Detection of Occult Metastatic Melanoma by Urine Chromatography

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SUMMARY

By using ion-exchange column chromatography with effluent monitoring using the stable, free radical $\alpha,\alpha$-diphenyl-$\beta$-picrylhydrazyl as a colorimetric reagent, we have demonstrated the occurrence of elevated levels of five peaks in the urine of patients with metastatic disease. The tentative assignment of two of the peaks as 3,4-dihydroxyphenylalanine and as 3-methoxy-4-hydroxyphenylalanine has been made. Three remain unknown. The correlation of these peaks with the clinical status of melanoma patients shows that, while the individual excretion pattern of these compounds may be variable, the sustained occurrence of one or more of them in a patient’s urine is evidence of recurrent or continuing disease. The excretion levels appear to be proportional to the tumor burden. The results with a group of 39 melanoma patients having Stage II or Stage III disease indicate that this chromatography technique provides earlier evidence of liver metastases than does the liver scan, may detect occult metastases generally, and has detected tumor in clinically enlarged lymph nodes. This method, in its present form, does not detect small pulmonary lesions earlier than chest X-ray or tomography do or brain metastases earlier than do brain scan or computerized axial tomography. The technique is clinically useful for the diagnosis of melanoma patients and in their follow-up while under treatment.

INTRODUCTION

A general problem in the management of the cancer patient is the detection of clinically occult metastatic disease. For this, there is at present no general solution. Useful approaches to this problem have, however, been developed for certain tumors, in particular those that perform specialized biochemical functions. Malignant melanoma has for over 1 century served as the archetype for such tumors, and a variety of chemical methods have been proposed for the detection of the intermediates of melanin synthesis, the melanogens.

It has long been recognized clinically that some patients with far-advanced melanoma void a urine that is dark or that darkens upon standing. This melanuria or, more properly, melanogenuria occurs in only a small proportion of melanoma patients, usually terminal, who survive long enough to develop the requisite tumor burden. The occurrence in the blood or urine of compounds that are involved in melanin synthesis (or possibly in pigment hydrolysis) has long provided a theoretical model for an approach to detecting occult metastases and estimating the tumor burden in the melanoma patient.

The identification of a number of purported melanogens using paper and thin-layer chromatography has been reported by Duchon and Matous (6). They state that DOPA and its metabolites, including indoles as well as other Thormählen-positive melanogens, are specifically associated with melanoma. Gan et al. (8) have reported a diazo-based colorimetric test for measuring urinary melanogens. Türler and Käser (16), using an alumina column and fluorecence detection, found elevated urinary DOPA levels in patients with melanoma, neuroblastoma, and pheochromoblastoma. Eichom et al. (7), using a similar method, reported that in patients with neuroblastoma there were elevated levels of DOPA, dopamine, HVA, and DOPAC. These elevated levels receded toward normal after treatment. Voorhees (17) found that, among several melanogens, only DOPA appeared to be consistently elevated in melanoma patients, but in only one-half of her cases. Hinterberger et al. (9) have reported elevated levels of free catechols in the urines of melanoma patients that increase with progression of the disease.

Agup et al. (1) have reported on the excretion of 5-S-cysteinyldopa in the urine of patients with melanoma. They eluted this material from an alumina column and assayed it fluorometrically. A similar procedure was used to assay DOPA plus dopamine. From their results with 24 melanoma patients, they concluded that the determination of urinary cysteinyldopa is a useful follow-up procedure for such patients, and their data showed a positive correlation between the levels of cysteinyldopa and DOPA plus dopamine. They report, for 1 patient, increasing levels of the cysteinyldopa with clinical progression of disease, but they do not compare the diagnostic utility of the assay with conventional test methods. Utilizing the stable, free radical DPPH, which reacts with the melanogens described by Duchon and Matous (and a number of normal urine constituents as well), we have described (15) an alternative assay for melanoma urines.

1 This research was supported in part by Grant CA-13761 from the National Cancer Institute. This is a publication of the Melanoma Clinical Cooperative Group.

2 Reprints available from either author.

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3 The abbreviations used are: DOPA, 3,4-dihydroxyphenylalanine; HVA, homovanillic acid; DOPAC, dihydroxyphenylacetic acid; DPPH, $\alpha,\alpha$-diphenyl-$\beta$-picrylhydrazyl; MOPA, 3-methoxy-4-hydroxyphenylalanine; GLC, gas-liquid chromatography.
M. S. Blois and P. W. Banda

The improved knowledge of the identity of the compounds occurring in melanoma urines and analytical methods of greater sensitivity and specificity, heretofore, seem not to have resulted in a procedure that meets the requirements of routine clinical application. In short, they have not apparently provided clinical information more conveniently, reliably, or earlier in the progression of the disease than existing methods of laboratory diagnosis. We report here on our clinical experience with a technique utilizing ion-exchange column chromatography with DPPH detection, a combination which we call the DPPH analyzer (3). This is a preliminary report based on observations of patients studied in a melanoma clinic over a 2-year period. There remain many chromatographic peaks of unknown identity. Some of the reaction mechanisms involved in the DPPH detection remain unknown, and there may yet be additional peaks that are melanoma related. The clinical correlations, however empirical they remain, may prove of interest to physicians who are faced with the difficult problem of staging melanoma patients prior to surgery or for monitoring patient response to chemotherapy, immunotherapy, or radiotherapy.

MATERIALS AND METHODS

The DPPH analyzer used has been previously described (3). Elution of the column is carried out with a programmed series of sodium citrate buffers at increasing pH. The sloping base line of the chromatograms arises from the pH dependence of the DPPH absorbance. The current instrumentation uses 0.9- x 35-cm columns of A-9 resin (Bio-Rad Laboratories, Richmond, Calif.), the detector monitors differential absorption (525 to 460 nm) in a 6-mm Glenco Model 56V flow cell (Glenco Scientific, Inc., Houston, Texas), and 0.5-mI sample volumes are injected onto the columns.

Urine samples are aliquots from a 24-hr collection that were kept under refrigeration during collection without added preservatives. Aliquots were acidified to pH 2.50 with HCl and then stored at —75° pending analysis. The mean sample size is approximately 1.5 ml of urine, which is taken to dryness and redissolved in a smaller volume of citrate buffer (pH 2.50). The exact urine volume to be used in the run is determined from the decolorization of DPPH by a urine aliquot (15), in order to load the columns with a constant level of reducing equivalents.

Melanoma urines were obtained from patients being cared for or seen in consultation at the University of California (San Francisco, Calif.) Melanoma Clinic. Urine samples have also been received from participating institutions of the Malignant Melanoma Clinical Cooperative Group (Dr. T. B. Fitzpatrick, Chairman, Massachusetts General Hospital, Boston, Mass.), of which our clinic is a member.

RESULTS

Some of the general features of the chromatograms obtained with the urine of melanoma patients have been described earlier (3, 4). The chromatogram of a normal urine sample is shown in the upper portion of Chart 1, while that of a patient with disseminated malignant melanoma is shown in the 2 lower diagrams. This patient presented a not uncommon clinical problem. He had a large and neglected primary melanoma of the skin when first seen. After excision of this primary, he was given a complete metastatic work-up and the test results were equivocal (elevated lactate dehydrogenase and alkaline phosphatase, normal liver and brain scans, and normal chest X-ray). The middle chromatogram shown is of the urine obtained at this time. Four peaks not present in normal urines are seen. Four months later, a repeat urine chromatogram showed further increases in all of these peaks (by factors of 2 to 4 times) and the emergence of a 5th peak. About 1 month later, the patient died. Autopsy revealed widely disseminated melanoma. The peaks that we consider to be abnormal are indicated by the lettered arrows. The identity of the compounds responsible is presently considered to be: Peak A, unknown, but appears to be a component found in the Prota synthesis (13) of 5-S-cysteinyldopa; Peak B, provisionally DOPA; Peak C, tentatively identified as MOPA; Peaks D and E, unidentified, but known not to be dopamine, vanillylmandelic acid, 5-hydroxyindoleacetic acid, HVA, DOPAC, or VLA, which have distinctly different elution

Chart 1. Top, chromatogram of normal urine; middle and bottom, those of Patient 31 (PT. 31) showing increase of melanogens during disease progression. Peaks A through E are the abnormal peaks discussed in the text. The numbered peaks mark the elution position of the following standard compounds: 1, uric acid; 2, dihydroxymandelic acid; 3, vanillylmandelic acid; 4, HVA; 5, 5-hydroxyindoleacetic acid; and 6, creatinine.
times. Evidence regarding the identification of Peaks A, B, and C is discussed below.

It had been recognized earlier that the urines of some patients with disseminated melanoma reacted strongly with DPPH and that the degree of reaction increased with the further spread of their disease (15). The clinical value of the DPPH test on unfractionated urine was limited, however, by the overlapping range of values between disseminated cases and normals due to the reaction of DPPH with many common urinary constituents. The development of the DPPH analyzer has allowed the melanogens to be separated from other metabolites in the urine, while detecting both normal and abnormal classes of compounds. It is now possible to follow the progressively increased excretion of individual melanogens as the tumor spreads. Chart 2 shows a series of the urines of a patient with progressively disseminating melanoma and the associated clinical and laboratory findings.

A group of 4 melanoma patients with enlarged regional lymph nodes were studied. Two of these patients were clinical Stage II and the other 2 were Stage III. 1 of the latter because of direct extension of axillary nodal tumor into the chest wall and the other because of remote, small cutaneous metastases. All 4 showed 1 or more abnormal peaks in the chromatogram, and none had evidence of disease beyond that described. These patients all underwent lymphadenectomy, and the patient with chest wall involvement had all accessible tumor excised in continuity. Subsequent urine chromatography showed a striking decrease of the abnormal peaks in 2 of the patients and complete disappearance in the other 2. Of the 2 patients whose abnormal peaks failed to disappear completely, the reduction in size for each was greater than two-thirds. While the increased excretion of melanogens with clinically worsening disease can only be considered as an association, the results with these 4 patients implies a casual relationship between tumor burden and melanogen excretion.

Presented in Table 1 is a summary of the melanogen excretion patterns for a group of 39 patients known to have recurrent or progressing melanoma according to conventional diagnostic methods.

In evaluating the urine chromatograms, we have for convenience scored the peak heights as follows: a peak height of at least 2 cm on the recorder paper (and reproducible on a repeat run), +: greater than 2 cm and less than 8 cm, + +: greater than 8 cm but not extending off scale, + + +; and off-scale values, + + + +. This method of reporting is not precise, since peaks of distinctly different sizes are grouped and reported as though they were equal. The + + + + class, for example, contains peaks that may be 10-fold different in size, while the difference between the + and + + + + classes may be up to 100-fold. The direct measurement of these components is discussed below. With several of these patients serial chromatograms were run, but the melanogen patterns shown in the table were those obtained at or near the same time that the other laboratory or clinical data used in staging was obtained.

The data in Table 1 help to reconcile some of the earlier reports by other investigators, who have described the association of individual and usually single melanogens with a patient's clinical state. It is evident from these data that no single melanogen is characteristic of the tumor. The excretion of Peak C is the most striking and the most common (in 26 of 33 positive urines), but diagnosis based upon this alone would miss several tumors that were proven histologically. The occurrence of DOPA (Peak B) (present in 11 of 33 positive urines) is in general agreement with the findings of Voorhess (17) that DOPA was elevated in only one-half of her cases. Our results disagree with her conclusion that DOPA is most frequently elevated in the urine of melanoma patients. Peak A was present in 23 of 33 positive urines. The unidentified Peaks D and E were present in 5 and 12 cases, respectively (of 33). Other peaks, such as at the position of HVA, have been strikingly elevated in some patients, but the overall correlation with the extent of clinical disease was more erratic. It is probable too that dietary artifacts are more serious with these compounds than with the 5 peaks we have chosen.

**DISCUSSION**

It is known from the work of Pauling et al. (12), using GLC, and from Scott et al. (14), using liquid-column chro-
Table 1
Melanogen excretion patterns for 39 patients with clinically disseminated melanoma

The metastatic sites believed involved at the time of the urine chromatography and the evidence of involvement is given. The method for scoring the peaks is discussed in the text.

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* EMJ, computerized axial tomography.
Matography with UV detection, that each method yields in excess of 300 resolvable peaks in normal urines. The number of DPPH-positive peaks in urine obtained from the cat-ion-exchange column chromatography is approximately 50, with perhaps up to an equal number detected on a column designed for more basic constituents. Therefore, within any 1 of the eluted DPPH-positive peaks, more than 1 chemical species can be expected to be present. At least 1 of these must be DPPH reactive; others may be as well.

The detection of 5-S-cysteinyldopa is at present ambiguous in our chromatography system. Prota (13) has described this amino acid as occurring in the biosynthesis of the pigment found in red hair (phaeomelans) and has reported its synthesis. It is formed in the reaction mixture of DOPA, cysteine, and mushroom tyrosinase (13). When we acidify this reaction mixture after a 1-hr incubation and apply it to the DPPH analyzer, we find at least 6 peaks. Four peaks are relatively minor, 2 of which represent residual cysteine and DOPA. One of the major peaks corresponds in position to Peak A, while the other major peak elutes close to the position of MOPA. In the absence of cysteine, the reaction mixture yields only residual DOPA upon chromatography. The identification of Peak B as DOPA is based on the following evidence: the elution time corresponds to that of DOPA; when DOPA is added to a urine sample showing Peak B, this peak is enhanced without change in line shape; and in patients receiving L-DOPA for treatment, a large peak appears in this position. The identification of Peak C was made by isolating the eluate containing this peak, derivatizing the sample, and passing it through the GLC. The principal GLC peak obtained corresponded to that produced by an authentic sample of MOPA that had been similarly derivatized when cochromatographed with it. Confirmation of these identifications, and a determination of which product in the Prota reaction mixture corresponds to cysteinyldopa, awaits mass spectrographic studies.

A number of compounds may be detected with the DPPH analyzer (3) including indican, ascorbic acid, uric acid, dihydroxymandelic acid, vanillylmandelic acid, DOPAC, VLA, HVA, and 5-hydroxyindoleacetic acid. Their elution positions do not correspond to the unknown peaks (D and E). Dopamine does not appear in the chromatograms shown, since chromatography of the catecholamines requires a different ion-exchange resin than presently used.

In its present state this technique, using the criteria given, affords considerable clinical information, and it is the clinical correlations of these melanogen excretion patterns that we wish to emphasize here.

We do not find the varying excretion patterns of Table 1 surprising for 2 reasons. The synthesis of melanin pigment requires that the melanocyte assemble an elaborate organelle, the melanosome, with an intact enzymatic apparatus and possibly with a control mechanism using 1 or more inhibitors. The development of this apparatus may be arrested in the neoplastic melanocyte at any 1 of several stages, from which one might expect to find the accumulation of pigment-related metabolites of several types and in varying proportions. Just as the pigmented state of melanomas varies, so may the excreted melanin intermediates. It seems likely that the anatomical site of a tumor nodule will influence the mix of products that will arrive in the urine. The blood-brain barrier may influence which melanogens will be found; 2 patients with brain metastases alone (confirmed at autopsy) excreted neither DOPA nor MOPA in detectable amounts. Tumors lying the the tributaries of the portal circulation may be expected to expose their products to the effects of hepatic conjugation, a possible origin of MOPA by hepatic methylation of DOPA.

The quantitative measurement of the compounds responsible for the abnormal peaks awaits their positive identification, since the stoichiometry of the DPPH reduction varies for different compounds (2). The quantitative measurement of the abnormal compounds will permit determining the normal range of these compounds in urine. We do not expect that the compounds responsible for the observed abnormal peaks need be entirely absent from normal urines but rather that they may occur at levels below those found for patients with active disease. Since creatinine is detected by the DPPH method and produces a peak in the chromatograms, it serves as an internal standard that is useful in quantitative measurements.

The natural history of melanoma and the limitations of noninvasive patient work-up make difficult the accurate determination of the diagnostic sensitivity and specificity. One can, however, estimate this accuracy, in particular, for certain clinical applications. As a control group, we used samples from patients seen in the Melanoma Clinic who met the following criteria: a history of a primary melanoma, previously excised with the deepest level of tumor invasion being Clark Level II (5), and no evidence of disease. This class of patients is reported to have only a few percentages of recurrence and to have 100% 5-year survival (5, 18). The use of such a population as controls would tend to increase the apparent rate of false positives, but it would more closely represent the age and sex distribution of the Stage II and III patients. In this control group, there were a total of 25 patients and 33 samples.

Among this control group the chromatograms had only 2 peaks at the + level (of 165 possible) and none greater than this. In these 33 presumed normal samples, peaks were present with the following distribution (95% of them being at the + level): A, 13; B, 5; C, 3; D, 10; and E, 3. In the samples from 39 patients with known disease, the distribution (Table 1) is: A, 23; B, 11; C, 26; D, 5; and E, 12. From this, we conclude that the discriminatory power of Peak C is greatest: it was falsely present in only 3 normal samples (in no instance greater than +) and present in 26 disease samples (in one-half of them, either + + + or + + + + +). By the same reasoning, Peak E (unknown) has the next greatest discriminating value, followed by Peak B. Peak A discriminates rather poorly with disease, as does Peak D, but both are provisionally retained because of striking occurrences in individual cases.

Because of the differences in discriminating power of the different peaks we have formulated a revised decision rule. Chromatograms are considered abnormal if any one of the following occur: Peak C is present at the + level (or greater); Peak E is present at the + level (or greater); Peak B is present at the + + level (or greater); Peak A is present at the + + level (or greater). Applying this rule to the control group, we find 7 “abnormal” urines corresponding to a
false-positive rate of 20%. In the disease group, the same
decision rule leads to an overall false-negative rate of 23%.
Among the control group, 6 patients with abnormal results
had multiple tests; in 5 of them, the other tests were normal.
Repeat urine chromatography on the disease group led in
every instance (except when the patients received therapy)
to further abnormal results, either of the same degree or of
greater abnormality. In reporting results clinically, we cur-
cently record those test results barely meeting the condi-
tions of the above decision rule as equivocal and request
repetition of the test; results with larger peaks or meeting
combinations of the minimum criteria are reported positive.

A source of false positives that must be excluded is drug
interference. Specific interferences from α-methyl-DOPA
and L-DOPA have been noted; these drugs must be avoided.
Other drug metabolites have been noted. They characteris-
tically appear as large peaks, and those observed to date
have had different elution times from the melanoma-related
peaks.

As would be expected, the overall false-negative rate is
sensitive to the patient mix: it would be larger with a higher
proportion of patients having small cutaneous metastases
(which we miss) and lower if one had more patients with
liver metastases (which appear to be detected reliably).
More to the point is the utility of the test in revealing disease
not diagnosable from physical examination or chest X-rays.
If we omit from the disease group those patients with only
small cutaneous metastases, s.c. nodules, enlarged lymph
nodes, or lung metastases, the false-negative rate drops to
about 12% (Patients 20, 24, 26, and 39 of 34 patients). In 7
patients with liver metastases confirmed by biopsy or au-
topsy within 90 days of the urine testing, there were no false
negatives.

Using whole-body 67Ga scanning, Milder et al. (11) re-
ported the detection of liver metastases in 4 of 7 patients
with known liver involvement. Melanomas larger than 2 cm
were detected in 75% of cases (for all sites), while those less
than 2 cm were detected only 17% of the time. Lunia et al.
(10), using technetium-99m-sulfur colloid liver scans, report
for liver metastases (for a variety of tumors) a false-positive
rate of 17% and a false-negative rate of 25%, with histopath-
ological confirmation. These authors also comment on the
unreliability of detection when the tumors are less than 2 cm
in diameter. From our experience, it appears that the urine
chromatography is a more reliable and earlier means of
detecting liver metastases than liver scanning. In 6 patients,
elevated melanogens were detected at or near the time
when liver scans were normal, with the scans becoming
positive within 1 to 3 months or with other evidence of liver
involvement becoming available.

With brain metastases, the earliest indications in our pa-

tients have been neurological signs or symptoms; the most
useful confirmatory tests have been the brain scan or com-
puterized axial tomography (EMI scanner). We had 2 pa-
tients who were believed to have brain metastases only and
both showed elevated melanogens (Patients 6 and 11). One
patient was considered to have bone metastases alone
(confirmed by biopsy), and the urine chromatography
appeared to be normal. There is insufficient evidence to attri-
butate this false negative either to insufficient tumor bulk or
to some peculiarity of this metastatic site.

In clinical use, we have found this technique to yield
information frequently not otherwise available. Since it is
less costly than isotopic scanning and is hazard-free, we
find it useful for the periodic, routine monitoring of disease-
free but high-risk patients. In cases where the usual diag-
nostic methods give conflicting clues, a fairly common situ-
ation, we find that serial measurements of the melanogens
gives a useful measure of the extent of disease. For patients
with stable, metastatic disease receiving chemotherapy, se-
rial melanogen determinations appear to be promising in
evaluating the response to therapy. In one-half of the pa-
tients with positive urines, the melanogen results affected
patient management because of earlier availability or they
assisted in resolving conflicting diagnostic clues. In the
other half, the results served to confirm the diagnostic
impression made by the other findings.

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Melanoma Detection by Urine Chromatography


Detection of Occult Metastatic Melanoma by Urine Chromatography

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