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Anti-peptide antibodies detect oncogene-related proteins in urine

(immunodiagnostic/synthetic antigen/hybridoma/monoclonal antibody/breast cancer)

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ABSTRACT **Antisera to a number of synthetic peptides predicted from nucleic acid sequences of oncogenes have been used to screen 483 urine samples of cancer patients, pregnant women, and normal controls for the presence of immunologically related proteins. Increased levels of oncogene-related proteins are found during neoplasia and pregnancy. The differential detection of these oncogene-related proteins indicates that panels of monoclonal antibodies may provide a convenient noninvasive means of detecting, classifying, and staging a wide variety of malignancies and may be useful in following fetal development during pregnancy.**

The expression of oncogenes in human malignancies (1–6) suggests that a thorough understanding of the gene products may provide sufficient insight into the molecular basis of these diseases to open new avenues for diagnosis and therapy. The presently known oncogenes encode a limited number of proteins, forming cooperating families that transform a wide variety of cells (7–13). It is likely that the number of proteins functionally related to known oncogenes greatly exceeds those already described.

To help clarify the relationship between oncogene-encoded and/or oncogene-related proteins and disease states, we have studied concentrated urine samples with polyclonal and monoclonal antibodies directed against conserved regions of the protein products of *sis*, *ras*, and *fes* oncogenes. The antisera readily detected eight oncogene-related proteins that were 5- to 50-fold more concentrated in the urine of cancer patients and pregnant women than in normal controls. Furthermore, unique patterns of expression were detected in various malignancies or during different gestational periods of pregnancy.

MATERIALS AND METHODS

Synthesis of Peptides. Synthetic peptides were produced by using modifications (14) of solid-phase synthesis described by Merrifield (15). The sequence of the peptide was confirmed by high-pressure liquid chromatography and amino acid analysis.

Production of Antisera. The synthetic peptides were coupled to keyhole limpet hemocyanin using *m*-malamidobenzoyl-*N*-hydrosuccinimide as the coupling reagent as described (16). The conjugate was diluted to 1 mg/ml with phosphate-buffered saline (PBS) at pH 7.4, and it was mixed with an equal volume of complete Freund's adjuvant. Each mouse (strain 129 GIX$^+$) was immunized intraperitoneally with ≈50 μg of peptide conjugate. Two weeks later, an equal amount of peptide mixed with an equal volume of alum (10 mg/ml) was injected intraperitoneally. At least 1 month later, the mice were injected intravenously with the synthetic conjugate diluted in PBS. The *fes* polyclonal antisera were from mice immunized three times. The hybridomas were generated from spleen cells taken from mice 5 days after the third injection. Hybridomas were formed by fusing the spleen cells with P3 × 63.431 nonsecreting myeloma cells by using polyethelene glycol (17). The hybridomas were screened by ELISA (18). Ascites fluid was raised in pristane-primed 129 GIX$^+$ × BALB/c mice.

Immunoblots. Cell extracts were made by resuspending trypsinized cells (5×10^7 cells per ml) in PBS (pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 3% Trasylol. After sonication, the insoluble material was pelleted and the supernatant was stored at −70°C. The urine was concentrated using a 10-kDa exclusion filter (Amicon). After dialysis against PBS, the urine was stored at −70°C. For immunoblot analysis, extracts of ≈10^6 cells or the equivalent of 1 ml of urine were reduced with 2-mercaptoethanol prior to being loaded on a 5–17% polyacrylamide gel and electrophoretically transferred to nitrocellulose. After blocking with PBS containing 3% bovine serum albumin, 0.1% Triton X-100, either mouse sera diluted 1:500 or ascites fluid diluted 1:2000 was incubated with the nitrocellulose overnight at 4°C. After washing three times with 0.1% Triton X-100/PBS, the nitrocellulose was incubated with rabbit anti-mouse IgF (1:500) for 90 min at 25°C. The nitrocellulose was washed three times and incubated with ^{125}I-labeled protein A (≈10^6 cpm/ml). Binding was visualized with Cronex intensifying screens as described (19).

RESULTS

Characterization of Peptides, Antisera, and Oncogene-Related Proteins. Anti-peptide antibodies are particularly suited for detecting proteins immunologically related to sequenced oncogenes (19–28). Since they are sequence specific, anti-peptide antibodies can be directed toward highly conserved regions of proteins to maximize the probability of identifying related molecules that may have similar functions. Because immune recognition of proteins by anti-peptide antibodies need not be highly dependent on antigen conformation, it is possible to identify proteins that are not detected by anti-protein antibodies, the bulk of which are directed against determinants unique to the folded protein (29). Finally, the binding of anti-peptide antibodies is relatively insensitive to alteration or fragmentation of the target antigen, such as might occur in bodily fluids or secretions.

The peptides used for this study were selected because they represent highly conserved regions of their respective oncogene families. The ras peptide is the Ha-*ras* sequence located 37–59 amino acids downstream from the threonine residue autophosphorylated by p21 encoded by v-Ha-*ras* (30) or v-Ki-*ras* (31). The sequence is identical in human c-Ha-*ras* (32) and c-N-*ras* (33), and it differs from c-Ki-*ras* (34) by one

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Abbreviation: PDGF, platelet-derived growth factor.

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conservative amino acid change. The sequence of platelet-derived growth factor 2 (PDGF-2) used to generate the *sis* monoclonal antibodies is located at the amino terminus of the chain (residues 1–18) and is homologous to the first 12 amino acids of the other chain (PDGF-1) of PDGF (35–37). The fes peptide constitutes residues 754–769 of the 85-kDa fusion protein (38) and is 79–94 amino acids downstream from the major tyrosine phosphorylation site.

The antisera to these conserved sequences react with proteins in a wide variety of transformed cell lines (unpublished data). Antibodies against the sis peptide detect a 20-kDa protein in SSV-transformed NRK cells (6) as well as a sis-related protein of ≈56 kDa in a wide variety of cell lines. Antibodies against the ras peptide detect a major protein of ≈21 kDa and a minor protein of ≈30 kDa in cell extracts. The antiserum against the fes protein detects the 85-kDa gag–fes fusion protein in mink lung cells transformed by the Snyder–Theilen strain of feline sarcoma virus as well as a 40-kDa protein in these cell lines.

In Fig. 1, the reactivity of these antisera with urinary proteins from a variety of patients is demonstrated. The sis antisera detects proteins of 56, 31, and 25 kDa in urine concentrates (Fig. 1*A*). The antibody binding to all three proteins is blocked by prior incubation with the sis peptide (Fig. 1*B*) but not by incubation with the ras peptide (Fig. 1*A*). The concentrations of the detected proteins are 5- to 50-fold higher than in normal individuals (see below). All urine samples contain the three sis-related proteins except for the sample from the patient with a lymphoma, which is missing the 56-kDa protein (lane 4). In Fig. 1*C* the various ras-related proteins detected in urine samples are displayed. Proteins of ≈100, ≈55, and ≈21 kDa are detected (Fig. 1*C*, lanes 2–4). Again, the specificity of the antiserum is demonstrated by the blocking of the activity by preincubation with the ras peptide (Fig. 1*D*) but not by preincubation with the sis peptide (Fig. 1*C*). The 55-kDa ras-related protein is different from the 56-kDa sis-related protein (see below) and displays different reactivity patterns for each sample. The protein is not detectable in Fig. 1*C* (lane 1) (gastric cancer), while four bands of almost equal intensity are seen in lane 2 (38-week pregnancy). A strongly reactive doublet is visualized in lane 3 when urine from a patient with breast cancer is probed. A minor band at ≈35 kDa is associated with high concentrations of the 55-kDa protein. In lane 4, a single 55-kDa band is detected. Proteins of ≈21 kDa are detected in all four lanes of Fig. 1*C*. These smaller proteins are present at similar concentrations, although the mobility of the protein in Fig. 1*C*, lane 1, is slightly slower. This altered mobility may be significant because of the effect of changes at amino acid 12 on the mobility of *ras*-encoded proteins (3). The binding detected at 25 kDa is difficult to interpret because of comigration with antibody light chain. In Fig. 1*E*, the 35- and 40-kDa fes-related proteins are shown. The binding is blocked by preincubation with the immunizing fes peptide (Fig. 1*E*, lane 1) but not by incubation with the ras peptide or with peptides representing the homologous sequences in erbB or in abl proteins (lanes 2–4). In summary, the three antisera described above specifically detect eight different proteins in urine, three sis-related proteins ($p56^{sis}$, $p31^{sis}$, and $p25^{sis}$), three ras-related proteins ($p100^{ras}$, $p55^{ras}$, and $p21^{ras}$), and two fes-related proteins ($p40^{fes}$, and $p35^{fes}$).

Detection of Oncogene-Related Protein in Urine of Cancer Patients. In Table 1, the frequencies of increased oncogene-related proteins detected in a study of 51 control urine samples and 172 samples from patients with a variety of malignancies is listed. The amount of oncogene-related proteins in the urine was estimated by using immunoblots of serially diluted samples as standard curves (Fig. 2*B*). Samples containing >20-fold increases of $p55^{ras}$ or 5-fold increases of the other oncogene-related proteins are listed. These increased levels of $p55^{ras}$, $p40^{fes}$, and $p35^{fes}$ were detected in ≈20% of urine samples from patients with active breast cancer. In Fig. 2*A* (lanes 1–3), the results of using the anti-ras monoclonal antibody to probe three samples taken at 1-month intervals from a patient with active breast cancer are shown. The data shown in Fig. 2*B* demonstrate that this protein is present at levels that are increased at least 50-fold. In addition to $p55^{ras}$, $p40^{fes}$ and $p35^{fes}$ are also detected in all three samples from this patient (Fig. 2*A*, lanes 4–6). In contrast, the patient did not have increased levels of the three sis-related proteins (lanes 7–9). This pattern of expression was not found in the urine in any of the other normal or cancer

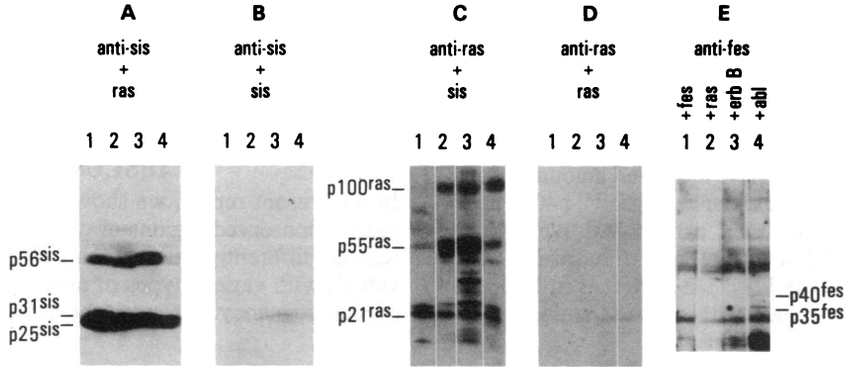

FIG. 1. Identification of oncogene-related proteins in urine. The equivalent of 1 ml of unconcentrated urine was applied to each lane of 5–17% polyacrylamide gel. The samples were probed with antisera to synthetic peptides predicted by *sis* (*A* and *B*), *ras* (*C* and *D*), or *fes* (*E*) oncogenes. Twenty microliters of ascites fluid (*A–D*) or mouse plasma (*E*) was preincubated for 30 min at 37°C with 100 μg of ras peptide [*A*, *D*, or *E* (lane 2)], sis peptide (*B* and *C*), fes peptide (*E*, lane 1), erbB peptide (*E*, lane 3), or abl peptide (*E*, lane 4). After preincubation and dilution (1:1000) in 3% bovine serum albumin/0.1% Triton X-100/PBS, pH 7.4, the antisera were used to probe the respective urine samples. The urine samples were obtained from patients with the following diagnoses: multiple myeloma (lane 1, *A* and *B*), gastric cancer (lane 2, *A* and *B*; lane 1, *C* and *D*), 35 weeks pregnant (lane 3, *A* and *B*), lymphoma (lane 4, *A* and *B*), gastric cancer (lane 1, *C* and *D*), 36 weeks pregnant (lane 2, *C* and *D*), breast cancer (lane 3, *C* and *D*), 39 weeks pregnant (lane 4, *C* and *D*), breast cancer (*E*). Exposure times were 2 hr (*A–D*) and 16 hr (*E*). The somewhat faster mobilities for $p56^{sis}$ in *A* (lanes 1 and 2) are due to excess albumin, while the distortion of $p31^{sis}$ and $p25^{sis}$ in *A* (lane 1) is due to excess light chain. The altered mobility of $p21^{ras}$ in *C* (lane 1) has not yet been investigated, nor has the increased detection of the two proteins at the bottom of *E* (lane 4). Overexposure of the gel pictured in *E* reveals $p40^{fes}$ and $p35^{fes}$ (lanes 2–4). Separate gels were run on samples in *A* and *B*, in *C* and *D*, and in *E*. At the time of urine collection, all cancer patients had clinically active disease with the exception of the urine used in *C* and *D* (lanes 3). This patient was without clinical signs of disease. Three months later, the malignancy showed obvious recurrence in this patient.

Table 1. Detection of increased levels of oncogene-related proteins in urine from cancer patients and from pregnant women

Diagnosis	*n*	$p100^{ras}$	$p55^{ras}$	$p21^{ras}$	$p56^{sis}$	$p31^{sis}$	$p25^{sis}$	$p40^{fes}$	$p35^{fes}$
Normal	51	26	0	20	8	12	20	4	6
Breast*	17	12	6	12	0	13	19	6	6
Breast†	24	25	**21**	29	8	12	25	**21**	**21**
Bladder	21	15	5	34	**34**	24	34	0	0
Prostate	16	32	0	12	**31**	25	37	0	0
Other‡	94	20	2	36	10	30	44	0	5
1st trimester	31	23	10	36	6	9	23	12	16
2nd trimester	60	20	13	32	25	28	45	2	13
3rd trimester	169	15	11	50	38	34	47	11	24

Numbers listed under each oncogene-related protein represent the percentage of samples that contained ≥20-fold elevations over detectable levels for $p55^{ras}$ or >5-fold elevations over detectable levels for all other proteins listed. Boldface numbers are significant for cancer patients.

*Patients in remission with no evidence of disease.

†Patients with active breast cancer.

‡Urine originated from patients with the following diagnoses: basal (n = 2), leukemia (n = 10), colon (n = 4), gastric (n = 5), Hodgkin's (n = 7), kidney (n = 4), melanoma (n = 9), molar pregnancy (n = 2), myeloma (n = 6), ovarian (n = 3), testicular (n = 3), lung (n = 14), lymphoma (n = 14), cervix (n = 11).

patients, except in one breast cancer patient who was without clinical symptoms at the time the initial study was done. Three months after the first urine sample was collected, the patient developed obviously recurrent disease. At this time, the levels of all three proteins were slightly higher than when the patient was presumed to be in remission (Fig. 3, lanes 1 and 2) and significantly higher than the low levels found in most normal individuals (lanes 3–8).

Different reactivity patterns were found with other patients. In contrast to the increased levels of the ras- and fes-related proteins found in urine of breast cancer patients, bladder and prostate cancer patients frequently secrete increased levels of the 56-kDa sis-related protein (Table 1). This protein is detected in the absence of the ras- and fes-related proteins described above. In addition to the 56-kDa sis-related protein, these patients frequently have increased levels of the 31- and/or 25-kDa sis-related proteins. High levels of the smaller proteins are also found frequently in urine from patients with lung and cervical cancer as well as in patients with non-Hodgkin lymphomas. In these latter patients, the increased 31- and/or 25-kDa sis-related proteins are found in the absence of the 56-kDa protein (Fig. 1*A*, lane 4). Thus, in the urine samples from cancer patients, three unusual patterns have been observed. A subset of the breast cancer patients have increased levels of $p55^{ras}$ in conjunction with $p40^{fes}$ and $p35^{fes}$. In contrast, a subset of patients with bladder and prostate cancer excrete increased amounts of all three sis-related proteins in the absence of $p55^{ras}$, $p40^{fes}$, and $p35^{fes}$. Finally, a subset of lung cancer and lymphoma patients excrete increased levels of only the smaller sis-related proteins. As shown in Figs. 1–3 as well as in Table 1, patterns of expression correlate with diseased states better than excretion of high levels of a single oncogene-related protein. In apparently normal individuals, increased levels of these proteins are rarely detected (Table 1).

Detection of Oncogene-Related Proteins in the Urine of Pregnant Women. In Table 1 increased levels of oncogene-related proteins in 260 urine samples of pregnant patients are also listed. As with the subset of breast cancer patients, very high levels of the $p55^{ras}$ are detected in the urine of a subset of patients at various intervals throughout the pregnancy. The frequency of detecting sis-related proteins increases as the pregnancy proceeds. Multiple urine collections were obtained from many of the patients. The levels of $p55^{ras}$ could change dramatically through the course of the pregnancy. In contrast to the relatively constant levels detected in multiple samples from normal or breast cancer patients, in pregnancy, the concentration of $p55^{ras}$ proteins could increase >15-fold in 1 week. As shown in Fig. 4, $p55^{ras}$ dramatically increased in the final 2 weeks of pregnancy. The concentration of the three sis-related proteins was approximately the same throughout the last month of pregnancy, and $p35^{fes}$ was detected in the final 2 weeks while $p40^{fes}$ was detected only in the final week. Postpartum (6 weeks) urine samples continued to contain increased concentrations of these sis-related proteins, although the ras- and fes-related proteins returned to normal low levels (data not shown).

DISCUSSION

In the present report, we show that three antisera, directed against conserved regions of oncogene-encoded sequences, reacted differently with proteins found in the urine of individuals with various types of neoplasia or at different times during pregnancy. Although the present study demonstrates

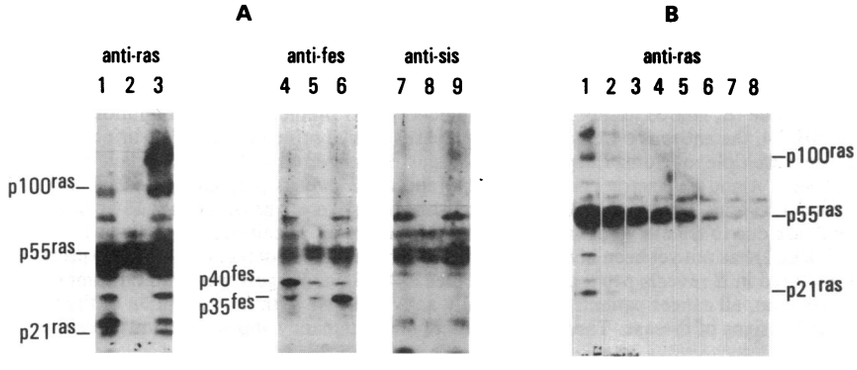

FIG. 2. Detection of $p55^{ras}$, $p40^{fes}$, and $p35^{fes}$ in urine from a breast cancer patient. (*A*) Urine samples (sample 1, lanes 1, 4, and 7; sample 2, lanes 2, 5, and 8; sample 3, lanes 3, 6, and 9) were collected at monthly intervals from a patient with active breast cancer. (*B*) Aliquots of the same sample used in *A* (lanes 3, 6, or 9) were applied at the following equivalents: lane 1, 1000 μl; lane 2, 500 μl; lane 3, 250 μl; lane 4, 125 μl; lane 5, 60 μl; lane 6, 30 μl; lane 7, 15 μl; lane 8, 7.5 μl. Samples were prepared and probed with antisera to *ras* (*A*, lanes 1–3; *B*), *fes* (*A*, lanes 4–6), or *sis* (*A*, lanes 7–9) as described in Fig. 1 except there was no preincubation with synthetic peptides.

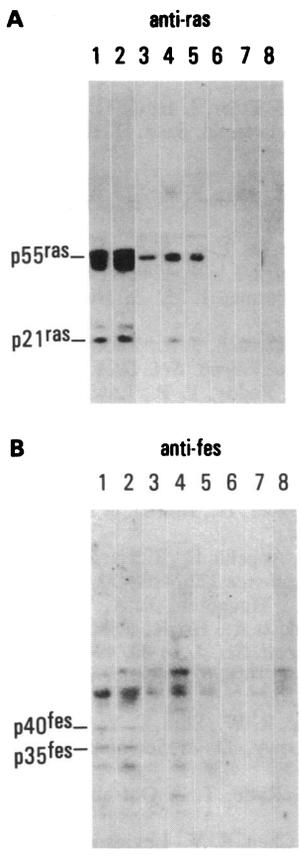

FIG. 3. Detection of ras- and fes-related proteins in multiple urine collection from normal individuals and from a recurrent breast cancer patient. Urine samples from a patient with recurrent breast cancer (lanes 1 and 2) or from normal individuals (lanes 3–8) were prepared and probed with antisera to ras (*A*) or fes (*B*). Urine used in lane 1 was from a patient in clinical remission from breast cancer. Urine used in lane 2 was from the same individual 3 months later when the tumor reappeared. Samples from a normal female collected 3 days apart (lanes 3–5), from a normal female collected 12 hr apart (lanes 6 and 7), and from a normal male (lane 8) were simultaneously probed.

the widespread excretion of these proteins in most individuals, increased levels are detected in certain cancer patients. The differences between normal controls and cancer patients are probably underestimated by the semi-quantitative methods used here. When quantitative methods are developed for studying these proteins, a clearer distribution between normal controls and disease states should emerge. The source of the increased amount of proteins in the urine of these patients is unknown, but it could originate from the tumor itself or from cells responding to the malignancy.

The proteins described in this report are immunologically related to oncogene proteins based on the highly specific reactivity of the various anti-peptide antisera. However, of the eight proteins described, only two ($p21^{ras}$ and $p31^{sis}$) represent oncogene-encoded proteins that have been described (24, 36, 39–41). The previously described $p21^{ras}$ protein has GTP binding activity (39), and analysis of yeast mutants suggests it is involved in adenylate cyclase control (40). Thus, $p21^{ras}$ is intimately involved with cell division, and therefore it is not surprising that the protein is readily detected in most urine samples. Indeed, hybridization studies using probes for Ha-*ras* or Ki-*ras* have shown readily detectable levels in mouse embryos throughout development (42, 43). Similarly, increased levels of transcripts specific for Ha-*ras* or Ki-*ras* have shown readily detectable levels in mouse embryos throughout development (42, 43). Furthermore, antisera to *ras* gene products have also detected

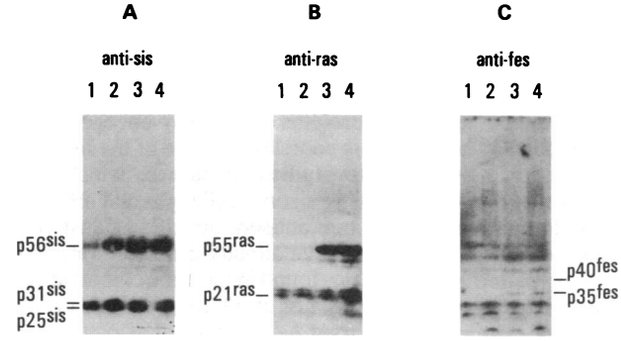

FIG. 4. Detection of oncogene-related proteins in urine during pregnancy. Four urine samples from the same individual collected at 1-week intervals during the final month of pregnancy were probed with antisera to sis (*A*), ras (*B*), or fes (*C*). Overexposure of *C* demonstrates the presence of $p35^{fes}$ (lanes 3 and 4) and $p40^{fes}$ (lane 4). The protein migrating slightly faster than the light-chain band (*C*, lanes 1–4) or at the bottom of the gel (*C*, lanes 2–4) was detected with some of the mouse antisera to the fes peptide. In addition, a protein of 150 kDa was also detected with some of the mouse antisera to the fes peptide (data not shown).

increased expression in tumor tissues (3). In this report, the most striking increase in this protein was found in the urine of patients with malignancies, of which only a limited number of samples have been tested; thus, the significance of increased urinary $p21^{ras}$ is yet to be determined.

The other previously described (41) protein that is detected in urine is the $p31^{sis}$ protein, which is one of the chains of the PDGF. Although the PDGF-2 chain is only 18 kDa when isolated from platelets (35), comparison of the human c-*sis* sequence (44, 45) with v-*sis* (24) indicates that the 18-kDa protein originates from a larger precursor protein. Indeed, analysis of a partially purified platelet extract reveals a protein of ≈31 kDa (19). Since PDGF has potent mitogenic activity and is released from platelets at the site of tissue injury, one of the physiological functions of PDGF is thought to be wound healing. In addition, PDGF-like material is secreted from a number of transformed cell lines (46, 47), and secretion appears to be developmentally regulated in smooth muscle cells (48). Thus, $p31^{sis}$ like $p21^{ras}$ may be physiologically important, and thus it is not surprising that it is present in the urine in normal and abnormal states.

In addition to the oncogene-encoded proteins of expected molecular size, additional proteins were detected in this study. It is not likely that their presence is due to spurious cross-reactivities because they are uniquely elevated in the urine of patients with certain cancers as well as in pregnant women. Furthermore, the reaction of the antibody with these proteins is inhibited specifically with the appropriate synthetic immunogens. Since the peptides used as immunogens represent conserved sequences among oncogene families, these additional proteins may represent members of these gene families. The expression of these genes may come under coordinate control during neoplasia or pregnancy. Regardless of the origin of these proteins, their unique expression in patients with certain types of neoplasia makes them important markers for further study.

Another significant finding in the present study was the detection of oncogene-related proteins in the urine of women during pregnancy. Since the products of oncogenes are thought to play a critical role in cell growth and development, it is not surprising that they are detected during pregnancy. The detection of the proteins in a temporal fashion, however, suggests that as additional antibodies are prepared, a set of reagents specific to stages of fetal development may be generated. Such reagents could be used to follow biochemical events in fetal development by simply monitoring the moth-

er's urine. Of course, it remains to be shown that the source of these proteins is, in fact, the fetus.

The data presented in this report represent early results from an ongoing research program in which many different site-directed antibodies against proteins encoded by oncogenes are being studied. The process is complex because of the factorial when multiple antigens are studied in cancer, which is a collection of diseases involving distinct cell types and different stages. Remarkably, only three antisera directed against conserved regions of oncogenes have already generated different reactivity patterns with proteins found in the urine of individuals with different types of neoplasia or at different periods during pregnancy. The results reported here suggest that patterns of reactivity will turn out to be more important than the presence of single oncogene-related proteins in a particular type of cancer. Finally, there may be genetic components controlling the secretions of certain oncogene-related proteins in apparently healthy people (unpublished observations). Analysis of these genetic components could aid in understanding the basis of predisposition to cancer.

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