Evaluation of Micro-Leukocyte Adherence Inhibition as an Immunodiagnostic Test for Pancreatic Cancer


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Abstract

Carcinoma of the pancreas is the fifth most lethal cancer in humans. Its almost uniform mortality rate is largely related to the rarity with which a diagnosis is established early in the course of the disease. In this study, we have evaluated the leukocyte adherence inhibition (LAI) assay as a specific immunodiagnostic test for the presence of pancreatic cancer. One hundred thirty micro-LAI assays were performed on 104 individuals that included 23 pancreatic carcinoma patients, 29 patients with benign disease (14 with acute pancreatitis), 26 patients with nonpancreatic gastrointestinal cancers, and 25 normal healthy volunteers. Using an LAI index of ≤0.20 as a cut-off value, 19 of 23 patients with pancreatic cancer had a positive test result in the micro-LAI assay. In contrast, 3 of 81 control patients also gave a positive test result. The LAI response of the pancreatic carcinoma patients appeared to be immunologically specific inasmuch as the leukocytes of these patients did not respond to a control colon carcinoma tumor extract. Thus, the micro-LAI assay is able to detect specifically pancreatic cancer and to discriminate between pancreatic cancer, acute pancreatitis, other forms of cancer, and the normal state. While these results suggest an immunodiagnostic potential for the micro-LAI assay in pancreatic cancer, this potential can be realized only after a much larger number of patients are evaluated and concomitant technological improvements are made in the assay that will result in a more standardized response.

Introduction

Carcinoma of the pancreas is the fifth most lethal cancer in humans, and its incidence is increasing (1). The almost uniform mortality associated with pancreatic cancer is primarily related to a lack of means of establishing a diagnosis early in the course of the disease inasmuch as the signs and symptoms associated with pancreatic carcinoma (obstructive jaundice, weight loss, and anorexia) are all relatively late events in the natural history of this disease (2, 4). Furthermore, the poor prognosis is secondarily related to the difficulty in establishing a proper differential diagnosis when patients present with these vague symptoms. Current techniques are for the most part invasive and are most sensitive in patients with more advanced disease (5). In search of a moderately accurate and specific blood test, investigators have turned to assays of cell-mediated immunity.

Cytotoxicity (10), leukocyte migration inhibition (3, 6), and LAI (8, 15) have been used to detect the presence of specific forms of gastrointestinal cancer on the basis of measuring a specific antitumor response to tissue-type-specific antigen(s). The LAI assay was described originally by Halliday and Miller (9) in 1972 and has been modified to an automated microassay by Leveson et al. (12). Our laboratory has used the micro-LAI assay to determine whether specific diagnostic retrieval in pancreatic carcinoma can be improved. On the basis of 97 LAI tests, we reported recently (14) that the micro-LAI assay is able specifically to detect pancreatic cancer and discriminate between pancreatic cancer and acute pancreatitis, other forms of cancer, and the normal state. These results suggested an immunodiagnostic potential for the LAI microassay in pancreatic cancer. In this study, we summarize the results after 130 tests and evaluate whether the means of expressing the results has an effect on the diagnostic ability of the test.

Materials and Methods

Subjects Tested. Individuals donating a blood specimen for LAI testing could be assigned to 1 of 4 groups: Group 1, patients with pancreatic cancer; Group 2, patients with benign conditions; Group 3, patients with malignant disease other than pancreatic cancer; and Group 4, normal volunteers. Results were excluded from this study if the patient was within 2 weeks postsurgery, had received chemotherapy or radiation therapy 30 days prior to the date of testing. The LAI tests were performed on both “coded” and blind specimens.

Patients with Pancreatic Cancer. This group consisted of 23 patients with carcinoma of the pancreas. Diagnosis was confirmed by histological examination of the tumor. Six patients had primary disease with or without metastases to the regional lymph nodes, and 17 patients had primary disease with either hepatic or distant metastases at the time the blood sample was taken.

Patients with Benign Disease. There were 29 patients in this group. Fourteen of these patients had acute pancreatitis. The balance of these patients had benign conditions that were not related to the pancreas.

Patients with Malignant Disease. There were 26 patients in this group with cancers other than pancreatic cancer.

1 Presented at the International Workshop on Leukocyte Adherence Inhibition, May 15 to 17, 1978, Buffalo, N. Y. This work was supported by USPHS Grant CA-23646 and Contract N01-CM-43794 from the Department of Surgical Oncology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N. Y. 14263.

2 Presenter. To whom requests for reprints should be addressed.

3 The abbreviation used is: LAI, leukocyte adherence inhibition.
Nineteen of these patients had nonpancreatic gastrointestinal cancers.

**Normal Volunteers.** This group consisted of 25 individuals, most of whom were staff either in our laboratory or in other departments at Roswell Park Memorial Institute. While the mean age and the male to female ratio of the first 3 groups were comparable, the normal volunteers were younger.

**LAI Technique.** The micro-LAI technique has been described previously (12) and is presented in detail as part of this workshop (7). The following is a brief description.

**Tumor Extracts.** Crude membrane preparations of pancreas and colon carcinoma were used in this study. Crude membrane antigens were prepared by finely mincing tumors with scissors and resuspending them in twice their volume of phosphate-buffered saline (0.01 M NaH₂PO₄ / H₂O / 0.14 M NaCl), pH 7.2. The tissue was homogenized in a Tekmar Tissumizer at medium speed, and the supernatant was collected after centrifugation for 20 min at 200 × g. The supernatant was dialyzed against 200 times its volume of phosphate-buffered saline for 3 days with 3 buffer changes. Protein estimations of final antigen preparations were made with the microbiuret method (11), including 0.5% deoxycholic acid for solubilization of membranes.

**Preparation of Peripheral Blood Leukocytes.** A 20-ml sample of heparinized venous blood was collected in vacutainer tubes (Monoject; Sherwood Medical Co., St. Louis, Mo.). The blood was then transferred to a plastic syringe (Plastipak; Becton-Dickinson, Rutherford, N. J.) and incubated upright for 1 hr at 37°. A leukocyte-rich plasma fraction was decanted into a plastic centrifuge tube and centrifuged for 8 min at 200 × g. The resultant pellet was treated in Tris-buffered ammonium chloride (Boyles solution) for 10 min at 4° to lyse erythrocytes. The Boyles solution was neutralized with medium (Roswell Park Memorial Institute Tissue Culture Medium 1640, plus 10% fetal calf serum; Grand Island Biological Co., Grand Island, N. Y.) and spun for 8 min. The leukocytes were washed 2 more times, and the cell concentration was adjusted to 5 × 10⁶ cells/ml with a Neubauer hemocytometer. Viability was assessed by exclusion of 0.2% trypan blue. In all cases, viability was greater than 95%.

**LAI Microassay.** Briefly, the test is carried out on No. 3034 microtest plates (Falcon Plastics Co., Oxnard, Calif.). The plates are divided into 4 quadrants. One quadrant contains medium, and the other 3 contain membrane preparations at 3 protein concentrations, 0.1, 0.05, and 0.01 mg/ml. The results are graphed at an antigen concentration of 0.05 mg/ml, since the optimal LAI response was obtained at this concentration. One drop of membrane preparation or medium was instilled into each well with a 1-ml syringe and a 25-g × 0.625-inch needle (Sherwood Medical Co.). One drop of leukocyte suspension (5 × 10⁶ cells/ml) was then instilled into each well in a similar manner. Plates were incubated at 37° under 5% CO₂–95% air for 1 hr, after which they were gently washed in 0.9% NaCl solution. Excess fluid was removed by inverting the plates. The residual adherent cells were fixed in absolute methanol for 10 min and exposed to Giemsa stain for a further 10 min. The stain was then washed off, and the plates were allowed to dry.

**Cell Counting.** Cell counts were performed with an automated differential scanning system (Cytotally Model 900; Artek Corp., Farmingdale, N. Y.) attached to an inverted microscope (Wild, Heerbrugg, Switzerland) by a CC-TV camera (Hitachi-HV, Hitachi, Japan). Cells were enumerated on the basis of size and density, the former parameter ranging from 0 to 30 in Quadrant 1. The microtest plate was inspected at ×40 with the scan area on the TV screen adjusted to the area of the bottom of each well. The cell counts in each experiment were converted to an adherence index by the following equations:

\[
\text{LAI} = \frac{\text{Experimental counts} - \text{Mean control count}}{\text{Mean control count}}
\]

\[
\Delta \text{LAI} = \text{LAI Colon Antigen(s)} - \text{LAI Pancreas Antigen(s)}
\]

This index is represented graphically as a mean of 13 replicate determinations at each dose.

**Statistical Analysis.** In a given test, the mean leukocyte adherence index of each donor to the specific (pancreas adenocarcinoma) and nonspecific (colon adenocarcinoma) antigen preparations was compared at each dose with Student's t test. Initially, a response was considered positive if the mean index for the specific antigen(s) was significantly lower (p < 0.05) than the index for the nonspecific antigen. Upon completion of the coded study (Charts 1 and 2), it was calculated that the upper limit of the 90% confidence interval for the specific mean adherence index of the pancreas carcinoma patients was approximately −0.20. Thus, this value has been subsequently used as a cut-off for determining positive and negative tests. The validity of this cut-off value is supported by the lower 90% confidence limits obtained for the benign disease and cancer control patients.

It is apparent that the cut-off point is somewhat arbitrary and is based on the size of the patient population. Therefore, the value used for cut-off will be revised as further results are obtained.

The means of the adherence indices of the donor groups were compared with Student's t test.

**Results**

**Coded Study.** Whole-blood specimens were obtained from patients with pancreatic carcinoma, patients with benign disease (acute pancreatitis), patients with nonpancreatic gastrointestinal malignant disease, and normal volunteers. These specimens were 'coded' in the laboratory, and the isolated peripheral blood leukocytes from these individuals were tested against crude membrane extracts of pancreas and colon carcinoma tumors. The results of 71 coded tests (dots) are summarized on Charts 1 and 2. On Chart 1, it is evident that the mean adherence index of leukocytes from 16 pancreatic carcinoma patients is −0.34 ± 0.09 (S.D.) in the presence of the pancreatic carcinoma membrane antigen. In comparison, the mean adherence indices of leukocytes from benign and malignant disease controls and from normal controls are above 0 in response to the same antigenic preparation. The difference between experimental and control groups is statistically significant (p < 0.0001) by the unpaired Student's t test. Conversely,
Blind Study. To eliminate any possibility of experimenter bias, a blind study was performed. Whole-blood specimens were centralized in a clinic, coded by a research nurse, and then brought to the laboratory. Fifty-nine LAI tests were performed, and the LAI index was calculated for each test. The clinical information was then matched with the LAI index and is presented in Charts 1 and 2 (open squares).

The results of the blind study mirrored the results of the coded study. With the exception of 2 false-positive tests, the adherence index of all pancreatic carcinoma patients was below —0.20 and the adherence index of all controls was above —0.20.

Expression of Results. The 3 variations of the LAI test (hemocytometer, test tube, and microtest) are not performed in an identical manner and, furthermore, do not use the same formula to evaluate the degree of adherence inhibition. While the hemocytometer and microtest versions of the LAI assay use a medium control as a base line to establish the number of adherent leukocytes, the test tube version of the LAI assay uses a tumor extract of a different tissue type to establish a base line and to calculate the degree of nonadherence. The use of a nonhistologically related control (colon carcinoma) in this study permitted an expression of results based on the difference in the LAI response between the specific (pancreas carcinoma) and nonspecific (colon carcinoma) tissue extracts. These results are presented in Chart 3.

In Chart 3 it is evident that the mean ΔLAI of leukocytes from the pancreatic carcinoma patients is 0.46 ± 0.32 (S.D.). In comparison, the mean ΔLAI of leukocytes from all control subjects was not greater than 0.15. Thus, the LAI index expressed as the difference between the response to a specific and nonspecific tumor extract can also be used to discriminate between experimental and control populations.

Summary

Table 1 summarizes the results of 104 different individuals evaluated by the micro-LAI assay. Using —0.20 as a cut-off adherence inhibition was not observed with leukocytes from pancreatic carcinoma patients in the presence of the colon carcinoma membrane antigen (Chart 2). Thus, the micro-LAI assay was able to discriminate between pancreatic cancer, acute pancreatitis, other cancers, and the normal state.

On the basis of the distribution of the LAI values of the population of patients with pancreatic cancer, benign pancreatic disease, unrelated gastrointestinal neoplastic disease, and nonneoplastic disease, an LAI value of —0.20 was selected as a positive (Chart 1). This cut-off value represents the upper limit of the 90% confidence interval for the coded experimental group. On the basis of this cut-off value, there were 4 false-positive controls (3 patients) and 1 pancreas patient with a false-negative value.

![Chart 1](image1)

**Chart 1.** Summary of all LAI assays performed with leukocytes from (A) pancreatic carcinoma patients (—0.23 ± 0.06; n = 35); (B) benign control patients, 14 with acute pancreatitis (+0.2 ± 0.13; n = 31); (C) malignant control patients, primarily gastrointestinal cancers (+0.11 ± 0.06; n = 28); and (D) normal healthy volunteers (+0.06 ± 0.10; n = 32) and tested against a pancreas tumor extract. The mean ± S.D. is given for each group (pancreas patients versus control; p < 0.0001 by unpaired Student’s t test). ◇, coded study; □, blind study.

![Chart 2](image2)

**Chart 2.** Summary of all LAI assays performed with leukocytes from (A) pancreatic carcinoma patients (0.134 ± 0.172; n = 35); (B) benign control patients (0.114 ± 0.168; n = 31); (C) malignant control patients (0.129 ± 0.191; n = 28); and (D) normal healthy controls (0.086 ± 0.114; n = 32). ◇, coded study; □, blind study. Mean ± S.D. is given for each group.

![Chart 3](image3)

**Chart 3.** LAI pancreas study from (A) pancreatic carcinoma patients (0.46 ± 0.32; n = 35), (B) benign control patients (0.01 ± 0.28; n = 27), (C) malignant control patients (0.12 ± 0.26; n = 28), and (D) normal healthy controls (—0.02 ± 0.06; n = 29). Summary of all LAI tests expressed as the difference in response to a nonspecific antigen(s) (colon carcinoma) versus a specific antigen(s) (pancreatic carcinoma) for all individuals outlined in Chart 1. Mean ± S.D. is given for each group.
The presence of tissue type-specific reactivity associated with the peripheral blood leukocytes of pancreatic cancer patients confirms the observations made by Rutherford et al. (15), who evaluated 3 pancreatic carcinoma patients; by Taguchi (16), who evaluated 7 pancreatic carcinoma patients; and by Thomson et al. (17), who evaluated 11 pancreatic carcinoma patients. While Taguchi could detect pancreatic cancer by the tube LAI assay, he could not discriminate between pancreatic cancer and acute pancreatitis. In contrast, this study and the study of Thomson et al. were able to distinguish between the benign and malignant states. The reason for this discrepancy is not known.

The presence of tissue type-specific reactivity associated with the peripheral blood leukocytes of pancreatic cancer patients was observed both when the results were expressed in the form of an index with a medium control and when the results were expressed as the difference in response (Δ) to a specific (pancreatic carcinoma) and nonspecific extract (colon). Using 0.30 (13) as a cut-off for the latter method (ΔLAI), a comparison of individual results indicated that 30 of 35 tests were concordant. In 2 of 5 discordant tests, the ΔLAI value would have indicated a positive result. In both of these cases, stimulation rather than inhibition of adherence was observed. These observations suggest that both methods of expressing results are valid and should be routinely used, particularly when stimulation of adherence is observed. Discordant results should automatically mandate a repeat test of the same blood specimen.

The choice of an LAI index of −0.20 as a cut-off point is based on a statistical evaluation of these data. However, it is somewhat arbitrary and dependent on the size of the experimental population. As our experimental group has become larger, we have observed that the mean adherence index has changed from $-0.30 \pm 0.107$ to $-0.23 \pm 0.05$. Furthermore, there has been a concomitant change in the mean adherence index of the control population. As a consequence, the cut-off point could be changed from −0.20 to −0.15. The change in the mean adherence index may be due to the fact that 4 different sources of pancreatic carcinoma antigen(s) were used in this study. While this would suggest that there is a common pancreatic tissue type membrane antigen(s), the variable response obtained with different tissue extracts of common histological type raises problems with regard to the routine use of this test as an immunodiagnostic assay. This problem may be overcome in the long term by studies that concentrate on the isolation and identification of the antigen(s) that is recognized in the LAI microassay. An enriched soluble antigen(s) may result in a response that is both characteristic of a given leukocyte population and constant over a long period of time. In the short term, our solution to this problem has been to accept an LAI index of ≤0.20 as a definitive positive result and an LAI index between −0.15 and −0.20 as a marginally positive response that warrants a repeat test.

The results presented in this study suggest that the micro-LAI assay has a good potential as an immunodiagnostic test for pancreatic cancer. Its ability to discriminate between benign and malignant states of pancreatic disease suggests a future for this test in aiding a clinician to make a differential diagnosis when patients present with vague symptoms

### Table 1

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* LAI index = 0.20.

b In our initial report (14), one patient was placed in this group on the basis of a pathological evaluation performed at an outside hospital. Upon autopsy, tissue specimens revealed the presence of a cholangiocarcinoma. The results are now listed as a false-positive (gastrointestinal cancer).

point, the results indicate that 19 of 23 pancreatic cancer patients specifically recognized the pancreatic membrane antigen. In this group, there were 6 patients with primary disease or primary disease with regional node involvement. All 6 of these patients specifically recognized the pancreatic carcinoma antigen. Furthermore, 3 false-positive results were obtained with leukocytes from 80 control patients.

### Discussion

The almost uniform death rate from pancreatic carcinoma appears to be related primarily to the fact that this cancer cannot be detected early enough and secondarily to the difficulty clinicians face in making a proper differential diagnosis when a patient presents with vague symptoms of anorexia, jaundice, and pain, with a resultant loss of valuable time. Furthermore, it is probable that a diagnostic procedure of reasonable accuracy might identify patients with pancreatic neoplasia at an earlier stage, increasing the frequency with which resectable lesions would be found at surgery. It has been the Japanese experience in gastric cancer that early diagnosis has increased cure rates from below 10% to nearly 50%. In search of a moderately accurate blood test, we have evaluated the ability of the micro-LAI assay to detect specifically pancreatic cancer.

The results of this study and our previous study (14) indicate that specific antitumor immunity could be detected in patients with pancreatic carcinoma. The micro-LAI, as applied to pancreatic cancer, appears to be immunologically specific and reproducible. This is based on the observation that peripheral blood leukocytes of 19 of 23 patients with histologically defined pancreatic cancer responded to a membrane preparation of pancreatic cancer by a decrease in adherence to a coated-plastic surface, whereas only 3 of 80 controls (benigns, other cancers, and normals) showed a similar decrease in adherence. Conversely, patients with pancreatic cancer showed no response to a control membrane preparation of colon carcinoma.

The presence of tissue type-specific reactivity associated with the peripheral blood leukocytes of pancreatic cancer patients confirms the observations made by Rutherford et al. (15), who evaluated 3 pancreatic carcinoma patients; by Taguchi (16), who evaluated 7 pancreatic carcinoma patients; and by Thomson et al. (17), who evaluated 11 pancreatic carcinoma patients. While Taguchi could detect pancreatic cancer by the tube LAI assay, he could not discriminate between pancreatic cancer and acute pancreatitis. In contrast, this study and the study of Thomson et al. were able to distinguish between the benign and malignant states. The reason for this discrepancy is not known.

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The choice of an LAI index of −0.20 as a cut-off point is based on a statistical evaluation of these data. However, it is somewhat arbitrary and dependent on the size of the experimental population. As our experimental group has become larger, we have observed that the mean adherence index has changed from $-0.30 \pm 0.107$ to $-0.23 \pm 0.05$. Furthermore, there has been a concomitant change in the mean adherence index of the control population. As a consequence, the cut-off point could be changed from −0.20 to −0.15. The change in the mean adherence index may be due to the fact that 4 different sources of pancreatic carcinoma antigen(s) were used in this study. While this would suggest that there is a common pancreatic tissue type membrane antigen(s), the variable response obtained with different tissue extracts of common histological type raises problems with regard to the routine use of this test as an immunodiagnostic assay. This problem may be overcome in the long term by studies that concentrate on the isolation and identification of the antigen(s) that is recognized in the LAI microassay. An enriched soluble antigen(s) may result in a response that is both characteristic of a given leukocyte population and constant over a long period of time. In the short term, our solution to this problem has been to accept an LAI index of ≤0.20 as a definitive positive result and an LAI index between −0.15 and −0.20 as a marginally positive response that warrants a repeat test.

The results presented in this study suggest that the micro-LAI assay has a good potential as an immunodiagnostic test for pancreatic cancer. Its ability to discriminate between benign and malignant states of pancreatic disease suggests a future for this test in aiding a clinician to make a differential diagnosis when patients present with vague symptoms.
Immunodiagnosis of Pancreatic Cancer

of pain, weight loss, and jaundice. Furthermore, its ability to discriminate between pancreatic cancer, other cancers, and normal volunteers suggests that the LAI test may be useful in establishing a diagnosis in asymptomatic individuals early in the course of the disease. However, this potential will be realized only after a much larger number of patients have been evaluated and there are concomitant technological improvements in the assay that will result in a more standardized response.

Acknowledgments

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References


Discussion

Dr. McCoy: With the 2 LAI-negative pancreatic cancer patients, if one would split the specimen and perform 2 tests on that blood, would you then be able to detect positives? In other words, are those 2 patients ever positive?

Dr. Goldrosen: The 2 patients that gave negative results were part of the blind study, and I had no choice in terms of patient selection.

Dr. McCoy: I think my point ought to be emphasized a little bit more. There may be some real advantage in actually taking a patient specimen, splitting it, and repeating it. Did you do some repeats? If so, how were these repeats done?

Dr. Goldrosen: What I usually prefer to do in terms of repeat tests is to evaluate an individual who has any suspicion of pancreatic cancer as soon as the patient is admitted to a hospital and then evaluate the patient a second time within a week prior to the patient going to surgery, rather than to take a patient's specimen on 1 day and split it in 2.

Dr. Catanzaro: Can you tell be how you are going to identify your patients with early pancreatic cancer?

Dr. Goldrosen: What we are working towards is a test that can detect pancreas, colon, and gastric cancer. What we are doing now is trying to screen all patients who come to a GI clinic and see if we can pick up a reasonable number of patients with gastrointestinal disease.
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