Generation of Anti-p53 Fab Fragments from Individuals with Colorectal Cancer Using Phage Display

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Although many individuals with malignancy develop Abs against p53, little is currently known of the structural features, V gene usage, and degree of somatic mutation of these Abs. Such information is critical to any meaningful understanding of the nature and significance of this humoral immune response to p53. We have constructed phage display libraries from six individuals with colorectal cancer and a demonstrable serum immune response against p53. Following panning with recombinant p53, a total of 43 binding Fab were identified. Four of these Abs bound with high affinity to wild-type denatured p53 ($1.19 \times 10^{-8} - 1.57 \times 10^{-8}$), as determined by BIAcore analysis, and were highly specific for both recombinant and cell line-derived p53, as determined by ELISA and immunoprecipitation. Epitope mapping showed they were reactive with the N terminus of human p53 between residues 27 and 44. Sequence analysis showed that the heavy chains were derived from the V_H1 gene family, and the light chains from V_L4. The pattern of replacement and silent mutations in the Fab sequence indicated that negative selection had occurred in the framework regions of all the V_H genes. We show that lymphocytes from individuals with cancer represent a valuable source of high affinity human Abs against p53. This approach provides an opportunity to examine the genetic structure of these naturally occurring Abs, and to draw inferences regarding the nature of the immune response that produced them. Abs identified in this way have a number of potential therapeutic applications. ***The Journal of Immunology*****, 1999, 163: 2276–2283.**

The p53 gene is mutated in more than 50% of human tumors (1). Point mutations in the central DNA binding domain are the most frequently observed mutation (2, 3), and result in loss of function due to conformational changes (4). The $t_{1/2}$ of the mutated protein is usually increased, resulting in accumulation of p53 in tumor cells. This accumulation of mutant protein is implicated as a factor in the development of an immune response to the protein in some cancer patients (5).

Anti-p53 serum Abs have been detected in up to 30% of individuals with cancer, and a range of different tumors (5–9). Of those patients with a humoral response to p53, it has been found that most have Abs against the immunodominant amino- and carboxyl-terminal regions (10). The secondary structure of these regions remains unchanged in mutant forms of the protein, suggesting that the immune response is more likely to be due to accumulation of the protein rather than to the appearance of novel antigenic determinants induced by mutation. However, several studies have identified patients who have anti-p53 Abs and yet have no identifiable accumulation of p53 in their tumors. This suggests that other mechanisms may be involved in the formation of the anti-p53 Ab response (11–13).

mAbs to p53 have been invaluable in investigating the function of p53 and its role in tumorigenesis. Epitope-mapping studies have shown that the majority of these mAbs have a similar pattern of reactivity to that observed in anti-p53 Ab preparations made from reactive human sera (14, 15). Relatively few murine mAbs have been found that are reactive with mutant or wild-type conformation-specific epitopes (16–18).

Molecular approaches for the generation of mAbs offer several advantages over traditional methods such as EBV transformation or hybridoma technology. In part, this is because in humans, these traditional methods often result in a bias toward certain B cell populations and the creation of cell lines that are unstable or producing only low levels of Ab (19). In contrast, molecular genetic approaches allow the use of genetic material from any source of available B lymphocytes to create random combinations of cloned heavy and light chain Ig genes. Recombinant Abs generated in this manner from human lymphocytes have shown specificities against a range of different Ags, including HIV Ags (20), autoimmune Ags (21, 22), and tumor Ags (23). Furthermore, these Abs have provided valuable information on the nature of the immune response occurring in the donor (21, 23).

Previous studies of the immune response against p53 in cancer patients have relied on serum analysis. These studies have yielded important information on the clinical significance, epitope dominance, and the role of protein over expression in the development of the anti-p53 immune response. However, several critical questions remain unanswered. To date, no human anti-p53 mAbs have been isolated either by conventional cell immortalization methods or molecular biological procedures. Hence, no information is available on human anti-p53 Ab V gene usage, the degree of somatic mutation, and structural features of the anti-p53 Abs. Such information is critical to any meaningful understanding of the nature and significance of the humoral immune response to p53.

This study reports the isolation of 14 anti-p53 Abs from a combinatorial library constructed with lymph node tissue from an individual with colorectal cancer. The nucleotide sequence and gene usage of these Abs were examined. Four anti-p53 Abs were examined in greater detail and found to have high affinity and be reactive to the amino-terminal region of the protein. This approach to isolating anti-p53 Abs could be used to generate Abs against a wide range of different p53 epitopes. Such Abs may be useful in

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functional studies of the protein as well as in the development of anti-idiotypic vaccines.

Materials and Methods

Patient data

After obtaining informed consent, blood and tissue samples were collected from 100 individuals seen at St. Vincent's Hospital (Sydney, Australia) from 1993–1997 who were undergoing resection of colorectal cancer.

Clotted blood was centrifuged at 2000 × *g* for 10 min and serum stored in aliquots at −70°C before use. Samples from 50 healthy individuals were obtained from Dr. Paul Kelly, Garvan Institute for Medical Research (Sydney, Australia) and used as controls in all ELISA and immunoprecipitation experiments. A fresh pericolic lymph node in the region of the tumor was harvested from colectomy tissue and frozen in liquid nitrogen before RNA extraction (23).

Immunohistochemical detection of p53

Sections of paraffin-embedded tumor tissue from each individual were subjected to immunohistochemical analysis of p53, as previously described (13). Tumor tissue was considered to have accumulated p53 when the average of 10 high powered fields showed greater than 5% of tumor cells with nuclear staining, in the absence of staining in the stromal cells and normal epithelium.

Production of recombinant p53

Recombinant p53 was expressed and purified as described previously (13). Briefly, a cDNA clone of wild-type p53 in the expression vector pET19b was transfected into *Escherichia coli* strain BL-21(DE3λ) (Novagene, Madison, WI). p53 was recovered from inclusion bodies, solubilized by denaturation, and purified from crude bacterial lysates using Ni_2^+ resin. This purification process results in the recovery of wild-type, denatured p53. Mutations in p53 result in unfolding of the protein, and hence the conformation of mutant p53 is similar to that of the wild-type denatured p53. The purity of p53 was assessed by PAGE in the presence of 10% SDS (SDS-PAGE) and then immunoblotting. The protein concentration was determined using the BCA assay kit (Pierce, Rockford, IL) with reference to a standard curve generated with BSA.

Detection of anti-p53 serum Abs

Wells of a microtiter plate (Polysorb, Nunc, Denmark) were coated with purified recombinant p53 (5 μg/ml in PBS) overnight at 4°C. Coated wells were washed three times each with 200 μl of PBS and then blocked with PBS/2% (v/v) BSA for 1 h at 20°C. Patient serum samples ($n = 100$) were diluted 1/100 in PBS and then applied in duplicate to the p53 and incubated for 1 h at 20°C. Binding Abs were detected with an alkaline phosphatase-conjugated goat anti-human IgG Fc-specific Ab (0.5 μg/ml in PBS/2% BSA; Jackson ImmunoResearch, West Grove, PA). The reactivity of each patient to p53 was expressed as a value relative to a standard curve generated from control serum known to contain anti-p53 Abs, as described previously (13). Serum activity was compared with a healthy group of volunteers ($n = 50$) and considered positive for anti-p53 Abs when the anti-p53 score was >2 SDs above the mean of the normal group.

The isotype of Abs in reactive sera was assessed using the above protocol, except that the anti-human IgG Fc-specific Ab was replaced with mouse anti-human IgG (IgG1, IgG2, IgG3, and IgG4; Dako, Carpenteria, CA) isotype-specific Ab (1 μg/ml) and detected with an alkaline phosphatase-conjugated goat anti-mouse Ig Ab (0.5 μg/ml in PBS/2% BSA; Jackson ImmunoResearch). The anti-p53 serum titer was defined as the highest dilution of serum that generated a signal of 3 times above background.

Library construction and biopanning

Pericolic lymph nodes were ground to a fine powder in liquid nitrogen, and total RNA was extracted using standard procedures (24). IgG1 κ-chain Fab libraries were constructed in the phagemid vector MCO1, as described previously (25). Briefly, Ig genes were amplified by RT-PCR using primers specific for human κ and IgG1 Ig genes, followed by digestion with *Sac*I/*Xba*I or *Spe*1/*Xho*I, respectively. The products were then cloned sequentially (light chain then heavy chain) into the MCO1, and the combinatorial libraries were electroporated into *E. coli* XL1-blue cells (Stratagene, La Jolla, CA) and packaged with VCS-M13 helper phage (Stratagene) to give the primary Ab phage library.

The size of the library was calculated from a proportion of clones taken after electroporation ($n = 20$ for each library) of the final heavy and light chain construct. A diagnostic PCR amplifying the V region of the heavy and light chain and BstN1 finger printing (see below) were used to calculate the number of clones with unique heavy and light chain inserts. On this basis, the total library size was estimated.

Wells of a microtiter plate were coated with recombinant p53, as described above, washed with PBS, then blocked with 2% (v/v) BSA/PBS. Aliquots of the phage Ab libraries (10^{12} CFU in 100 μl) were applied to each well and incubated at 20°C for 2 h. Excess phage were washed from the plate with six washes with PBS/0.1% (v/v) Tween, followed by two washes in PBS. Adherent phage were then eluted with 100 μl of 0.1 M glycine, pH 3, for 10 min at 20°C, and neutralized with 10 μl of 1 M Tris, pH 8. Eluted phage were reamplified for the next round of panning, as described previously (23). The panning procedure was conducted five times. An aliquot was taken from the eluted output from each round of panning and used to infect the *E. coli* nonsuppressor strain HB2151 for the production of soluble Fab. Infected bacteria were plated onto LB agar with 50 μg/ml of carbenicillin, and single colonies were picked for soluble Fab production.

Analysis of soluble Fab reactivity by ELISA

Initial small scale cultures were grown overnight from a single colony at 37°C in 2YT broth with 2% (v/v) glucose and 50 μg/ml of carbenicillin (2YT/glu/carb). These small scale cultures were then diluted 1/100 in 2YT/glu/carb and grown at 37°C to an OD of 0.8. The resultant larger cultures were then centrifuged and resuspended in 2YT containing 1 mM isopropyl β-D-thiogalactoside and 50 μg/ml of carbenicillin and grown for an additional 16 h at 30°C. Following centrifugation, the supernatant from these final cultures was assessed for anti-p53 Fab by ELISA.

Culture supernatant was incubated for 2 h at 20°C in duplicate wells of an ELISA plate that had been coated with p53 and blocked with 2% BSA, as described above. After washing with PBS, 100 μl of the anti-*myc* mAb, 9E10 (0.5 μg/ml in PBS/0.5% BSA, from ATCC cell line CRL-1729), was added to each well, and incubated at 20°C for 1 h. This Ab detects the *myc* tag on the C terminus of the heavy chain. The wells were again washed, and HRP-conjugated goat anti-mouse Ig Ab was added (0.5 μg/ml in PBS/2% (v/v) BSA; Jackson ImmunoResearch). After further washing, color was developed with 100 μl of TMB substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the reaction was stopped with 50 μl of 1 M H_2SO_4. Clones were considered positive where the OD was more than 3 times the signal seen in wells not coated with p53. In each ELISA, a negative control without 9E10 was used to assess background signal.

To determine the effect of Ag concentration on reactivity, anti-p53 Fab were reanalyzed using p53 coated at concentrations from 0.015 to 10 μg/ml. Furthermore, to demonstrate Ag-specific inhibition of binding, 100 μl of bacterial supernatant containing 0.5 μg of Fab was incubated with 5 μg p53 (50 μg/ml in PBS) for 1 h before application to wells of a p53-coated ELISA plate (1 μg/ml).

Because reactivity measured with the 9E10 Ab may be due to the binding of unassociated heavy chain protein alone, the involvement of the light chain in binding to p53 was confirmed by an ELISA with an anti-κ-specific Ab. A biotinylated goat anti-human κ-specific Ab (0.2 μg/ml in PBS/2% (v/v) BSA; Rockland, Gilbertsville, PA) was used as the second Ab instead of the 9E10 Ab, and followed by HRP-conjugated streptavidin (0.05 μg/ml in PBS/2% (v/v) BSA; Dako).

The cross-reactivity of Fab with other Ags was assessed by ELISA using a similar method to that described for p53. The following Ags and concentrations were used: ErbB2 extracellular domain (5 μg/ml; gift from Ruth Lyons, Garvan Institute), MUC1 (5 μg/ml; gift from Dr. Ian McKenzie, Austin Research Institute, Melbourne, Australia), carcinoembryonic Ag (CEA) (5 μg/ml), insulin (5 μg/ml), tetanus toxoid (1 μg/ml; CSL, Melbourne, Australia), BSA (1 μg/ml; Sigma-Aldrich, Castle Hill, Australia), and keyhole limpet hemocyanin (1 μg/ml; Sigma-Aldrich).

Inhibition of serum Ab reactivity by Fab

A competitive binding assay was used to determine the relative specificity of p53 Fab and Abs in patient serum. Wells were coated with p53 (0.1 μg/ml), as described, then incubated with either patient serum (10% v/v in PBS) or PBS alone for 45 min at room temperature. Fab 163.1, 163.5, 163.17, and 163.24 (1 μg/ml in PBS) were then added to an equal volume of serum (10% (v/v)) or PBS, applied to wells, and incubated for an additional 60 min. The wells were washed thoroughly, and bound Fab was detected with 9E10 and alkaline phosphatase-conjugated goat anti-mouse Ig Ab, as above. The effect of nonspecific inhibition was controlled for by the use of serum from patient 100, which did not contain anti-p53 Abs. The percentage inhibition of Fab binding that occurred due to the presence of anti-p53 Abs in serum was calculated as the signal obtained with Fab alone minus the signal obtained with the Fab plus serum, expressed as a percentage of the Fab alone signal.

Table I. *Clinicopathological details, anti-p53 serum titer, and Ab library sizes of patients selected for the study*

Patient ID	100	107	149	163	357	790
Sex	M	F	M	M	F	M
Tumor site	Sigmoid colon	Sigmoid colon	Rectum	Sigmoid colon	Rectum	Sigmoid colon
Dukes' Stage	B	B	C	B	C	C
Tumor differentiation	Poor	Poor	Moderate	Poor	Moderate	Moderate
p53 overexpressed	Yes	Yes	Yes	Yes	No	Yes
Anti-p53 titer (IgG)	0	512	1,024	8,192	512	16,384
IgG isotype	NA	IgG1	IgG1	IgG1	IgG1	IgG1
Library size	1.3×10^6	1.7×10^6	1.6×10^7	4.5×10^7	2.4×10^7	3.0×10^7

Analysis of Fab reactivity by immunoprecipitation

The colorectal cancer cell line HT29, which contains mutant p53, was used to assess the reactivity of Fab with human p53 from eukaryotic cells. Approximately 10^7 cells were lysed in TNES buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture (Boehringer Mannheim, Castle Hill, Australia), and 1 mM PMSF) and then cell debris were removed by centrifugation at $10,000 \times g$ for 10 min. Approximately 250 μg of the total lysed protein was used in each immunoprecipitation. Either the mouse anti-DO-7 (0.5 μg; Dako) or the bacterially expressed Fab (1 μg) was added to the lysate and incubated for 1 h at 4°C. The anti-*myc* 9E10 Ab (1 μg) was then added to the mixture containing Fab and incubated for 1 h at 4°C. At this point, 20 μl packed volume of protein A-Sepharose (Zymed Laboratories, San Francisco, CA) was added to all tubes and incubated for an additional hour at 4°C. The protein A-Sepharose was washed four times with PBS, and subjected to 10% SDS-PAGE under denaturing and reduced conditions. Proteins were transferred to polyvinylidene difluoride membrane by electroblotting, blocked with 10% skim milk powder, and probed with a goat anti-p53 Ab specific for the N-terminal region of the protein (Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by an HRP-conjugated donkey anti-goat Ig Ab (Jackson ImmunoResearch), and then the blots were developed using chemoluminescent substrate (DuPont NEN, North Sydney, Australia). A Fab specific for tetanus toxoid, protein A-Sepharose alone, and an extract only control were included in each experiment as negative controls.

Epitope mapping

A set of deletion mutants derived from human p53 (26) was supplied by Dr. David Lane (University of Dundee, Dundee, Scotland). The deletion mutants used were Hup53, 3M (residues 1–393), 3R (1–223), 4U (1–106), 11 (27–393), and 18 (44–393), as described by Nissim (26). Briefly, cultures of *E. coli* (BL21 DE3λ) containing the constructs were grown to an OD 0.8. The cells were lysed in bacterial lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 50 mM NaCl, 1% Nonidet P-40, and 1 mM PMSF), and 50 μl of the lysate was subjected to SDS-PAGE and electroblotting, as described above. Bacterial expressed Fab was incubated with the membrane for 1 h at 20°C and then washed with PBS. Bound Fab was detected with 9E10 and HRP-conjugated goat anti-mouse. Negative controls were as described above.

Sequence analysis

The V region of selected clones was sequenced using a cycle sequencing kit, according to the manufacturer's specifications (Promega, Madison, WI). Miniprep DNA was prepared by alkaline lysis, and both strands of DNA were sequenced using primers outside the V region. The primers used for sequencing the light chain were 5′-AA GAC AGC TAT CGC GAT T (OmpA leader sequence) and 5′-ATG AAG ACA GAT GGT GCA GC (5′ end of the κ C region), and the heavy chain 5′-CTA CGG CAG CCG CTG GAT TG (PelB leader sequence) and 5′-GGA AGT AGT CCT TGA CCA G (5′ end of the IgG C_H1 region). The heavy and light chain V region for Fab clones was matched to available V genes, D genes, and J genes using the DNA plot alignment package and V base sequence database.

Using the method of Chang and Casali (27), the frequency of replacement mutations (R)[2] in the CDR and framework (FR) for each of the p53 Abs was calculated with respect to its closest germline gene. The probability that R mutations were occurring at a frequency above or below the expected random frequency was calculated in a binomial distribution model, using the expected number of R mutations in the germline gene, the actual number of observed R mutations in the Fab sequences, and the probability of R mutations localizing to the CDR or FRs (27, 28). Amino acids from 1–94 of the heavy chain (29) and 1–95 for the light chain were used for the analysis of R mutations. Amino acid residues occurring as a result of primer sequence in the FR1 region were excluded from the analysis. A p value of less than 0.05 indicated that the R mutations had occurred in a nonrandom fashion.

Fab purification

Soluble Fab was precipitated with ammonium sulfate (35% (w/v), resuspended in 5 ml of PBS, and then purified by IMAC affinity chromatography (Qiagen, Chatsworth, CA). Eluted fractions containing Fab were pooled and then fractionated by size exclusion chromatography (Superdex 200; Pharmacia, Piscataway, NJ) in HBS buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Nonidet P-40). Purity was assessed by SDS-PAGE and silver staining.

BIAcore analysis of selected Fab

Recombinant p53 was coupled to a CM5 chip using standard amine immobilization protocols. The chip was activated using 50 mM *N*-hydroxysuccinimide and 200 mM *N*-(dimethylaminopropyl)-*N*′-ethylcarbodimide. Recombinant p53 at 100 μg/ml in PBS diluted 1/10 in sodium acetate (1 M, pH 4.8) was injected at a flow rate of 10 μl/min. No greater than 400 resonance units were coupled to the chip for affinity analysis.

All measurements were conducted in HBS buffer. For the analysis of affinity, concentrations of Fab ranging from 10–200 nM were injected for 90 s at a flow rate of 30 μl/min over two flow cells, one with coupled p53 and the other without. Dissociation was measured over 90 s by the injection of HBS buffer. The chip was regenerated with 20 μl of 1 M glycine, pH 2, at 30 μl/min flow rate. The resonance unit of the blank flow cell was subtracted from the p53-coupled cell, and the affinity constants were calculated using the BIAevaluation 3 software package for a global fit.

Results

Patient serum analysis and Ab library construction

Of the 100 patients with colorectal cancer screened for Abs against p53, 17 were found to have anti-p53 Abs. From the patients found to have p53-reactive serum, six were selected for further study, including one patient with no detectable anti-p53 Abs as a negative control. In addition, each of the patients was assessed for the predominant IgG isotype reactive with p53. It was found that all of the individuals selected had predominantly IgG1-reactive anti-p53 Abs. IgG1K Ab libraries were therefore constructed from the pericolic lymph node tissue taken from these six colorectal cancer patients. The size of the Ab libraries from each of the constructed individuals, together with clinical data, serum, and reactivity against full-length p53, is shown in Table I.

Anti-p53 Fab selection

Each Ab library was subjected to five rounds of panning against recombinant p53. A 20–100-fold increase in the number of eluted phage was observed in rounds four and five.

No Fab with reactivity against p53 were identified from 32 phage clones isolated from each library after each of the first three rounds of panning (total number of clones analyzed = 960). The

[2] Abbreviations used in this paper: R, replacement; CDR, complementarity-determining region; FR, framework; CEA, carcinoembryonic Ag.

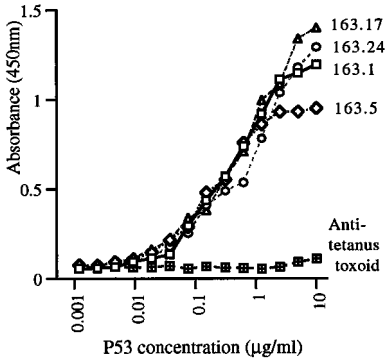

FIGURE 1. ELISA of reactivity of Fab against p53. The reactivity of Fab against varying concentrations of p53 (wild type denatured) coated onto an ELISA plate. Following incubation with Fab in bacterial supernatant, Fab binding to p53 were detected with 9E10 mAb, followed by an HRP-conjugated goat anti-mouse Ig Ab. An anti-tetanus toxoid Fab expressed under the same conditions as the p53 Fab was used as a negative control.

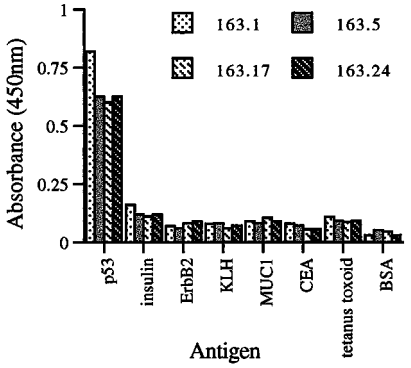

FIGURE 2. Lack of cross-reactivity of Fab. Binding of Fab with other Ags, as assessed by ELISA. Binding Fab in crude bacterial supernatant were detected with 9E10 mAb, followed by a goat anti-mouse-specific HRP-conjugated Ab. The signal obtained for p53 was at least 4 times greater than that observed with all other Ags.

library from patient 163 was found to have 1 of 32 p53-reactive clones from round four, and 42 of 128 p53-reactive clones from round five. No positive clones from rounds four or five (96 clones analyzed from output phage) were identified from patient Ab libraries 100, 107, 149, 357, or 790 (192 phage clones analyzed from each library).

The 43 p53-reactive clones isolated from library 163 were analyzed by restriction enzyme digestion, and five clones were eliminated from further analysis on the basis of lacking a heavy chain of the correct size. All clones had light chain inserts of the expected size. The remaining 38 clones were DNA fingerprinted from V region PCR products using the frequent cutting restriction enzyme BstN1. This allowed the identification of four unique heavy chain BstN1 profiles that paired with five unique light chain profiles, giving a total of 14 clones with unique heavy and light chain combinations (results not shown). Four clones with unique heavy chain were epitope mapped and analyzed for reactivity against recombinant p53, cell line-derived p53, as well as for cross-reactivity with other Ags (clones 163.1, 163.5, 163.17, 163.24). The nucleotide sequence of the 14 clones with unique heavy and light chain combinations was determined, the deduced amino acid sequence generated, and the mutation pattern analyzed.

Confirmation of anti-p53 Fab reactivity

The reactivity of clones 163.1, 5, 17, and 24 with varying concentrations of p53 is shown in Fig. 1. The reactivity of the Fab against p53 was also demonstrable using a sheep anti-human κ-chain-specific Ab (results not shown), indicating that the fully assembled Fab fragment, including light chain, was responsible for the p53-binding activity. When preincubated with excess p53 before ELISA, the signal was reduced by between 11 and 27% of the levels observed in the standard protocol (results not shown). Furthermore, the four clones showed no reactivity against other Ags, including ErbB2, MUC-1, CEA, tetanus toxoid, insulin, keyhole limpet hemocyanin, and BSA (Fig. 2).

Inhibition ELISAs using serum from the individual from whom the Fab were raised (individual 163) showed that Abs in that serum were able to partially inhibit the binding of the four Fab analyzed. Percentage inhibition for each of the Fab was 163.1 − 58 ± 6%; 163.5 − 50 ± 3%; 163.17 − 50 ± 4%; 163.24 − 64 ± 1%. In parallel experiments, control sera that did not contain anti-p53 Abs (individual 100) had either no effect or increased the binding of Fab to p53.

The ability of the Fab to detect p53 in bacterial lysates was assessed by Western analysis (Fig. 3*A*). The Fab were able to detect p53 in the lysate, but did not appear to react with other proteins. In addition, it was found that each of the Fab was able to immunoprecipitate mutant p53 from the human colorectal cancer cell line HT-29 (Fig. 3*B*).

Epitope mapping

Epitope mapping of the Fab clones 163.1, 5, 17, and 24 showed that all were reactive with full-length human p53 (residues 1–393), as well as the deletion constructs 3M (1–223), 3R (1–187), 4U (1–106), and 11 (27–393). None of the clones were reactive with the 18 construct (residues 44–393), indicating that the Fab were

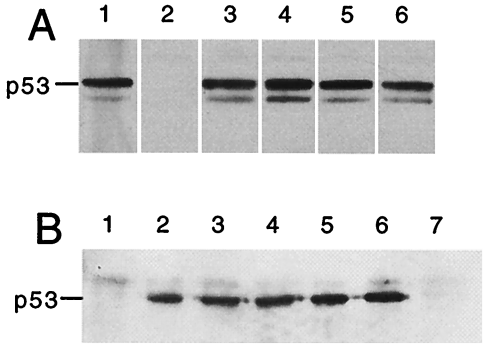

FIGURE 3. Immunoblot analysis of Fab binding to p53. *A*, Binding of Fab clones to recombinant p53 (wild type denatured) in bacterial lysates. The binding of DO-7 was detected with an HRP goat anti-mouse (*lane 1*). The human anti-p53 Fab (163.1, 5, 17, 24; *lanes 3–6*) and human anti-tetanus Fab (negative control; *lane 2*) were detected with 9E10 mAb, followed by HRP goat anti-mouse Ig Ab. The smaller of the two bands represents a degradation product of p53. *B*, Immunoblot analysis of immunoprecipitates from lysates of the human colorectal cancer cell line HT-29 (contains mutant p53). Immunoprecipitation was performed using the human Fab anti-tetanus toxoid (*lane 1*); the anti-p53 mAb DO-7 positive control Ab (*lane 2*); Fab from clones 163.1, 5, 17, and 24 (*lanes 3–6*, respectively); and protein A with lysate alone (*lane 7*). Following immunoprecipitation and electroblotting, the blots were incubated with a goat anti-p53 Ab, followed by an HRP-conjugated donkey anti-goat Ab. The higher and fainter band in all lanes represents secondary Ab binding to protein A. Immunoprecipitations of either protein A-Sepharose, 9E10 and protein A, or HT29 lysate with 9E10 and protein A were also blotted and probed. A signal similar to *lane 7* was observed in all cases (results not shown).

Table II. *The most homologous germline sequence is shown together with the number of nucleotide mutations*

Clone No.	VH Gene	D Gene	J Gene	Nucleotide Mutations in the V Region[a]	VK Gene Family	J Gene	Nucleotide Mutations in the V Region[a]
163.16	DP-7	ND	JH4b	43/294 (14.6)	DPK24	JK2	10/305 (3.2)
163.23	DP-7	ND	JH4b	43/294 (14.6)	DPK24	JK2	10/305 (3.2)
163.22	DP-7	ND	JH4b	44/294 (15)	DPK24	JK2	11/305 (3.6)
163.1	DP-7	ND	JH4b	44/294 (15)	DPK24	JK2	11/305 (3.6)
163.15	DP-7	ND	JH4b	45/294 (15.3)	DPK24	JK4	3/305 (1)
163.20	DP-7	ND	JH4b	52/294 (17.7)	DPK24	JK2	14/305 (4.6)
163.5	DP-7	ND	JH4b	52/294 (17.7)	DPK24	JK2	18/305 (5.9)
163.7	DP-7	ND	JH4b	51/294 (17.3)	DPK24	JK4	7/305 (2.3)
163.6	DP-7	ND	JH4b	51/294 (17.3)	DPK24	JK4	6/305 (2)
163.9	DP-7	ND	JH4b	50/294 (17)	DPK24	JK2	14/305 (4.6)
163.2	DP-7	ND	JH4b	52/294 (17.7)	DPK24	JK4	4/305 (1.3)
163.14	DP-7	ND	JH4b	52/294 (17.7)	DPK24	JK2	14/305 (4.6)
163.24	DP-7	ND	JH4b	54/294 (18.5)	DPK24	JK2	0/305 (0)
163.17	DP-7	ND	JH4b	54/294 (18.5)	DPK24	JK4	2/305 (0.6)

[a] The number of nucleotide mutations in the V region/total number of nucleotides (%).

reactive with an epitope between residues 27 and 44 (inclusive) of human p53.

Affinity and sequence analysis

The dissociation constants for the Abs 163.1, 5, 17, and 24 were 1.19×10^{-8}, 1.5×10^{-8}, 1.57×10^{-8}, and 1.38×10^{-8}, respectively. The χ^2 values were all less than 1 when using the model for 1:1 interaction with a drifting baseline.

For each of the 14 clones sequenced, the closest germline gene match and the percent nucleotide difference from this gene are shown in Table II. A comparison of the V region of the 14 Fab clones showed that the clones had greater than 95% homology with each other and appeared to share the same V gene, D gene, and J gene combination (Table II). The V region of these clones consisted of the V gene DP-7 (V_H1–46) from the V_H1 gene family, and the J gene, J_H4b. No D segment gene could be assigned to these clones with confidence due to the lack of homology with known D gene sequences, although all clones had similar D regions. All the heavy chains of these clones had extensive mutations throughout the V gene region. The percentage difference between the heavy chain V gene and the matched germline V gene ranged from 14.6–18.5%. The mutations were frequent, not only in the CDR regions, but also throughout FR1 and FR3 regions. There were relatively few mutations in the FR2 region. The light chain partners of the 14 clones had greater homology with the matched V gene than the heavy chain, with the percentage of mutations ranging from 0 to 5.9%. The light chain partners of these clones used the same light chain V gene DPK-24 in combination with either the JK2 or JK4 gene.

Mutation analysis

The deduced amino acid sequences from the 14 clones were used to determine the R and silent mutations within FR and CDR (Fig. 4), and these values were used to calculate the probability that R mutations in FR or CDR were not random. Random mutations, either R or silent, occur evenly throughout a given sequence, while Ag-driven responses are often localized and result in a higher or lower proportion of R mutations depending on the selection pressures defined by Ag selection (27). The probability that the mutations in the FR and CDR regions arose as a result of Ag-driven selection is shown in Table III.

All the heavy chains derived from the DP-7 V gene had *p* values less than 0.05 as a result of a lower proportion of R mutations in the FR than was expected. This suggests Ag-driven B cell selection, with suppression of R mutation in the FR. In contrast, the frequency of R mutations in the CDR region of the same clones was no greater than may be expected at random. Analysis of the matched light chain sequences showed that only clones 163.16 and 163.23 had a significantly different number of R mutations. In these instances, the pattern mirrored the heavy chain, with negative selection occurring in the FR, but no clear evidence of Ag-driven selection in the CDR1 and CDR2 regions.

Discussion

In this study, combinatorial Ab gene libraries and phage display have been used to isolate high affinity human Fab fragments with specificity for the p53 tumor suppressor gene product. The isolated Fab bound to the amino-terminal region of P53 between residues 27 and 44, and were reactive with both recombinant wild-type p53 and a mutant form of p53 immunoprecipitated from colorectal cancer cells. The isolation of recombinant anti-p53 Abs of unspecified affinities has previously been reported from a synthetic Ab gene library (26). However, this study represents the first report of the isolation of anti-p53 Abs from an individual with a demonstrable Ab response to p53. As such, it provides important information on the gene usage and epitope specificity of the immune response to p53 seen in humans with malignancy. It also provides a reproducible strategy for the exploitation of these potentially useful immunotherapeutic agents.

The well-recognized occurrence of anti-p53 Abs in the serum of some individuals with colorectal cancer provided an opportunity to more closely examine the specificity of this response to an important tumor suppressor gene product. Because of the combinatorial approach used in Ab phage display, it is possible to generate Fab containing pairings of light and heavy chains that were not present in the original source tissue. In numerical terms, the clonal expansion and active synthesis of Ig will mean that mRNA from antigenically stimulated B cells will predominate in the RNA extracted from source tissues. In practical terms, panning against Ag will favor the reestablishment of pairings that have evolved in the face of Ag selection. Nevertheless, the possibility remains that the Abs identified in this study may be neo-Abs that do not accurately reflect the Abs responsible for the anti-p53 activity seen in serum. This is a possibility that is not possible to eliminate experimentally. However, recent experience has shown that Ab phage display can successfully isolate specific Abs from individuals with demonstrable serum Ab responses to a variety of Ags. These include infectious agents such as *Haemophilus influenzae* b (30), and self Ags important in autoimmune disease, including the acetylcholine

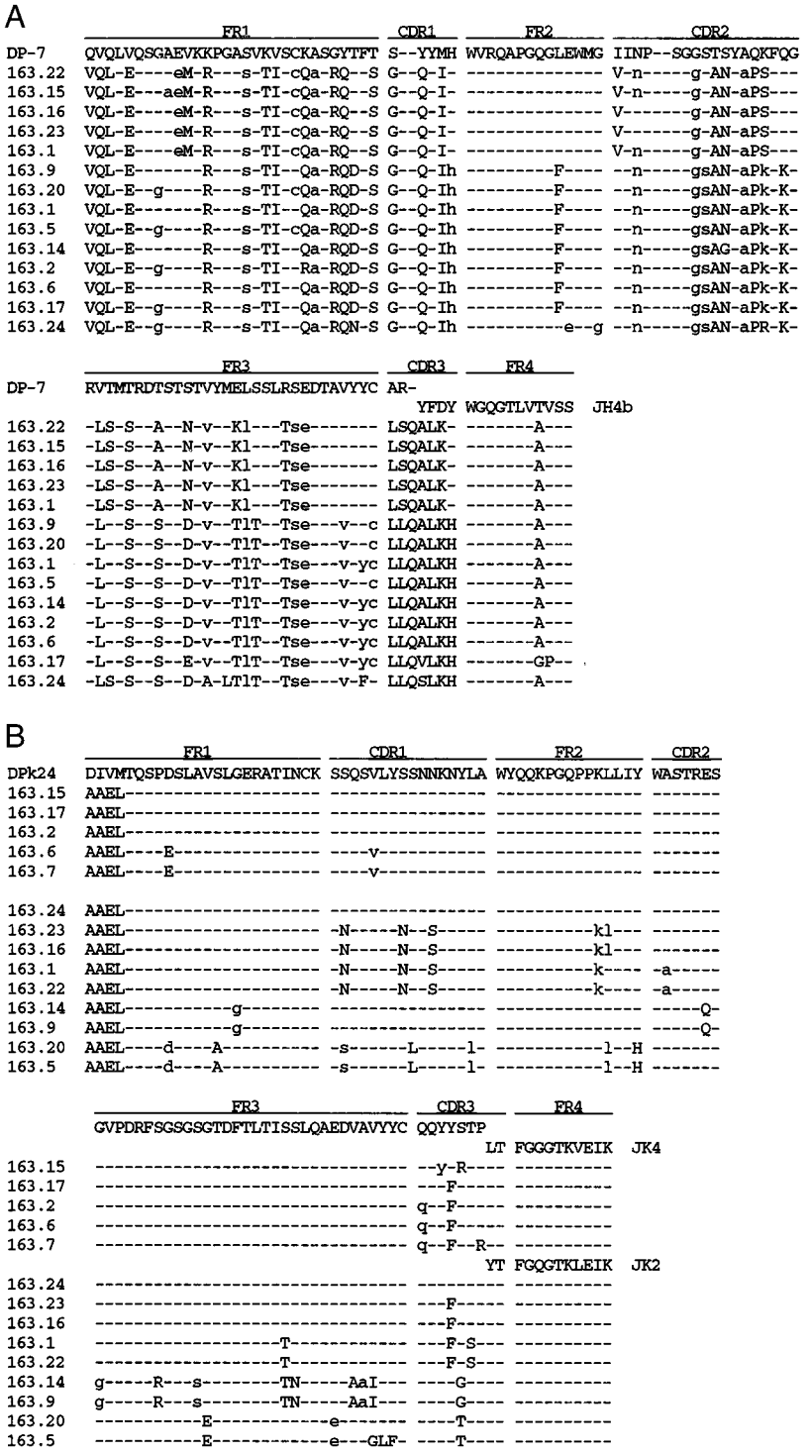

FIGURE 4. Sequence analysis of anti-p53 Fab genes. Deduced amino acid sequence of heavy (*A*) and light (*B*) chain clones reactive with p53. R (uppercase) and silent mutations (lowercase) are shown with respect to the most homologous germline sequence.

receptor (21), anti-thyroglobulin Abs (31), and anti-neutrophil cytoplasmic Abs (32). Importantly, Fab isolated in this manner have been shown to accurately reflect the in vivo Ab response. In the case of *H. influenzae*, V gene combinations of isolated Fab were shown to mirror the V gene repertoire used in response to natural infection (30). Recombinant anti-neutrophil cytoplasmic Abs isolated from an individual with ulcerative colitis shared the same disease-specific epitope reactivities as serum Abs (32). Likewise, several studies have shown that the epitope specificity of isolated Fab largely represents the major specificities of Abs present in serum (for example, 90% inhibition of serum acetylcholine receptor Abs (21) and 92% inhibition of anti-thyroid peroxidase Abs (33)). We found that isolated Fab were able to block up to 65% of the anti-p53 Ab activity seen in patient serum. Given that serum responses to p53 probably arise against multiple epitopes, this level of inhibition is significant. However, it provides only indirect evidence that the Fab were constructed from Ig genes responsible for the production of the naturally occurring Abs.

Selection of an appropriate tissue source of Ig genes is an important factor in Ab library construction (34), and several groups have previously reported the use of enriched sources of lymphocytes in the isolation of disease-specific Abs. For instance, Ig

Table III. *Variable gene mutational analysis*[a]

	Heavy							Light						
Clone No.	Total No. of R Mutations	FR R Mutations (expected)	CDR R Mutations (expected)	FR R:S Ratio	CDR R:S Ratio	p(FR)	p(CDR)	Total No. of R and S Mutations	FR R Mutations (expected)	CDR R Mutations (expected)	FR R:S Ratio	CDR R:S Ratio	p(FR)	p(CDR)
163.16	35	15 (23.12)	8 (7.13)	15:8	8:2	**0.02**	0.13	6	0 (1.97)	4 (1.05)	0:2	4:0	**0.04**	0.09
163.23	35	15 (21.34)	8 (6.40)	15:8	8:2	**0.02**	0.13	6	0 (2.55)	4 (2.05)	0:2	4:0	**0.04**	0.09
163.22	36	15 (21.34)	8 (6.58)	15:8	8:3	**0.01**	0.13	7	1 (2.97)	5 (2.38)	2:0	5:1	0.07	0.07
163.1	36	15 (21.34)	8 (6.58)	15:8	8:3	**0.01**	0.13	7	1 (2.98)	5 (2.38)	1:1	5:1	0.07	0.07
163.15	37	15 (21.94)	8 (6.76)	15:9	8:3	**0.01**	0.14	2	0 (0.85)	1 (0.68)	0:0	1:1	0.33	0.44
163.20	38	16 (21.34)	7 (6.95)	16:9	7:6	**0.01**	0.16	10	3 (4.25)	7 (3.41)	3:3	2:2	0.19	0.19
163.5	38	16 (22.53)	7 (6.9)	16:9	7:6	**0.01**	0.16	13	6 (5.53)	2 (4.43)	6:3	2:2	0.21	0.09
163.17	39	16 (23.12)	7 (7.13)	16:10	7:6	**0.01**	0.16	5	3 (1.70)	1 (1.36)	1:0	2:2	0.32	0.30
163.6	36	16 (21.34)	7 (6.58)	16:9	7:6	**0.03**	0.16	4	1 (1.70)	1 (1.36)	1:0	1:2	0.32	0.30
163.9	37	16 (21.94)	7 (6.77)	16:8	7:6	**0.02**	0.16	11	5 (4.68)	2 (3.75)	5:4	2:0	0.23	0.15
163.2	36	16 (22.53)	7 (6.58)	16:9	7:6	**0.03**	0.16	2	0 (0.85)	1 (0.68)	0:0	1:1	0.33	0.45
163.14	36	16 (21.34)	7 (6.58)	16:9	7:6	**0.03**	0.16	11	5 (4.68)	2 (3.75)	5:4	2:0	0.23	0.15
163.24	42	19 (24.90)	8 (7.68)	19:8	8:5	**0.02**	0.15	0	0 (0)	0 (0)	0:0	0:0	1.0	1
163.17	39	16 (23.14)	5 (7.13)	16:9	7:6	**0.01**	0.16	2	0 (0.85)	1 (0.68)	1:1	0:0	0.33	0.44

[a] The total number of replacement (R) and silent (S) mutations in the FR and CDR regions 1 and 2 of each heavy and light chain genes. Bold text indicates clones with a nonrandom distribution of R mutations.

genes from thyroid-infiltrating lymphocytes have been used to successfully isolate Abs against thyroid peroxidase (35). The use of such enriched sources of Ig genes may obviate the need for the production of large libraries ($>10^8$) to isolate specific Abs (36, 37).

In this study, libraries were constructed from pericolic lymph nodes draining a colorectal tumor, because it was considered that this tissue was more likely to represent an enriched source of anti-p53 Abs. To further increase the likelihood of isolating specific Fab, we selected individuals with a demonstrable IgG1 response to p53 protein. In this regard, it is of note that all those Fab with high affinity for p53 were derived from the individual with a high serum Ab titer against p53, and that no Abs were isolated from the one individual without a demonstrable serum response. We are less able to explain the failure to isolate anti-p53 Abs from the remaining four individuals with lower but detectable serum Abs. It is possible that this outcome may reflect the presence of lower affinity Abs, less readily isolated by the rounds of in vitro selection. It is also possible that the Ig-producing cells were absent from the sampled lymph node, or in numbers too low for effective isolation from the gene library. Finally, it is possible that the Abs from different individuals may recognize conformations of the target protein not available in the panning strategies used in this study. The further evaluation of this latter issue may allow the isolation of a broader repertoire of Abs.

This study has, for the first time, provided an opportunity to examine the genetic structure of naturally occurring p53 Abs, and to draw inferences from that structure regarding the nature of the immune response that produced them.

Nucleotide sequencing showed that the V genes of the p53 Fab had undergone extensive mutation (6.1–18.5%), a finding that was highly unlikely to be explained on the basis of polymerase-induced errors in the PCR (38). In fact, this frequency of V gene mutations is higher than that reported for class-switched germinal center and memory B cells (up to 4%) (39, 40). It provides strong support for the contention that the isolated Abs reflect the occurrence of a specific Ag-driven humoral immune response in these individuals. The particularly high mutation frequency may perhaps reflect the chronic nature of Ag exposure in individuals with malignancy. Although the mechanism of p53 presentation to the immune system remains uncertain (5), it is clear that the process can develop early in the process of tumor development. For instance, serum p53 Abs have been reported in smokers several years before the detection of the malignancy (15). This suggests that antigenic p53 may be presented to the immune system throughout the course of the disease, and that this continual exposure may be responsible for the extensive somatic mutation rate in the V genes.

Statistical analysis of the frequency of R mutations in the V genes provides further evidence to support the contention that the isolated Fab arose as a result of Ag-driven selection. The negative selection for R mutations seen in the FR regions of V_H1 family Abs is a typical feature of affinity-matured Abs.

Isolated Abs used V region germline genes predominantly from V_H1. Several authors have recently analyzed the germline V_H gene usage of a large series of Abs produced by hybridomas or phage display, and directed against a wide range of exogenous and autoantigens (41, 42). Ohlin et al. (41) showed that V_H3 (47%), V_H1 (35%), and V_H4 (13%) were the most commonly used germline genes, with V_H6, V_H5, and V_H2 being used in 3, 2, and 0% of cases, respectively. Although definitive conclusions regarding the gene usage of anti-p53 Abs require further study, it is possible that the V_H gene usage is restricted to the V_H1 gene family, in particular the DP-7 gene (43).

The structural features of the Fab, and the inferences drawn from them, are supported by affinity analysis using surface plasmon resonance. The isolated Fab all showed relatively high affinity for wild-type denatured p53, again suggesting that they may represent the product of a specific Ag-driven immune response.

The successful isolation of stable and clonal Fab has also allowed a closer examination of the epitope specificity of naturally occurring p53 Abs. All Fab isolated in this study bound to residues 27–44 of p53, a region that is specific to primates in that it differs by 9 amino acids from the closest homologous p53 sequence (feline), and by 15 amino acids from the rat p53 protein (44). This region is particularly important as a site for interaction with transcription machinery, as well as viral proteins (45). To date, most human serum Abs and murine monoclonals against p53 have been shown to bind to a narrow range of immunodominant epitopes that span residues in the N-terminal region (10–25, 40–50), the central region (120–130, 205–215, 285–295), and the C-terminal region (345–393) (15, 46, 47). Very few Abs with binding specificity for the region between amino acids 27 and 44 have been isolated (18, 26). This may reflect unique specificities seen only in the human immune response, yet masked in the analysis of the polyclonal responses seen in serum.

Recent studies have suggested a number of important potential uses for recombinant anti-p53 Abs. For instance, murine anti-p53 monoclonals have recently been shown to inhibit engraftment of the murine sarcoma cell line MethA in mice by acting as an anti-idiotypic vaccine (48). Likewise, the intracellular expression of recombinant fragments of the murine Abs pAb 421 and DO-1 has provided important insights into the role of p53 in carcinogenesis (49, 50). Clearly, the use of human p53 Fab would have advantages over murine Abs when used as anti-Id vaccines, while their unique epitope specificity would also make them useful tools in functional studies. This study demonstrates that lymphocytes from individuals with cancer represent a unique and valuable source of such Abs, and outlines strategies for the successful exploitation of this important resource.

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