. These serum specimens had been stored for no longer than 7 days at 4 °C before simultaneous assay by both p53 immunoassays and by certain variations of the method—e.g., with the PAb240 capture antibody replaced by a monoclonal antibody against the irrelevant antigen lutropin (LH, clone 5301; Medix Biochemica) or omitting the primary capture antibody altogether and adding only diluent. The latter two assay configurations served as negative controls with which to reveal nonspecific interferences from serum, if any, in the original method.

Statistical Methods

Determination of descriptive statistics and Pearson correlation coefficients, and the analysis of 2 × 2 contingency tables by χ^2 tests, were performed by SAS version 6.02 software (SAS Institute, Cary, NC).

Results

The ability of an anti-p53 monoclonal antibody, directly coated onto microtiter wells, to function in a "sandwich-type" immunoassay was demonstrated for DO-1 (Fig. 1). Unlike PAb240 or PAb421 antibodies, in the presence of p53-containing solutions (15 mg/L given as an example) conjugates of DO-1 could generate high fluorescence counts whether directly coated to wells or incubated in wells precoated with GaMIg. The fact that DO-1 was as efficient as PAb240 in an assay configuration where immunocaptured p53 protein is detected by polyclonal CM-1 antiserum suggests that DO-1 does not mask a critical epitope recognized by the polyclonal rabbit antibodies. The assay of buffer A alone (sample or calibrator diluent) gave the background fluorescence for each assay design, the quantity of which was similar in all cases. As a consequence, the indirect PAb240 and direct DO-1 configurations had comparable signal-to-background ratios for a given p53 calibrator. When an antibody against human TSH was incorporated into the direct- and indirect-coating assays at concentrations equivalent to that used for the anti-p53 antibodies, the low fluorescence demonstrated the requirement for p53-specific IgG to generate signal. Separate experiments have shown that optimal coating amounts for DO-1 were 400 ng per well. Optimal incubation times were 3 h at 37 °C for the first incubation and 1 h at room temperature for the second incubation. Further optimization of the composition and amounts of other reagents used in the new assay are listed below. A diagrammatic representation of our assay is shown in Fig. 2.

As in the assay design for the immunofluorometric measurement of prostate-specific antigen (*33*), this newly developed assay includes the addition of goat serum to the diluent of the GaRIg-ALP to reduce background signal by neutralizing the anti-goat antibodies present in some sera. These antibodies would be especially problematic to the indirect microtiter well-coating methods used here, because of possible cross-linking of the GaMIg antibodies on the well surface and the subsequently added GaRIg-ALP enzyme-labeled antibodies, which

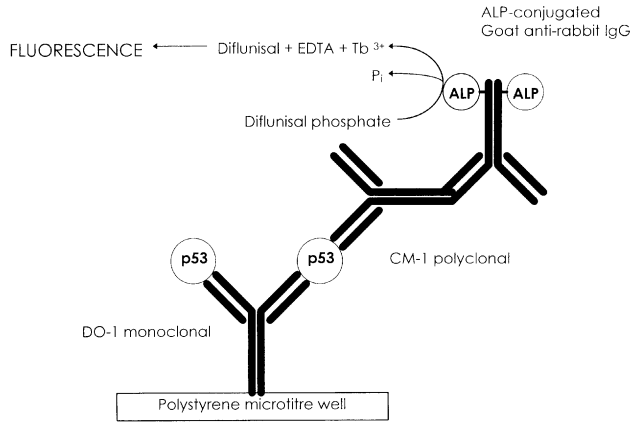

Fig. 2. Schematic of new p53 immunofluorometric assay configuration.

Reagent concentrations, incubation times, and washing steps are discussed in the text.

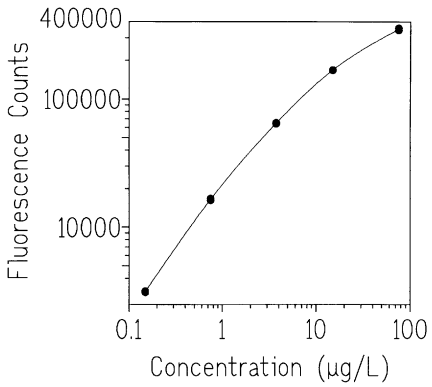

Fig. 3. Calibration curve of new assay.

would result in non-p53-dependent immobilization of labeled detection antibody to the wells and give false-positive signals. One remedy for this problem was the use of excess unlabeled goat IgG derived from the goat serum added to the GaRIg-ALP diluent. We suspect that saturation of free epitope-binding sites of nonspecifically immobilized human anti-goat antibodies by the nonlabeled IgG might therefore contribute to reducing background fluorescence.

Given the results and practical considerations mentioned above, the assay design selected, as depicted in Fig. 2, included the following features: microtiter wells coated overnight with 400 ng (100 µL) of DO-1 per well; twofold dilution of samples and calibrators in the wells (50 µL each) with buffer A containing 0.5 mol/L KCl, 10 mL/L mouse serum, and 5 mL/L Tween 20 detergent, with incubation for 3 h at 37 °C; addition of 100 µL of polyclonal CM-1 antiserum diluted 5000-fold in buffer A, with incubation for 1 h at 25 °C; addition of 12 µg/L (100 µL) GaRIg-ALP diluted in buffer A containing 0.5 mol/L KCl and 100 mL/L goat serum, with a 1-h incubation at 25 °C; and the addition of enzyme substrate and developing solution as described above for time-resolved fluorescence measurement. Fig. 3 displays a representative calibration curve.

When the new p53 immunoassay was applied to nine patients' sera supplemented with p53 protein from two different cell lysates (COLO 320HSR and Sf9), the recovery of p53 relative to that in buffer A (sample and calibrator diluent) ranged from 75% to 131% for the COLO 320HSR lysate and from 72% to 98% for the Sf9 lysate (Fig. 4). Because of the greater dilution of Sf9 lysate in each serum specimen, the total amount of p53 protein added to specimens via the Sf9 lysate was lower than that added to the same sera supplemented with the lysate from the colon carcinoma cell line. The background fluorescence was uniformly low in all sera and only slightly above that of buffer A (data not shown).

The detection limit of the assay, calculated as the

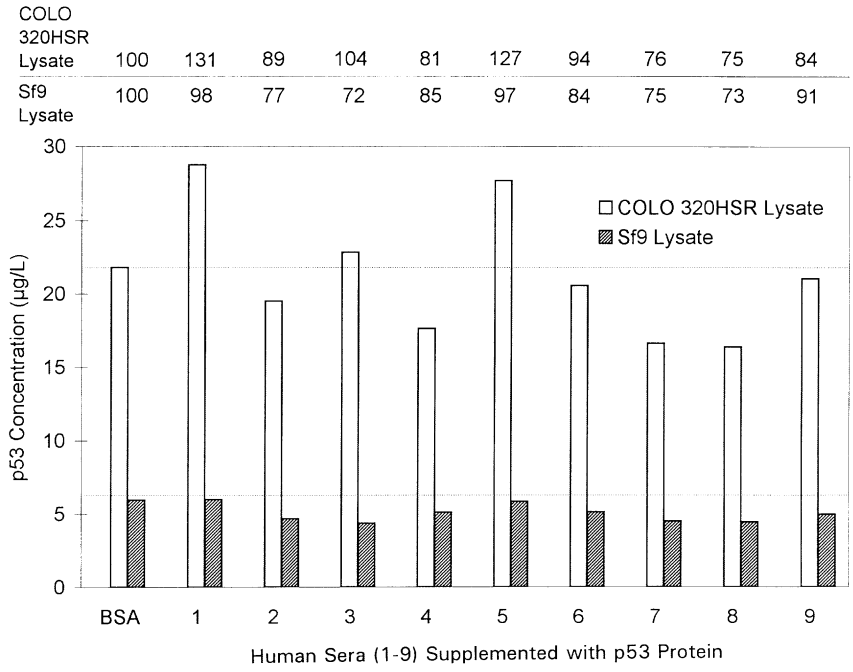

Fig. 4. Recovery of p53 protein from human sera.

p53 was added from lysates of COLO 320HSR cells, or from Sf9 cells infected with a recombinant baculovirus expressing the wild-type *p53* gene. The *broken lines* indicate 100% recovery for each lysate. Recovery from each cell lysate relative to that from buffer A is listed *above* chart.

Table 1. Descriptive statistics of clinical specimens assayed for p53 protein.

Specimen	n	p53 concentration Mean	SD	Median	Min.	Max.
Breast tumor tissue extracts[a]						
Original assay	198	0.90	6.08	0.10	0	83.33
New assay	198	0.60	1.80	0.18	0	19.88
Nondiseased breast tissue extracts[a]						
Original assay	70	<0.04	0.02	<0.04	0	0.05
New assay	68	<0.04	0.02	<0.04	0	0.08
Hospitalized patients' sera[b]						
Original assay	78	0.75	2.33	0.09	0	15.23
New assay	78	<0.04	0.05	0	0	0.38

[a] p53 concentrations expressed in μg/g of total protein.
[b] Noncancer patients, p53 concentrations expressed in μg/L.

p53 concentration that could be discriminated from zero with 99% confidence, was 0.5 units/L (~0.04 μg/L of p53). The range of p53 concentrations yielding a linear assay response was 2–1000 units/L (corresponding to 0.15–75 μg/L) . The calculated values for intraassay precision at p53 concentrations of 0.15, 0.75, and 4.5 μg/L were 11%, 9%, and 7%, respectively; the interassay precision at the same concentrations (10 measurements over 1 week) was 15%, 12%, and 8%, respectively.

Both the original and new immunoassay configurations were used to assay the cytosolic extracts of 198 breast tumors for p53 protein concentration, the distribution of which, determined by each of the two methods, is summarized in Table 1. Pearson correlation analysis indicated good correlation ($r = 0.93, P < 0.001$) between the methods (Fig. 5). A frequency distribution of p53 values obtained by the new assay of tumor extracts is shown in Fig. 6. When dichotomized according to an arbitrary cutoff of 0.38 μg/g of total protein (or 5 units/g as previously described) (*25*), the rates of positivity for p53 were 15% and 23% by the original and new methods, respectively. Regardless of the p53 assay method used to categorize the specimens, contingency table analyses revealed p53-positivity status to be strongly associated with negative status for estrogen receptors or progesterone receptors (Table 2).

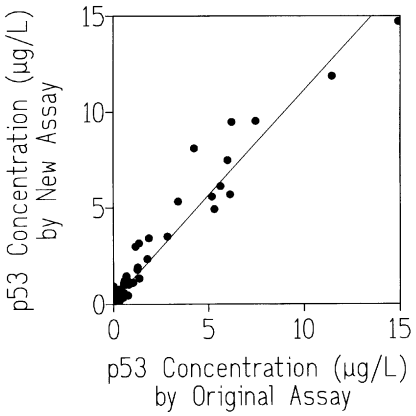

Fig. 5. Correlation between p53 concentrations measured from breast tumor cytosols by original and new immunoassay methods.

The assay of nondiseased breast tissue yielded p53 protein values far lower than those obtained from the analysis of breast neoplasms. This is illustrated by the minimal overlap between the p53 concentration frequency distributions generated by the assay of normal and diseased tissues by either assay design (Table 1

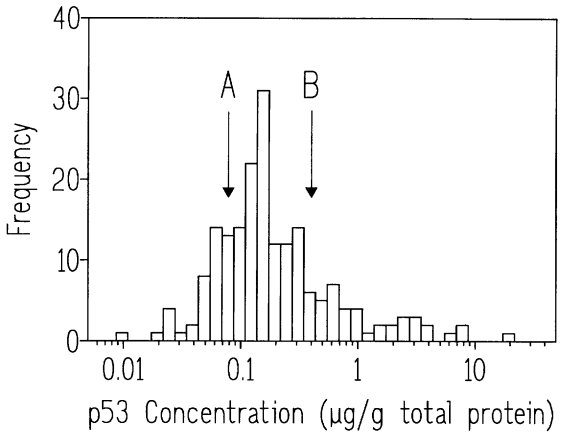

Fig. 6. Frequency distribution of p53 concentrations in breast tumor cytosols assayed by the new method.
A, 100th percentile of p53 values in nondiseased breast tissues; *B*, arbitrary cutoff for p53 overexpression (0.38 μg/g) in breast tumors used in this study.

Table 2. Relationships between p53 protein status and steroid hormone receptor status of breast tumor specimens.

Receptor conc, fmol/mg	No. of specimens (and %) p53 <0.38 μg/g	p53 ≥0.38 μg/g	P
Old assay			
ER < 10	47 (71.2)	19 (28.8)	
ER ≥ 10	122 (92.4)	10 (7.6)	<0.001
PR < 10	52 (75.4)	17 (24.6)	
PR ≥ 10	117 (90.7)	12 (9.3)	0.004
New assay			
ER < 10	41 (62.1)	25 (37.9)	
ER ≥ 10	112 (84.8)	20 (15.2)	<0.001
PR < 10	47 (68.1)	22 (31.9)	
PR ≥ 10	106 (82.2)	23 (17.8)	0.025

ER, estrogen receptor concentration; PR, progesterone receptor concentration.

and Fig. 6). Because the measured p53 protein values of all of the nondiseased breast extracts fell below the analytical detection limits of both assay techniques (~0.04 μg/L), the lack of a strong correlation ($r = 0.40$, $P < 0.001$) between the original and new methods was not surprising.

We were interested in investigating, in a prospective study, whether mutant p53 protein enters the blood circulation and, if so, whether its concentration in blood is related to the concentrations of p53 in the tumor. Matched tumor specimens and samples of both pre- and postsurgical sera are now being collected for this purpose. The ability to detect p53 in serum is dependent on a sensitive and specific analytical method. When we added p53 protein to serum, the recovery was almost complete (Fig. 4). We further investigated whether the analysis for p53 in undiluted sera from 78 hospitalized patients without cancer would yield negative results. In contrast to the new assay, which yielded results <0.15 μg/L for all sera tested except two (with values of 0.23 and 0.38 μg/L), the original assay gave values >0.15 μg/L in 29 specimens and >0.38 μg/L in 15. Moreover, 87% (65 of 78) of sera measured by the new assay had p53 values below the detection limit of 0.04 μg/L, compared with only 21% (16 of 78) when measured by the original method. Consequently, the correlation between the two methods for the analysis of these sera ($r = 0.67$, $P < 0.001$) was intermediate between those found for the analyses of breast tumors and nondiseased breast tissues. To determine whether these sera contained interfering substances, we assayed them in parallel by two methods in which another irrelevant capture antibody (anti-LH monoclonal) was utilized instead of PAb240 or no capture antibody was used. Application of these latter two modifications to the original assay configuration, in which GaMIg was immobilized on the solid phase, gave the results shown in Fig. 7. Clearly, these sera, which gave undetectable p53 values by the new assay configuration, contained substances that interfered with the original assay and generated a signal with both specific and nonspecific monoclonal capture antibodies (anti-p53 or anti-LH, or none at all). We hypothesize that the interfering agents act by cross-linking the coating GaMIg and detection GaRIg-ALP antibodies, which have been generated in goats. These interfering substances are probably human anti-goat antibodies, given that inclusion of goat serum into the assay buffer diminished but did not completely eliminate the problem (data not shown). With use of directly coated monoclonal anti-p53 mouse antibodies, this problem is eliminated.

The two methods (original and new) were also used to measure p53 in the cell lysates of various cell lines; the results are presented in Table 3.

Discussion

Investigations aimed at determining the role of *p53* in a given malignancy have generally followed one of two parallel paths: either identification of mutations in the *p53* coding sequence, or the demonstration of p53 protein accumulation within affected cells. In either approach, a wide variety of analytical techniques may be applied (*34*). Although direct sequencing provides unequivocal evidence of mutational events, indirect methods including single-strand conformation analysis and constant denaturant gel electrophoresis may also be informative for alterations within the *p53* gene. Complexity and cost have limited the ability to sequence all 11 exons of the *p53* gene in large numbers of

Table 3. p53 protein concentrations in cell lines.

		p53 conc, μg/g	
Cell line	Source	Old assay	New assay
COLO320 HSR	Colon	82.23	49.85
T-47D	Breast	20.46	27.51
DU-145	Prostate	5.70	7.88
MCF-7	Breast	22.95	45.12
pRNS-1	Prostate	107.64	93.05

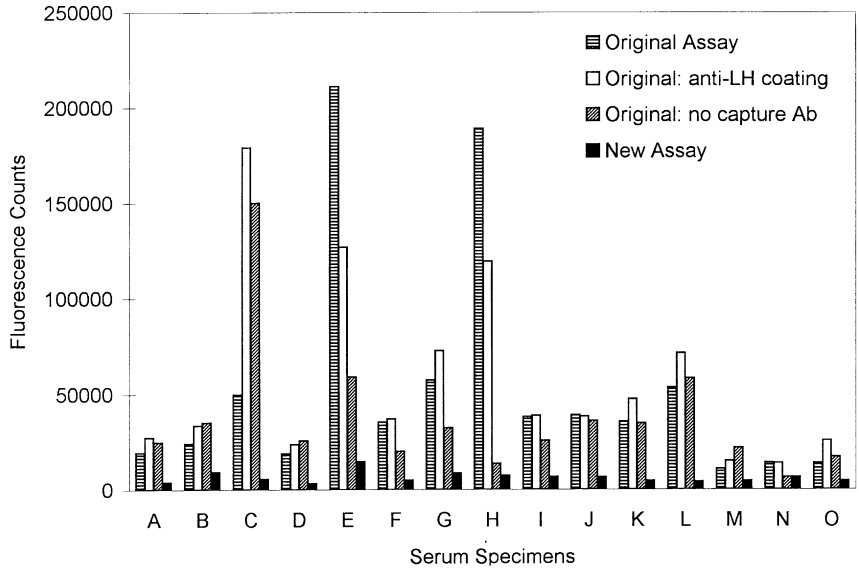

Fig. 7. Results of various methods for serum specimens displaying high p53 values (>0.38 μg/L) by the original method.

clinical specimens. Far more common have been attempts to show p53 protein overexpression, particularly by conventional immunohistochemistry, in studies questioning the clinical implications of *p53* alterations.

Immunohistochemistry also has its limitations for p53 protein detection in tissues (*35*). Most important is the lack of consensus with regard to the appropriate methodology and scoring system for the interpretation of staining patterns, which may vary considerably depending on the choice of antibodies and fixation methods (*36*, *37*), as well as interobserver variability in application of a given scoring system. We have suggested elsewhere (*19*) that ELISA-type methods for p53 may overcome many of these pitfalls by reporting quantitative results, which are more objective and possess further advantages that make them ideal for use in clinical studies. The requirement for p53 protein to bind simultaneously two antibodies enhances specificity, and washing tissue extracts from the wells after initial sample incubation reduces background signal; moreover, ELISAs demand less technical expertise and can be set up to analyze large batches of specimens.

The number of ELISA methods developed for p53 protein quantification remains small. In that developed by Midgley et al. (*21*), monoclonal antibody PAb421 was directly coupled to the microtiter well surface, and PAb421-bound p53 was detected by probing with polyclonal CM-1 rabbit antisera, subsequent addition of swine anti-rabbit antibody conjugated to horseradish peroxidase, and visualization with tetramethylbenzidine. In the process of evaluating new monoclonal antibodies against p53, the same group reported (*22*) that DO-1 was suitable for immunohistochemical or immunoblotting procedures and was functional in an ELISA in which DO-1 replaced PAb421. The latter assay has been applied extensively by these workers, including its use in comparing the relative merits of ELISA and immunohistochemistry for p53 analysis (*38*, *39*). More recently, a "sandwich" luminometric immunoassay for p53 protein, with a lower detection limit than conventional microplate ELISAs, has been described (*40*).

An immunoassay against p53 had also been reported by our group, with PAb240 and CM-1 as the capture and detection antibodies, respectively (*23*, *24*). Because PAb240 performed poorly when coated directly to plates, it was added instead to GAMIg-coated wells. With the introduction of newer monoclonal antibodies against p53, demonstrated to have utility for use in a wider range of histological specimens, and because of the difficulties encountered when assaying sera, we investigated making alterations in our original configuration of the immunoassay. Our detection system involved enzymatic hydrolysis of the ALP substrate diflunisal phosphate, which could form a ternary complex with EDTA and the lanthanide metal Tb^{3+}, such that the fluorescence (emitted at 615 nm) persists for milliseconds, far longer than the inherent fluorescence of most sample matrices. The same detection system is used in our new p53 immunoassay, as described here. The basic assay configuration in terms of choice and orientation of anti-p53 antibodies is similar to that in the method of Vojtesek et al. (*22*), but their method does not incorporate further measures for reducing background signal and therefore is less sensitive.

The most frequent specimen type to which p53 ELISA methods have been applied are tumor tissue extracts. Our new assay obtained a broad range of p53 values in breast tumor cytosols, but generally these values were higher than those obtained in normal breast tissues, in the great majority of which p53 was undetectable. We propose that the p53 values in the latter group of specimens represent the "normal" reference range for p53 protein in breast tissue. This reference range, to our knowledge, has not been reported previously, and the selection of a cutoff point for p53 positivity exceeding the 100th percentile of this normal range is a highly conservative choice. Categorizing the breast tumor extracts on the basis of a cutoff of 0.38 μg/g of total protein (5 units/g) gave a p53-positivity rate of 23%, in general accordance with our earlier observations in breast tumors (*24*, *25*) and with others who used ELISA (*41*) or various immunohistochemical techniques (*42*, *43*). The use of this cutoff point permitted the demonstration of the negative association between p53 status and estrogen or progesterone receptor status, which we have reported previously (*23–25*). The determination of negative hormone receptor status is generally thought to predict poor response to endocrine (i.e., anti-estrogen) therapy (*44*) and may be associated with an unfavorable prognostic outcome (*45*).

The analysis of sera from noncancerous hospitalized patients by our original method uncovered an unexpectedly high rate of p53 positivity. The same sera assayed in parallel by our new method exhibited mostly background amounts of fluorescence. Control assays confirmed that the high fluorescence signals for these sera by the original method were entirely due to nonspecific interference. The results of the assay of the 78 sera by the new method, designed to suppress such serum-associated nonspecificity, may constitute a reference range for hospitalized patients—for whom essentially all of the p53 values fell below 0.38 μg/L. Using a commercial kit (Oncogene Science), Greco et al. (*46*) reported similar concentrations of p53 protein in the sera of blood donors. In contrast, other workers (*47*) using a similar kit reported concentrations of p53 protein ≥2.30 μg/L in the sera of hospital control subjects. Clearly, specificity is an important consideration in assessing the applications of ELISAs to clinical studies involving p53.

The assay for p53 protein in cell lines raises several issues, of which we at present have only a limited understanding. For example, the assay of many cultured cell lysates diluted in a neutral buffer equivalent to PBS results in a nonlinear decrease in the p53 concentrations measured by either immunoassay, which implies that some components of the extracts

may bind p53 and release it only upon further dilution (unpublished data). Furthermore, we saw substantial differences between the p53 concentrations measured by the two methods depending on the cell line tested. Both the human colon adenocarcinoma cell line COLO 320HSR (*29*) and the ductal breast carcinoma cell line T-47D (*48*) are known to harbor p53 mutations that lead to the accumulation of mutant p53 protein. There was concordance between the methods when T-47D was measured, but not in the case of COLO 320HSR. The DU-145 prostate carcinoma cell line, demonstrated elsewhere to show strong nuclear staining for p53 and to harbor a double mutated *p53* gene (*49*), appeared to have lower p53 concentrations by both assays than did COLO 320HSR or T-47D. Having twofold higher p53 concentrations by the modified immunoassay, the MCF-7 breast adenonocarcinoma cell line has been demonstrated to express wild-type p53 protein sequestered cytoplasmically by unknown mechanisms (*50*). The highest concentrations of p53 protein observed by both assay methods occurred in pRNS-1 (*51*), a prostate cell line transformed by SV40 and stably expressing large T antigen, which can bind to and thereby increase the half-life of p53 protein. It will therefore be necessary to perform studies to identify p53-binding factors in cell lysates responsible for the nonlinear assay response to dilutions of cell lysates, and to determine the basis for the widely differing p53 concentrations in the lysates measured by the two assay methods.

We report here the development of a new specific and highly sensitive ELISA for quantifying p53 protein in biological fluids. Although this new assay is similar to the immunofluorometric procedure used in our laboratory in several earlier studies of p53 protein in breast tumor cytosols, it has a substantial advantage in being virtually free of interferences in human serum specimens. Recent data by other groups (*46*, *47*) and by an ongoing study in our laboratory suggest that p53 protein may be found in the serum of cancer patients, perhaps by its release from tumors that overexpress p53. The development of a specific assay for p53 protein in serum may therefore prove to be a fundamental tool, given the diagnostic, prognostic, and disease-monitoring implications of this finding.

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