Prognostic Import of Circulating Cancer Cells After Curative Surgery:*

A Long Time Follow up Study

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DURING the last three years we have studied 99 patients who had cancer, using 591 separate preparations, to determine the presence or absence of cancer cells circulating in the human blood stream.

One of the objectives of this study was to determine the prognostic significance of the presence of cancer cells circulating in the blood stream after supposedly curative surgical operation with or without the use of adjuvant chemotherapy.

A group of 17 patients was selected for intensive study. These patients were registered in the Adjuvant Cancer Chemotherapy Program of the Cancer Chemotherapy National Service Center, sponsored by the National Cancer Institute. Our patients are part of a large group composed of patients from many institutions who are being studied by a double-blind method to determine the effect of various chemotherapeutic agents. We selected this group in our institution because they have been more intensively followed than are our other patients with cancer.

It is to be emphasized, however, that neither the authors nor the technical personnel had any knowledge of the clinical progress of these patients until after the cytology studies had been completed and recorded. The blood samples were drawn by technicians, processed, screened and results recorded over periods of months without any knowledge of the clinical state of the patient.

Many technics and methods have been used in many different laboratories. None of these are easy and it takes a great deal of time and effort for an investigator and his team to develop a technic which is reliable in their hands. We worked for about a year using the blood of patients with advanced cancer, normal blood of patients without cancer and normal blood to which Hela cells were added before we began to believe that we could get reliable results. The technics we now use are outlined:

Preparation of Slides

1. Ten ml. of heparinized blood is placed in a 50-ml. siliconized centrifuged tube which is then filled with P.V.P. Tween 80® solution.** Invert tube several times.

2. The tube is centrifuged at 1,400 rpm for 8 minutes.

3. The supernatant fluid is aspirated to within ¼-inch of the sediment and dis-

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** P.V.P. Tween 80:

P.V.P. (Plasdone C)	10.0 Gm.
W.R. 1339	0.3 cc.
E.D.T.A. (Sequestrene)	1.0 Gm.
Tween 80	3.0 cc.
Normal saline q.s. ad	1,000 cc.

TABLE 1. *Summary of 4 Patients Who Have Consistently been Shown to be Free of Circulating Cancer Cells and Who are Clinically Free of Recurrence*

Patient, Primary Site	Date of Operation	Dates and Results of Cell Study	Clinical Status
P. C. Rectum	9-25-59	Neg: 10-22-59, 10-29-59,, 10-26-61	Clinically free 10-26-61 (25 mos. postop.)
M. S. Rectum	8-18-59	Neg: 12-11-59, 3-16-60	Died 4-11-60 of broncho-pneumonia (age 71). Free of cancer for 8 mos.
C. W. Rectum	10-16-59	Neg: 10-22-59, 10-26-59, 11-11-59, 4-14-60, 9-29-60, 2-2-61, 9-14-61	Free of disease 9-14-61 (23 mos.)
V. B. Breast	1-14-60	*S : 1-19-60 Neg: 2-25-60, 4-21-60, 12, 1-60, 3-2-61 4-27-61, 7-27-61 10-26-61	Free of disease 10-26-61 (15 mos.)

* S = Suspicious.

carded. Tween 80 solution is added to the 25 ml. mark, contents of tube are mixed by inversion. 2.0 ml. of 1.0% saponin is mixed with 20 ml. of Tween 80 in a separate tube, this mixture is then added to the first tube. The tube is inverted several times. At the end of a minute 6.0 ml. of 5.0% Calcium Gluconate is added and the tube inverted for complete mixing.

4. The specimen is centrifuged at 1,000 rpm for 5 minutes.

5. The supernatant fluid is aspirated to within ¼-inch of pellet. The cells are suspended in the small amount of fluid remaining by vigorously striking against palm of the hand.

6. Add 4.0 ml. of normal saline. Mix by shaking. Fill tube to 50 m. mark with Fixative No. 1.*** Invert several times.

7. After 10 minutes, filter through millipore filters with suction. The number of filters used depends upon the number of cells present. With many cells more filters are necessary so that the preparation is not too thick.

8. Each filter is carefully placed in a separate small dish containing Modified Carnoy's Fixative † for 10 minutes.

9. Place filters in suitable holders and stain with Papanicolaou or Shorr's Stain. Mount on 3″ × 2″ slides and cover with 50 × 50 mm. cover slides using Permount.

Staining Procedure

Shorr's Procedure

1. After fixation in Carnoy's solution, transfer slides into alcohol, 80%.

2. Rinse in alcohol, 70%, alcohol, 50% and distilled water, until clear.

*** Fixative No. 1:

Buffered formalin	700	cc.
Alcohol, 95%	300	cc.
Glacial Acetic Acid to give pH of 7.00		

Buffered Formalin: Dibasic sodium Phosphate 6.5 Gm.; Monobasic sodium phosphate 4.0 Gm.; Formalin, 40% 100.0 cc.; Distilled water q.s. ad 1,000 cc.

† Modified Carnoy's Fixative:

Alcohol, 95%	2,100	cc.
Chloroform	750	cc.
Glacial acetic acid	150	cc.

Store in tightly-stoppered brown bottle at room temperature.

3. Stain in dilute hematoxylin for 45 seconds.

4. Rinse in running tap water until clear (3–4 minutes).

5. Rinse in distilled water.

6. Place in ammonium hydroxide, 1.5% made in alcohol, 70% for 1 minute.

7. Rinse in distilled water, then alcohol, 50%.

8. Stain in Shorr Stain for 1 minute.

9. Rinse three times in alcohol, 50% using 3 separate changes.

10. Run through alcohols, 70%, 80% and three dishes of 95%.

11. Dehydrate and clear by running through: 1) n-Propanol—1 minute; 2) A mixture of equal parts of propanol and xylol; and 3) 2 dishes of xylol, 3 minutes each.

Papanicolaou Procedure

Steps 1 through 5 are the same as in the preceding Shorr's Procedure.

6. Rinse in alcohol, 50%.

7. Place in solution of ammonium hydroxide, 1.5% in alcohol, 70% for 1 minute.

8. Rinse in alcohol, 70% and run through alcohols, 80% and 95%.

9. Stain in OG-6 for 75 seconds.

10. Rinse in 3 dishes of alcohol.

11. Stain in EA-65 for 3 minutes.

12. Rinse in 3 dishes of alcohol, 95%.

13. Dehydrate and clear by running through: 1) n-Propanol—1 minute; 2) A mixture of equal parts of propanol and xylol; and 3) 2 dishes of xylol—3 minutes each.

Our preparations are screened by tech-

TABLE 2. *These Patients Were Treated by Surgery and Chemotherapy and Developed Clinical Recurrence. This shows a Comparison Between the Time Cancer Cells Were Found Circulating in the Blood and the Time at Which Clinical Recurrence Was Determined. All Are Known to be Living with Cancer or Dead from Cancer*

Patient, Primary Site	Date of Operation	Date Cancer Cells First Found in Peripheral Blood	Last Date Pt. Observed to be Clinically Free of Cancer	Date Pt. Found to Have Evidence of Clinical Recurrence	Comment
M. B. Cecum	10- 7-59	12-15-60	8-22-60	4-24-61	Suspect cells found 5 mos. and cancer cells 2 mos. before clinical recurrence observed.
M. M. Breast	1- 8-60	1-12-60	1- 8-60	2-19-60	Cancer cells found 1 mo. before clinical recurrence.
P. B. Lung	3-26-60	10-20-60	11- 2-60	2-13-61	Cancer cells found 3½ mos. before clinical recurrence observed.
V. K. Breast	4-20-60	4-27-60	7-29-60	8-16-60	Cancer cells found 3¾ mos. before clinical recurrence observed.
N. P. Cecum	9-29:59	10-14-59	9-29-60	2-15-61	Cancer cells found 16 mos. before clinical recurrence observed.
E. R. Sigmoid	3-22-60	3-20-60	3-22-60	9-28-60	Cancer cells found 6 mos. before clinical recurrence observed.

nicians who mark abnormal cells with an ink dot. These marked cells are then reviewed by the chief technician (junior author) and further reviewed by the senior author and at times by consulting pathologists and hematologists.

Four of the 17 patients had repeated analyses of their peripheral blood for cancer cells and none found and all have shown no clinical evidence of recurrence of cancer (Table 1).

Thirteen patients were found to have cancer cells circulating in their peripheral blood after attempts at cure by operation.

Six of these 13 patients have verified evidence of recurrence. They are alive with, or dead of cancer (Table 2). In these, cells were found in the peripheral blood one to 16 months before clinical recurrence was detected. Furthermore, and most importantly, five of six patients were observed clinically and were thought to be free of

TABLE 3. *Summary of 7 Patients Who Had Attempts at Curative Surgery Who Are Clinically Free of Cancer but Who Have Cancer Cells Circulating in Their Peripheral Blood*

Patient, Primary Site	Date of Operation	Dates and Results of Cell Study	Date Patient Observed to be Clinically Free of Cancer	Comment
E. S. Breast	10-30-59	Neg: 11- 3-59, 11-13-59, 12- 4-59 *S : 3-24-60 Neg: 4-21-60 Pos : 1- 5-61, 5- 4-61, 7- 6-61	10-30-61	Clear of cancer cells 1½ mos., then cells appeared Clinically free of cancer at 24 mos.
A. J. Breast	3-23-60	Pos : 3-30-60, 6-23-60, 8- 4-60, 10- -6-60, 3- 2-61, 5- 4-61, 8- 3-61, 9- 7-61	9- 7-61	Cancer cells found 8 times. Remains clinically free of cancer at 18 mos.
D. S. Breast	2-19-60	Pos : 2-26-60, 4-28-60, 6- 9-60, 8-24-61	8-24-61	Cancer cells found 4 times. Remains clinically free of cancer at 18 mos.
M. M. Breast	10-10-59	Neg: 12-10-59, 1-14-60 *S : 10- 6-60, 2- 2-61 Pos : 4- 6-61, 8- 9-61	8- 9-61	Clear of cancer cells 3 mos., then cells appeared. Clinically free of cancer at 18 mos.
A. H. Breast	10-13-59	Neg: 12-10-59, 1-14-60 *S : 3-10-60 Pos : 6- 2-60, 4- 6-61, 10- 5-61, 11- 2-61	11- 2-61	Clear of cancer cells 2 mos., then cells appeared. Clinically free of cancer at 24 mos.
J. M. Rectosigmoid	4-20-60	Neg: 4-27-60, 8-11-60 Pos : 10-13-60, 11-10-60, 12- 8-60, 4-13-61, 7- 6-61	10- 5-61	Clear of cancer cells 4 mos., then cells appeared. Clinically free of cancer at 18 mos.
T. H. L. Colon	4-20-60	Pos : 4-27-60 Neg: 9-22-60 *S : 2- 2-61 Neg: 4- 6-61, 6-29-61	7-10-61	Cancer cells present 1 week postop., then disappeared. Pt. clinically free of cancer at 15 mos.

* S = Suspicious.

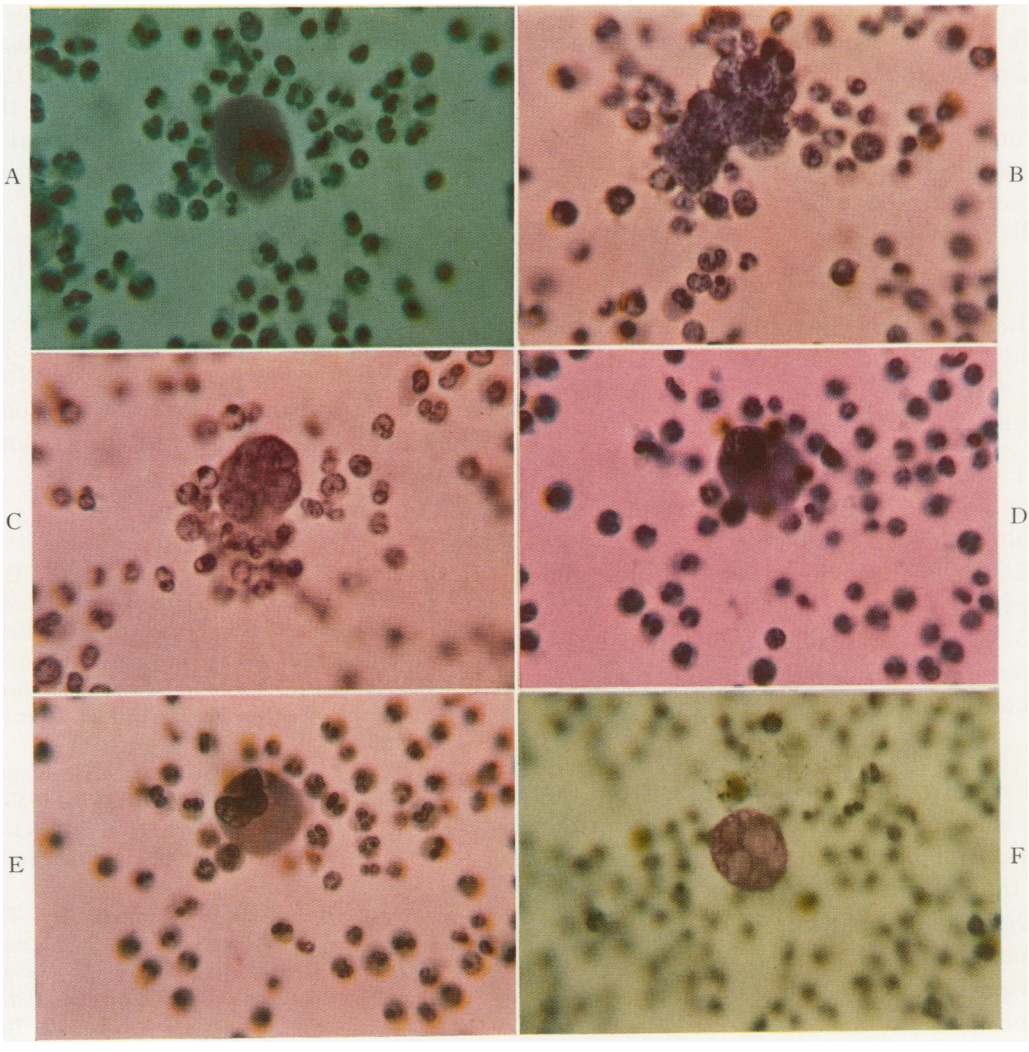

FIG. 1. Photomicrographs of examples of malignant cells found in peripheral blood of: A-E, Cancer of the Breast, F. Hypernephroma.

disease at the time of, or many months after we had demonstrated cancer cells in their peripheral blood. These patients all had multiple cell studies. Table 2 shows only the first date cells were found. All continued to show cancer cells on subsequent repeated studies.

Seven of the 13 patients who were found to have cancer cells in their peripheral blood have been found to be clinically free of disease for periods of 15 to 24 months (Table 3). Five of these patients had cancer of the breast and two cancer of the colon.

The pattern of the time of the appearance of cancer cells after original operation on these patients is interesting.

Four of these were clear of cells for periods of one and one-half to four months. Cells then appeared in their blood and have been found repeatedly on many subsequent examinations.

One patient showed cells one week after operation and was clinically free of cells

thereafter. Two of the seven have shown cells to be present on every determination.

Besides the difficulty in the technic of isolating and identifying the cells there is another difficulty which lies in the impossibility of presenting photographic evidence that the cells we find are cancer cells.

Summary

Review of the literature related to this study is beyond the scope of this paper. Brief reference to some of the background studies are as follows:

1. Fundamental observations: Ashworth [2]; Pool [11]; Engell [3]; Fisher [5]; Moore [8]; Malmgren [7]; and Roberts.[13]
2. Technic: Papanicolaou [10]; Seal [16]; and Alexander.[1]
3. Prognostic significance of circulating cancer cells: Engell [4]; Salgado [15]; Long [6]; Moore [9]; Potter [12]; Roberts [14]; and Watne.[17]

The significance of our work is to be inferred from the relationship between the presence or absence of cancer cells in the peripheral blood and the clinical progress of the patient.

None of the patients in whom we have been unable to find cells circulating in the peripheral blood show any evidence of clinical recurrence.

In all cases in which clinical recurrences have occurred we have found cancer cells circulating in their peripheral blood for periods up to several months before the clinical recurrence could be determined.

The third, and to us the most interesting group, is composed of those patients who are clinically well at the present but in whom we have consistently and repeatedly observed cancer cells circulating in their peripheral blood for as long as two years.

In this group there are five with cancer of the breast. These have been clinically free of disease for periods of 18 to 24 months. This cancer is notoriously treacherous and frequently occurs many years after apparent successful treatment. A two-year follow up is known to mean nothing in this disease. We intend to follow this group as long as possible, as well as the two with cancer of the colon. We predict that they will have clinical cancer if we follow them long enough.

The other two patients in this group who are still showing cancer cells had cancer of the colon. Although free of clinical cancer at 15 and 18 months, respectively, we believe that it is likely that they will develop recurrence at some future time and we intend to keep following them.

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Discussion

Dr. Warren H. Cole (Chicago): As Dr. Drye stated, it is quite difficult to diagnose these cells obtained from the blood stream as individual cells. When you get them in clumps it is much easier. As he has also implied, some workers, including even a few skilled cytologists, are doubtful that these are actually cancer cells; we do admit it will take years to prove their significance, whatever it is. I, personally, am quite sure that we are dealing with cancer cells, although we do know and realize that there will be a large percentage of error in our interpretation of cells. That is to be expected. We are also sure that the majority of cells circulating in the blood stream will die. They are killed by the host resistance, and of course as resistance decreases a larger number of cells will survive.

Even though Dr. Drye's series is a very small one, it still has some very significant data in it. You recall that smears from 13 of the 17 patients in this series were positive. There are three important features in that point: 1) that none of the patients having negative smears have as yet had recurrence; 2) that all patients developing recurrence had positive smears one to 16 months before the recurrence; and 3) that half the patients with positive smears already had recurrence.

Our incidence of positive smears is not as high as Dr. Drye's. We are using quite a different technic and there may be a difference there. In our total series of several hundred, we had only about 25 per cent positive smears in the curative group (i.e., the patients having operation with no residual tumor), and about 40 per cent in the incurables.

When we analyzed our entire series we did not find a significant difference in survivals in the two groups, although the deaths were higher in the patients having positive samples. However, when we analyzed the 97 pateints who were studied during the operation, we found something rather significant. This observation extends over a period between two and five years. Thirty-eight per cent of the patients having positive blood samples in this series observed during operation are dead, whereas only 20 per cent of those having negative blood samples are dead; accordingly, it appears that in a two- to five-year period of observation in operated cases the occurrence of cells is a bad prognostic sign. However, I believe that all of us working in this field will admit a longer period of observation is necessary before we can determine the true significance of these cells in the blood stream.

Dr. William T. Rumage (closing): The problem of identification, of course, is one that has occurred and does occur regularly and a problem with which all investigators in this field are very much concerned. I hesitate at this hour to turn out the lights once again but you might want to see some more of our slides. We are very proud of how well our technician does this particular technic.

(Slide) Dr. Drye showed you four breast cases. The first one on this series is a melanoma. The next one is also a melanoma. You can see the signet-ring appearance that has been described previously.

The next slide is from a patient with a cervical malignancy; and the next slide is one from a renal clear cell carcinoma. The next slide is also from the G.U. tract, from the bladder, a clump of cells. And then the last one is from a pulmonary malignancy.

Radical excision of a malignant growth all too often has proved to be ineffective in a patient with cancer even though the surgery was thought to be curative at the time. Therapeutic failure may be due to three reasons: 1) local recurrence by direct extension or a lymphatic spread; 2) the growth of distant metastases which existed at the time of surgery and were not recognized; or 3) the devel-