Demonstration of the Microtest Version of the Leukocyte Adherence Inhibition Assay

Martin H. Goldrosen, John H. Howell, and Edward D. Holyoke

Department of Surgical Oncology, Roswell Park Memorial Institute, Buffalo, New York 14263

Abstract

This paper gives a detailed description of the microtest leukocyte adherence inhibition technique, as well as the results obtained with blood specimens coded by impartial observers. Three coded blood specimens from patients with colon cancer, pancreatic cancer, and melanoma were tested against crude membrane preparations of pancreatic and colon adenocarcinoma tumors. No tissue type-specific reactivity was observed. The inability to demonstrate specific reactivity was due to extensive variability observed within each test. The extensive variability resulted from time constraints of the workshop that necessitated deviations from the normal procedure.

Introduction

Three coded whole-blood specimens were received from independent observers and evaluated in the microtest LAI assay against crude membrane preparation(s) of pancreatic and colon adenocarcinoma tumors. The primary objective was to demonstrate how the microtest LAI assay was performed, while the secondary objective was to detect tissue type-specific reactivity with coded blood specimens. The assays were performed by Dr. Anthony J. Russo and Joel Zalin.

Materials and Methods

Tumor Extracts. Primary or metastatic tumor tissue is obtained immediately following surgery or as autopsy material and is thoroughly washed in buffer. The crude membrane preparations are prepared immediately, or the tissue is stored at −80° until it is used. All steps of the procedure are performed at 4°C.

The tumors are finely minced with scissors (1-mm chunks), taking care to remove all tissue debris. Tumor chunks are resuspended in twice their volume of phosphate-buffered saline (0.01 M NaH₂PO₄, 0.14 M NaCl, pH 7.2) in a 50-ml centrifuge tube and subjected to 30-sec homogenizations in a Tekmar Tissumizer at medium speed. The homogenization is continued until there are no intact chunks are resuspended in twice their volume of phosphate-buffered saline and recentrifuged at 200 × g for 20 min. The supernatant is pooled and dialyzed against 200 times its volume of 0.005 M sodium phosphate low-ionic-strength buffer, pH 7.2, for 3 days with 3 buffer changes.

Protein estimates of final antigen(s) concentrations are made by the microbiuret method, including 0.5% deoxycholic acid for solubilization of membranes (1). Tumor antigens are stored at −80° for up to 6 months. Each vial contains 0.5 ml of tumor antigen(s) at a protein concentration of approximately 10 mg/ml. The contents of each vial are used once, and the balance is discarded.

Preparation of Peripheral Blood Leukocytes. A 20-ml sample of heparinized venous blood is collected in vacutainer tubes (Monoject; Sherwood Medical Co., St. Louis, Mo.). Blood is transferred to a plastic syringe (Plastipak; Becton-Dickinson, Rutherford, N. J.) and incubated in an upright position for 1 hr at 37°. The leukocyte-rich plasma fraction is decanted into a 12-ml conical tube and centrifuged for 8 min at 200 × g.

The resultant pellet is treated with Tris-buffered ammonium chloride (Boyle's solution) for 10 min at 4°C to lyse erythrocytes. The Boyle's solution is 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 at 200 × g. The leukocytes are washed 2 more times, and the cell concentration is adjusted to 5 × 10⁸ cells/ml with a Neubauer hemocytometer. Viability is assessed by exclusion of 0.2% trypan blue. In all cases, viability is greater than 95%.

LAI. The LAI microassay has been previously described (2, 3) and is outlined in Chart 1. The LAI assay is carried out on microtest plates (no. 3034; Falcon Plastics Co., Oxnard, Calif.). The plates are divided into quadrants containing 15 wells each. The tumor antigen(s) in 3 concentrations (0.1, 0.05, and 0.01 mg/ml) and control medium (Roswell Park Memorial Institute Tissue Culture Medium 1640 plus 10% fetal calf serum) are instilled into the wells of their respective quadrants (outlined in Chart 1) using a 1-ml tuberculin syringe and a 25-gauge x ½-inch needle (Sherwood Medical Co.). One drop of leukocyte suspension (5 × 10⁶ cells/ml) is instilled into each well in an identical manner. The plates are allowed to sit for 10 min and then are placed in an incubator at 37° under 5% CO₂ for 1 hr.

Nonadherent cells are removed by immersing the plates in a shallow dish containing 0.9% NaCl solution and gently moving them back and forth 10 times. The plate is removed from the shallow dish. Excess fluid is removed by inverting

---

1 Demonstration performed at the International Workshop on Leukocyte Adherence Inhibition, May 15 to 17, 1978, Buffalo, N. Y. This work was supported by USPHS Grants CA 22931 and CA 23646.

2 To whom requests for reprints should be addressed, at Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N. Y. 14263.

3 The abbreviation used is: LAI, leukocyte adherence inhibition.
the mean control count and are reproducible from experi-
mental counts—Mean control count
3 and 5 that are directly proportional to the magnitude of
test and the S.E. The mean cell counts of control quadrants
Culture Medium 1640; FCS, fetal calf serum: Exp. , experimental.
eral blood leukocytes. RPMI 1640, Roswell Park Memorial Institute Tissue
metal blood leukocytes. RPMI 1640, Roswell Park Memorial Institute Tissue
The index is represented graphically as a mean of 13
obtained in each well. Cells are enumerated in Quadrant 1
resulting in a mean control count of approximately 100
the plate, moving the plate in a vertical plane, and suddenly
moving the plate in a vertical plane, and suddenly
the vertical motion. The residual adherent cells are
fixed in absolute methanol for 10 min, exposed to
Giemsa stain for a further 10 min, and washed with metha-
ol until the Giemsa stain can no longer be seen. The plates
are allowed to dry thoroughly (overnight) before residual
adherent cells are enumerated.

Cell Counting. Cell counts are performed with an auto-
mated differential scanning system (Cytotally Model 900;
Artel Corp., Farmingdale, N. Y.) attached to an inverted
microscope (Wild) by a CC-TV camera (HV-16S; Hitachi,
Japan). The microtest plates are inspected at ×40 with the
scan area of the TV screen adjusted to the area of the
bottom of each well. Cells are enumerated in Quadrant 1
with the upper size limit adjusted between 20 and 35,
resulting in a mean control count of approximately 100
cells. The lower size limit and the upper and lower density
limits are set at zero. The fine focus on the inverted
microscope is adjusted until a maximum cell count is
obtained in each well.

Statistical Analysis

Individual Data. The highest and lowest value in each
quadrant is eliminated to get rid of “outliers,” and the cell
counts are converted to an adherence index by the follow-
ing equation:

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

The index is represented graphically as a mean of 13
replicate determinations at each dose. Ninety-five % con-
fidence limits for each mean are calculated using Student’s t
test and the S.E. The mean cell counts of control quadrants
are generally between 50 and 200 and have S. E.’s between
3 and 5 that are directly proportional to the magnitude of
the mean control count and are reproducible from experi-
ment to experiment. S. E.’s greater than 7 are indicative of
a test with extensive variability and mandate a repeat test.

Grouped Data. From all tests performed to date, it has
been calculated that the upper limit of the 95% confidence
interval of the mean adherence index of the patient popu-
lation with the specific antigen(s) is approximately —0.20
(3). This value has been subsequently used as a cutoff value
for determining positive and negative tests. It is apparent
that the cutoff point is somewhat arbitrary and is based on
the size of the patient population. Therefore, this value will
be revised as further results are obtained.

Critical Comments

Tumor cell membrane preparations may not resuspend
well at higher concentrations (10 mg/ml) and after thawing.
This results in a precipitate in the microtest well that
interferes with the enumeration of residual adherent cells.
Tumor cell membranes can ordinarily be resuspended by
continuous passage through a 3-ml syringe barrel attached
with a 18-gauge x 1.5-inch needle. Alternatively, the mem-
brane preparation can be initially stored in low-ionic-

strength buffer (5 mM sodium phosphate), resulting in less
aggregation. Leukocytes must be resuspended well after
each centrifugation step so that the cells are in the form of
a single-cell suspension, with a minimum number of aggre-
gates prior to plating.

For optimum efficiency, the assay should be performed
by 2 individuals, one handling the preparation of cells while
the other handles the preparation of the different dilutions
dilutions of antigen(s). Random wells of plates should be inspected
prior to their use. Plates with variable-size wells, dust
particles, or scratches should be discarded. Antigen(s) and
leukocytes are instilled into each well by holding the needle
and syringe at a 45° angle to the plate with the needle bevel
up. The smallest possible drop is formed and allowed to fall
into the center of the well before it has an opportunity to
roll on the side opposite the bevel. The drop should be
instilled from the same height into each well. The plates
cannot be disturbed during the plating process. This would
result in the dispersion of the cells to the periphery and an
uneven distribution. When the plates are moved in the
vertical position to remove excess fluid, care must be taken
to keep plates perfectly horizontal. Examination of the cells
in the control wells should reveal an even distribution of
cells with no residual membranous debris. If residual
debris exists, the plates can be rewashed with absolute
methanol and allowed to dry a second time.

Absolute counts between 100 and 150 cells in the control
quadrant represent an ideal number of cells. With absolute
counts greater than 300 cells/well in the control quadrant,
an adherence index with marked stimulation is observed.
To overcome this, the upper size limit control on the
Cytotally can be adjusted upward so that only larger cells
are counted. This step can be performed only if there are
no cellular aggregates and the same setting is used for all
the plates of one leukocyte source. Justification of this
arbitrary definition is based on the observation that the
responsive cell is a larger cell within the population being
evaluated (4) and the rationale that a constant definition is
used throughout the enumeration phase.
**Results and Discussion**

Table 1 summarizes the results obtained with the microtest version of the LAI assay when the 3 coded specimens were incubated with extracts of pancreatic and colon cancer and also states the patients' actual diagnoses. An LAI index greater than \(-0.20\) was obtained with the 3 coded specimens in the presence of the 2 tumor extracts, indicating a lack of recognition of the 2 tumor extracts by all 3 coded leukocyte specimens. While an LAI index greater than \(-0.20\) would be expected with the leukocytes from the melanoma patient (Sample 1) in the presence of the pancreatic and colon extracts, the anticipated result of an LAI less than \(-0.20\) was not obtained with the leukocytes from the colon patient (Sample 2) in the presence of the colon extract and similarly with the leukocytes from the pancreatic cancer patient (Sample 3) in the presence of the pancreatic extract. An analysis of the S.E.'s of the absolute mean control counts (Table 1) indicates a value greater than 6.0 in 5 of 6 tests, suggesting that the inability to demonstrate specific reactivity was due to extensive variability within each test. In our laboratory, these results automatically mandate a reevaluation of the same blood specimen before the result is accepted. This extensive variability arose because time constraints of the workshop forced us to deviate in a minimum of 2 crucial steps from our normal procedure. While the hemocytometer and the test tube version of the LAI assay are performed in 1 day and enumerated the following day, after microtest plates are thoroughly air-dried. For the purpose of the demonstration, our group set up and enumerated the plates on the same day and before they were thoroughly dried. Furthermore, time constraints did not permit us to use the "focusing technique" described in "Materials and Methods" to obtain a maximum cell count for each well.

The successful performance of the micro-LAI assay requires a certain degree of skill in the plating procedure so that equal numbers of evenly distributed cells are initially present in each well and strict adherence to the above protocol throughout every phase. After mastery of the plating technique, this microassay can be used as a valuable tool to characterize the immune response in different states of tumorigenesis, isolate biologically active tumor antigen(s), and diagnose the presence of specific forms of cancer.

**References**

Demonstration of the Microtest Version of the Leukocyte Adherence Inhibition Assay

Martin H. Goldrosen, John H. Howell and Edward D. Holyoke


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/2_Part_2/630

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.