Demonstration of Tube Leukocyte Adherence Inhibition Assay with Coded Samples of Blood

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Abstract
Heparinized samples of blood from three different patients were coded by impartial observers. The buffy coat leukocytes from the coded samples of blood were isolated and incubated separately with extracts of colon and pancreatic cancer in the tube leukocyte adherence inhibition assay. At the completion of the assay, the leukocytes from Patient 1 were equally nonadherent to both cancer extracts with a nonadherence index value of 8. By contrast, leukocytes from Patient 2 exhibited increased nonadherence to the extract of colon cancer ($p = 0.02$) with a nonadherence index value to colon cancer antigen of 89. Leukocytes from Patient 3 displayed increased nonadherence to the extract of pancreatic cancer ($p < 0.05$) with a nonadherence index value to pancreatic cancer antigen of 39. When the code was broken, Patients 1, 2, and 3 had diagnoses of malignant melanoma, colon cancer, and pancreatic cancer, respectively. Hence, this was a classical criss-cross experiment; the patient with malignant melanoma reacted to neither of the antigens, whereas the patients with colon and pancreatic cancer reacted to the sensitizing cancers which had unique organ-type specific neoantigens.

Introduction
The present paper describes the preparation of materials and reports our results of the demonstration of the tube LAI assay (2). Coded samples of blood were provided by impartial observers, Dr. Takasugi and Dr. McCoy, at the workshop on LAI.

Materials and Methods

Tumor Extracts. Tumor tissues from autopsy are stored at $-40^\circ$ until processed. Normal tissue and fibrous tissues are dissected away from the tumor material, and the tumor specimen is finely minced with sharp scissors. Approximately 20 g of the finely minced tumor are placed in ice-cold PBS and homogenized at short intervals of 30 to 60 sec over a period of 10 to 15 min in 5 volumes of PBS at approximately 40,000 rpm in a VirTis 45 homogenizer. The vessel containing the tumor is surrounded with ice water, and short bursts of homogenization are used to prevent any heating of the tumor sample. The homogenate is centrifuged at 20,000 $\times$ g for 30 to 60 min, and the supernatants are pooled and stored at $-40^\circ$ in 0.3- to 0.5-ml aliquots. Each aliquot is used only once, since repeated thawing and freezing of the PBS extracts bring about loss of activity. PBS extracts of tumor, even without thawing, often lose specific activity somewhere between 4 to 6 months; for this reason, we change to fresh tumor extracts before 6 months (2, 5).

Titration of PBS Tumor Extracts. In the workshop demonstration of the tube LAI assay, extracts of colon and pancreatic cancer were used at a protein concentration of $100 \mu g$/tube. In our laboratory, the 2 different tumor extracts were tested against PBL from control subjects to determine the protein concentration of each extract to be used. The extracts were considered to be appropriately titrated when the number of nonadherent leukocytes from control subjects to the 2 tumor extracts were similar and the number of nonadherent cells was somewhere between 30 and 70 with an average of 40 to 50 in about 10 subjects. With the 2 extracts appropriately titrated, PBL from patients with suspected cancer of the colon or pancreas were then tested against the extracts to show that the extracts possessed specific antigen activity (1, 3, 5).

PBL from control subjects are tested daily to check on the titration of the antigens since in the period of 3 to 6 months, when the extracts are being used, the nature of the extracts often changes, which results in either a slight increase or decrease of nonadherent PBL from control subjects to one of the antigens. If this occurs, a slight change in the concentration of the antigens may be required during their 3 to 6 months of use to maintain PBL from control subjects exhibiting similar nonadherence to the 2 tumor extracts.

Any combination of unrelated tumor extracts may be used; however, the proper titration of the 2 unrelated antigens is of key importance. The use of multiple different antigens on a random basis, while theoretically possible, makes the constant checks on the titration of the extracts difficult.

Tube LAI Assay. Samples of heparinized venous blood are collected in 2 green-stoppered vacutainer tubes (Becton, Dickinson & Co., Ltd., Rutherford, N. J.) and are incubated vertically at $37^\circ$ for 45 min. The resultant leukocyte-rich plasma fraction is aspirated and centrifuged at 200 $\times$ g for 5 min. The cell button is then suspended by repeated pipeting in 3.0 to 3.5 ml of isotonic Tris-buffered NH$_4$Cl solution (which is at $4^\circ$) and left for 15 min at room temperature to lyse the contaminating RBC. The procedure...
is terminated by the addition of 6.5 ml of Medium 199, the cells are centrifuged, and the supernatant is removed and discarded. The cells are washed twice with 10 ml of Medium 199 and resuspended at a concentration of $1 \times 10^6$ cells/ml of medium.

Antigen-induced LAI is performed in 20-ml Kimax test tubes (16 x 150 mm). Aliquots of 0.1 ml of a PBL suspension ($1 \times 10^7$ cells/ml) are placed in the glass test tubes. Then either 0.1 ml of Medium 199 or 0.1 ml of tumor extract is added to each tube. The mixture is brought to a final volume of 0.5 ml by the addition of Medium 199. The suspension in each tube is agitated, and the tubes are then incubated horizontally, so that the contents cover at least four-fifths of the length of the lower surface of each tube. The tubes are incubated at 37° in a humidified atmosphere of 5% CO$_2$-95% air. After 2 hr of incubation, the tubes are placed upright, and the 0.5 ml of medium at the bottom of the vertical tube is gently aspirated twice with a Pasteur pipet. A sample is placed immediately on a hemocytometer, and the nonadherent cells in the center square are counted. Each tube is handled in a precisely uniform manner, and each extract is plated in triplicate or quadruplicate. The results are expressed as NAI (2):

$$\text{NAI} = \frac{A - B}{B} \times 100$$

where $A$ is the number of nonadherent cells in the presence of specific antigen and $B$ is the number of nonadherent cells in the presence of the nonspecific antigen.

The difference in nonadherence to the 2 extracts is expressed by the NAI. With PBL from more than 95% of control subjects, the difference in nonadherence to 2 unrelated tumor extracts is less than NAI 30 (1, 3, 4). Hence, NAI > 30 is accepted as positive, and NAI < 30 is negative.

Results

Table 1 shows the results when coded samples of PBL provided by Dr. Takasugi and Dr. McCoy were incubated with extracts of colon and pancreatic cancer. PBL from Coded Sample 1 showed similar nonadherence to the extracts of colon and pancreatic cancer. PBL from Coded Sample 2 showed increased nonadherence when incubated with the extract of colon cancer in comparison to the extract of pancreatic cancer. The difference in nonadherence was statistically significant; however, NAI > 30 is accepted as positive even if the difference is not statistically significant. PBL from Coded Sample 3 showed increased nonadherence to the extract of pancreatic cancer with an NAI value of 39. Table 1 shows that the absolute number of nonadherent cells from different donors is variable, and it is the difference in nonadherence to 2 unrelated tumor extracts which determines if the PBL are reacting to 1 of the 2 tumor extracts. Hence, Patient 1 showed no reactivity to either colon or pancreatic cancer antigens. Patient 2 showed specific reactivity to the colon cancer antigen, whereas Patient 3 had tumor-specific immunity to the pancreatic cancer antigen. When the code was broken, Patients 1, 2, and 3 had malignant melanoma, colon, and pancreatic cancer, respectively.

Table 1 shows a classical criss-cross experiment, the patient without colon or pancreatic cancer showed no reactivity to either antigen, whereas the patients with colon cancer and pancreatic cancer reacted only to the sensitizing tumor antigen. Hence, the tumor-specific antigens of colon and pancreatic cancer are unique and show no cross-reactivity.

Discussion

The measurement of tumor-specific immunity by the tube LAI assay is rapid, noninvasive, and qualitative. Although the assay is simple in concept, success is dependent upon a number of factors: (a) daily performance of the assay is required to maintain a high level of skill; (b) the 2 extracts must be titrated on PBL from control subjects to select the optimum protein concentration of the extracts; (c) PBL from control subjects need to be tested daily to check that

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**Table 1**

<table>
<thead>
<tr>
<th>Coded sample</th>
<th>Colon cancer</th>
<th>Pancreatic cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Mean ± S.D.</td>
<td>No.</td>
</tr>
<tr>
<td>1</td>
<td>37.33, 37.45</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>58.40, 44.68</td>
<td>53 ± 13</td>
</tr>
<tr>
<td>3</td>
<td>28.25, 26.29</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

$^a$ NAI was calculated as described in "Materials and Methods." The NAI values with either colon cancer or pancreatic cancer extracts as the specific antigen are shown. NAI $\geq$ 30 is positive and is based on the fact that the NAI value of $>95\%$ of control subjects is less than 30 (Ref. 1-5) and the difference between the mean NAI of groups of patients with early cancer and control subjects is highly significant ($p < 0.001$).

$^b$ Students' t test was used to calculate whether the difference in nonadherence to the 2 different extracts was significant. Although the difference in nonadherence for the individual patients was statistically significant, in many assays where the NAI value is $\geq$ 30 the difference in nonadherence in the single test is not significant at $p < 0.05$.

$^c$ NS, not significant; S, significant.

$^d$ At the workshop, the NAI for Coded Sample 3 was reported as 30 to the extract of pancreatic cancer, which was a positive NAI; however, recalculation of the NAI in preparing this report showed that the NAI was 39 rather than 30. Both NAI values are positive.
the extracts remain appropriately titrated; (d) the experimenter after 1 month of experience in performing the tube LAI assay should be able to test PBL from control subjects with no more than 5% of the controls having NAI values ≥ 30. Otherwise there is an error in methodology.

References

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