

## Leukocyte Adherence Inhibition in Cancer Patients and the Repeated Tube LAI Assay

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**=Abstract=**The tube Leukocyte Adherence Inhibition (LAI) assays were performed to study immunological reactivity of patients with a variety of tumors (lung cancer, cervix cancer, stomach cancer and hepatoma). The result showed that leukocytes from cancer patients reacted only to the antigenic extracts of tumors of the same type and that the LAI response was directed to an organ type specific neoantigen.

After the initial tube LAI assay with normal lung tissue, the repeated tube LAI assay was performed with lung cancer extracts on the resultant cells adherent to the glass tube. In 8 patients with lung cancer, the changes in the number of nonadherent cells were all above 100%, while in 9 healthy control subjects they were all below 100%. NAIs (non adherence index) of the repeated tube LAI assay were significantly higher than those of the original tube LAI assay in patients with lung cancer ( $p < 0.01$ ).

**Key words:** *Leukocyte adherence inhibition, Human cancer, the repeated tube LAI assay*

### INTRODUCTION

Leukocyte Adherence Inhibition (LAI) assay was first introduced by Halliday and Miller in 1972. It has been recognized as a very useful immunodiagnostic tool in cancer patients since then. It has been used successfully to detect the cell mediated immune response in patients with breast CA, colon CA, lung CA, stomach CA, pancreatic CA, Hepatoma, cervix CA and laryngeal CA. (Armistead and Gowland 1975; Flores *et al.* 1977; Grosser and Thomson 1976; Halliday *et al.* 1977; Lopez *et al.* 1978; Marti and Thomson 1976; Maluish 1979; Rutherford *et al.* 1977; Tataryn 1978; Tataryn *et al.* 1979).

The LAI assay is based on the phenomenon that peripheral blood leukocytes of control subjects adhere to glass surface of the test tube, but in the presence of tumor specific antigen, peripheral blood leukocytes from the cancer patient lose their adherence to a glass surface. The mechanism has not been explained clearly.

HBsAg mediated LAI assay was reported previously (Seo 1988). However the LAI for tumor specific immunity has not been studied yet in Korea.

In the present study, tumor-specific immunity to organ-type specific neoantigens of lung cancer, cervix cancer, stomach cancer and hepatoma was studied by the tube LAI assay.

### MATERIALS AND METHODS

#### 1. LAI for Tumor Specific Immunity Donors of Leukocytes

Heparinized blood samples were withdrawn from 19 patients with lung cancer, 9 patients with cervix cancer, 6 patients with stomach cancer, 5 patients with hepatoma, and 23 control subjects. Peripheral blood leukocytes (PBL) were immediately prepared from these venous blood samples, and tested for leukocyte adherence inhibition by the tube LAI assay. The diagnosis was confirmed pathologically by examining specimens at surgery or biopsy. The tube LAI assay was performed without previous knowledge of the subject, and control subjects were tested simultaneously with the cancer patients.

### Tumour Extracts

Phosphate buffered saline (PBS) tumor extracts were prepared as described by Grosser and Thomson (1975). Tumor tissue samples were obtained at operation. Normal lung tissue and lung cancer samples were partly supplied by the Cancer Research Institute at Seoul National University.

The tumor and normal lung tissue were finely minced with scissors after removing the connective and necrotic tissues and homogenized in ice-cold phosphate buffered saline (0.01M phosphate buffer and 0.15M saline) at PH 7.3 for 15 minutes (Grosser and Thomson 1975; Thomson 1978). The homogenate was centrifuged at 20,000 x g for 30 minutes and the supernatant fluids were pooled and stored in small aliquots at -70 C. The protein concentrations of the tumor extract were determined by the method of Lowry *et al.* (1951) with bovine albumin as a standard.

### Preparation of Peripheral Blood Leukocytes

Peripheral blood mononuclear cells were prepared as described in the paper reported previously by the author (Seo 1988).

### Antigen-Induced Tube LAI Assay

The tube LAI assay was performed as described previously (Grosser and Thomson 1975). Antigen-induced LAI was performed in 20 ml Kimax test tubes (16 x 150 mm). In each experiment three test tubes were prepared. Aliquots of 0.1 ml of a PBL suspension ( $1 \times 10^7$  cell/ml) were placed in three test tubes. Then 0.1 ml of Dulbecco's modified eagle's medium or 0.1 ml of normal tissue extract or 0.1 ml of tumor extract was added to one of the three tubes. The mixture was brought to a final volume of 0.5 ml by the addition of DMEM. The suspension in each tube was agitated, and the tubes were then incubated horizontally, so that the contents covered at least four-fifths of the length of the lower surface of each tube. The tubes were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>-95% AIR. After 1 hr of incubation, the tubes were placed upright, and the 0.5 ml of medium at the bottom of the vertical tube was gently aspirated twice with a 10  $\mu$ l pipet. A sample was placed immediately on a hemocytometer, and the nonadherent cells in 4 squares were counted. Each tube was handled in a precisely uniform manner, and each experi-

ment was done in duplicate.

The results were expressed as NAI (Non Adherence Index).

The number of nonadherent cells varied from patient to patient and day to day considerably. Hence, tumor specific LAI reactivity could be determined only by the difference in LAI reactivity between two extracts; one being the specific tumor extract and the other the nonspecific tumor or tissue extract. The difference in reactivity to the two extracts was expressed as NAI.

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

Where A is the number of non-adherent cells in the presence of specific antigen (tumor extract), and B is the number of non-adherent cells in the presence of nonspecific antigen (normal tissue extract). NAI's of > 30 were positive and those of <30 were negative. This value was chosen arbitrarily on the basis that in previous studies more than 95% of control subjects had values less than 30 (Flores *et al.* 1977; Grosser and Thomson 1975; Lopez and Thomson 1977; Marti and Thomson 1976; Thomson *et al.* 1978).

### Repeated Tube LAI Assay

After the initial tube LAI assay with normal lung tissue extract, the test tubes were placed vertically and nonadherent cells were counted, and all of the nonadherent cell suspensions were discarded. Then, 0.4 ml of DMEM and 0.1 ml of lung CA extract were added to each tube. The tubes were placed horizontally so that the same side of the tube was covered by the medium, and the tube LAI assay was carried out again according to the method described above. The number of nonadherent cells was counted in the repeated tube LAI assay.

In order to express the change in the number of nonadherent cells calculated in the second assay, the number of nonadherent cells with the lung cancer extract in the repeated tube LAI assay was divided by the number of nonadherent cells with normal lung extract in the initial tube LAI assay, then multiplied x 100; i.e., the change in the number nonadherent cells was expressed as a percentage of ratio of nonadherent cells in the second tube LAI assay.

Table 1. Percentage of nonadherent cells

Groups	% of nonadherence without antigen	% of nonadherence with normal lung cell extract	% of nonadherence with lung CA extract
Healthy controls (n=15)	13.1±4.0*	14.7±4.1	15.2± 4.2
Patients with lung Ca. (n=19)	13.7±4.7	15.5±5.4	27.5±10.7

\*MEAN±S.D.

$$\text{Change of non-adherent cells (\%)} = \frac{\text{non-adherent cells in repeated tube LAI assay} - \text{non-adherent cells in initial tube LAI assay}}{\text{non-adherent cells in initial tube LAI assay}} \times 100$$

Another combination of extracts in the initial tube LAI assay and the repeated tube LAI assay were as follows. After the non-adherent cells by the initial tube LAI assay with normal lung tissue extract were discarded, the repeated tube LAI assay was done in paired glass tubes with normal lung tissue extract or lung cancer extract to calculate NAI in the repeated tube LAI assay. These results were then compared with the NAI in the original tube LAI assay.

#### Statistical Analysis

Statistical analysis was made by Student's t-test and ANOVA.

## RESULTS

### LAI assay for Tumor Specific Immunity

The mean nonadherence percentages of leukocytes incubated with extracts of normal lung tissue as the nonspecific antigen and with extracts of lung cancer as the specific antigen were summarized on Table 1. The results of the LAI assay in 15 healthy controls showed that the mean nonadherence percentages of leukocytes incubated with lung cancer extract, normal lung extract and without antigen were 15.2, 14.7 and 13.1 respectively. There were no significant differences among these 3 values ( $p > 0.05$ ). Likewise, the mean nonadherence percentages of leukocytes from 19 patients with lung CA incubated with normal lung extract and without antigen were 15.5 and 13.7, respectively. These values are similar to the mean nonadherence of

leukocytes from the 15 control subjects ( $p > 0.05$ ). By contrast, the mean nonadherence of the leukocytes from the 19 patients with lung CA was 27.5 when incubated with the extract of lung CA. This was significantly higher than the mean nonadherence of the same leukocytes incubated with the extract of normal lung tissue ( $p < 0.01$ ), or the mean nonadherence of the same leukocytes when incubated without antigen ( $p < 0.01$ ), and this was also significantly higher than the mean nonadherence of leukocytes from control subjects incubated with extracts of normal lung tissue or without antigen ( $p < 0.01$ ).

Figure 1 showed the distribution of the NAI values of 19 patients with lung CA and 15 control subjects. A comparison of the distribution of the NAI values of lung CA patients with control subjects revealed a significant difference.

The NAIs of the control subjects were less than 30, where most of the lung cancer patients have values of 30 or greater with lung cancer extract. The NAI was less than 30 in only two of the cancer patients, as shown clearly on Figure 1. A NAI value of 30 or greater was accepted as positive. The reason for accepting a NAI value of 30 or greater as positive is based on previous studies which showed that more than 95% of population of control subjects had values of 30 or less. Table 1 showed that the organ specific antigen did not appear to be represented in normal tissues since patients with lung cancer did not react with extracts of normal lung tissue.

To determine the specificity of the tube LAI, leukocytes from 9 patients with cancer of the cervix were tested against cervix CA extract as the specific antigen and stomach CA or hepatoma extract as the nonspecific antigen. These results were shown on Table 2. The mean per-

Table 2. LAI response to an organ-type specific neoantigen

Diagnosis of leukocyte donors	% Nonadherent cells to extracts of		
	Cervix cancer antigen	Stomach cancer antigen	Hepatoma antigen
Cervix CA. (n=9)	28.3 ± 4.9*	20.5 ± 4.8	19.4 ± 4.3
Stomach CA. (n=6)	25.5 ± 12.1	41.7 ± 13.2	27.1 ± 11.5
Hepatoma (n=5)	30.7 ± 17.2	32.3 ± 16.3	52.4 ± 9.5
Control subjects (n=8)	18.4 ± 4.9	17.3 ± 4.7	18.9 ± 4.7

\*MEAN ± S.D.

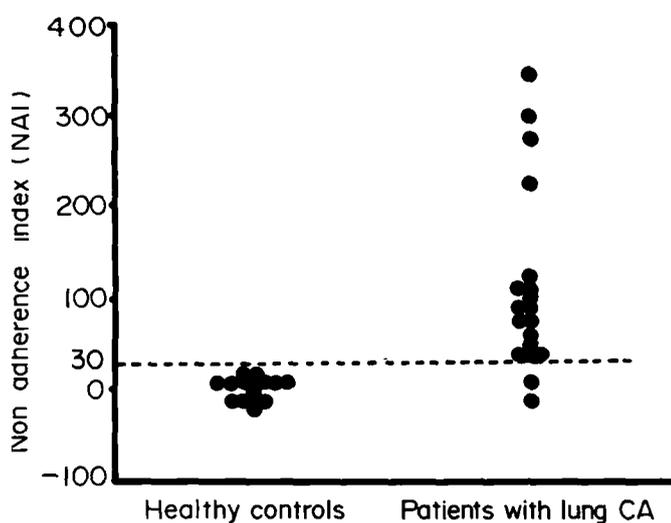


Fig.1. NAI in healthy controls and patients with lung CA.

centges of nonadherent leukocytes from the patients with cervix CA incubated with the extract of cervix CA, stomach CA, and hepatoma were 28.3, 20.5 and 19.4, respectively. In the patients with cervix CA, the mean percentage of nonadherent cells in the presence of the cervix CA extract was significantly higher than that in the presence of the extract of stomach CA or hepatoma ( $p < 0.01$ ).

Results expressed by NAI, clearly indicated the increased leukocyte adherence inhibition to the specific cervix CA antigen. Figure 2 showed that distribution of the NAIs in patients with cervix CA was quite different from the NAIs in the healthy controls.

NAIs in 8 healthy controls were all below 30, while all the NAIs in patients with cervix CA except one were above 30.

Leukocytes from 6 patients with cancer of the stomach were tested against stomach CA ex-

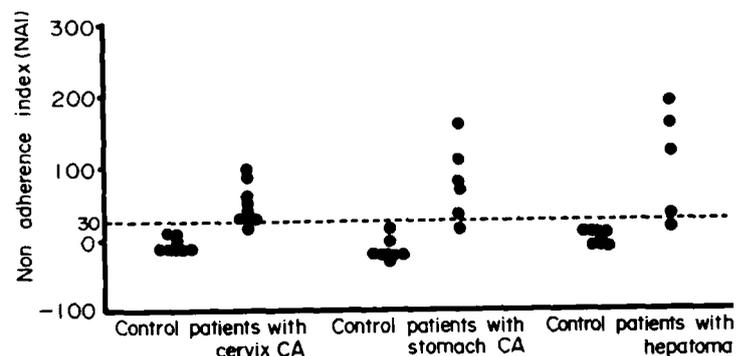


Fig.2. NAI in patients with cervix CA, stomach CA, hepatoma and the healthy control.

tract as the specific antigen and cervix CA or hepatoma extract as the nonspecific antigen. These results were shown on Table 2. The mean percentages of non-adherent leukocytes from the patients with stomach CA incubated with the extract of cervix CA, stomach CA, and hepatoma were 25.5, 41.7 and 27.1, respectively. Results in Table 2 indicated that in the patients with stomach CA, the mean percentage of nonadherent cells in the presence of the stomach CA extract was significantly higher than that in the presence of the extract of cervix CA or hepatoma ( $p < 0.01$ ).

Results expressed by NAI clearly indicated the increased leukocyte adherence inhibition to the specific stomach CA antigen. Figure 2 showed that distribution of the NAIs in patients with stomach CA was quite different from the NAIs in the healthy controls. NAIs in 8 healthy controls were all below 30, while five of the six NAIs in patients with stomach CA were above 30.

Leukocytes from 5 patients with hepatoma were tested against hepatoma extract as the

Table 3. Repeated LAI assay by lung cancer extract as the second antigen after the treatment of normal lung cell antigen in the patients of lung cancer

Patient's number	Original LAI			Repeated LAI		
	% Nonadherent cells to extracts of			% Nonadherent cells to extracts of		
	Normal lung tissue antigen	Lung cancer antigen	NAI	Normal lung tissue antigen	Lung cancer antigen	NAI
1	9.4	42.0	346	6.7	39.2	485
2	7.0	23.8	226	4.9	19.0	287
3	8.0	32.0	300	5.7	26.1	357
4	19.7	37.4	89.8	13.0	29.5	127
5	23.1	35.2	52.3	12.7	31.0	144
6	23.0	41.2	79.1	19.0	37.2	85.3
7	15.9	32.7	105.6	11.2	26.9	140
8	18.0	37.4	108	15.1	32.7	117

specific antigen and stomach CA or cervix CA extract as the nonspecific antigen. The mean percentages of nonadherent leukocytes from the patients with hepatoma incubated with the extract of cervix CA, stomach CA, and hepatoma were 30.7, 32.3, and 52.4, respectively. Result in Table 2 indicated that in the patients with hepatoma, the mean percentage of nonadherent cells in the presence of the hepatoma extract was significantly higher than that in the presence of the extract of stomach CA or cervix CA ( $p < 0.01$ )

Results expressed by NAI clearly showed the increased leukocyte adherence inhibition to the specific hepatoma antigen. Figure 2 showed that distribution of the NAIs in patients with hepatoma was quite different from the NAIs in the healthy controls.

NAIs in 8 healthy controls were all below 30, while four of the 5 NAIs in patients with hepatoma were above 30.

Leukocytes from healthy controls were tested against cervix CA extract, stomach CA extract or hepatoma extract. The mean percentages of nonadherent leukocytes from 8 healthy controls with cervix CA, stomach CA and hepatoma were 18.4, 17.3 and 18.9, respectively (Table 2). In contrast to the results in cancer patients as described above, there were no significant differences among the mean percentages of nonadherent cells in the presence of three tumor extracts ( $p > 0.05$ ). NAIs in the healthy controls didn't show antigen specific LAI reactiv-

ity to tumor extracts. The result shown in Table 2 and Figure 2 revealed that the LAI response was directed to an organ type specific neoantigen.

#### Repeated Tube LAI Assay

To minimize the nonspecific effect of normal lung extract on the nonadherence of leukocytes, the repeated tube LAI assay was done. In 8 patients with lung CA, when the repeated tube LAI assay was performed with lung CA extract on the resultant cells adherent to the glass tube after the initial tube LAI assay with normal lung tissue, the changes in the number of nonadherent cells were all above 100%. By contrast, in 9 healthy control subjects, when the repeated tube LAI assay was performed as described above, the changes in the number of nonadherent cells were all below 100%. These results were shown on Figure 3.

The original tube LAI assay was compared with the repeated tube LAI assay on Table 3 in patients with lung CA, and on Table 4 in the healthy controls. In 8 patients with lung CA, the NAI of repeated tube LAI assay was significantly higher than that of original tube LAI assay ( $P < 0.01$ ). By contrast, in 9 healthy controls there was no significant difference between the NAI of repeated tube LAI assay and that of the original tube LAI assay. ( $p > 0.05$ ). These results were shown on Figure 4.

Table 4. Repeated LAI assay by lung cancer tissue extract as the second antigen after the treatment of normal lung cell antigen in the healthy controls

Healthy control's number	Original LAI			Repeated LAI		
	% Nonadherent cells to extracts of			% Nonadherent cells to extracts of		
	Normal lung tissue antigen	Lung cancer antigen	NAI	Normal lung tissue antigen	Lung cancer antigen	NAI
1	14.7	16.4	11.6	11.0	10.0	9.1
2	10.6	12.7	19.8	6.7	7.8	16.4
3	10.8	13.4	24.0	7.4	8.8	18.9
4	11.5	10.0	-13.3	10.5	11.0	4.8
5	15.7	14.2	-9.6	8.7	6.2	-28.7
6	18.9	18.0	-4.8	14.0	12.4	-11.4
7	21.0	19.2	-8.6	15.2	16.8	10.5
8	9.4	10.1	9.4	6.2	4.8	-22.6
9	8.7	10.0	14.9	4.9	5.5	12.2

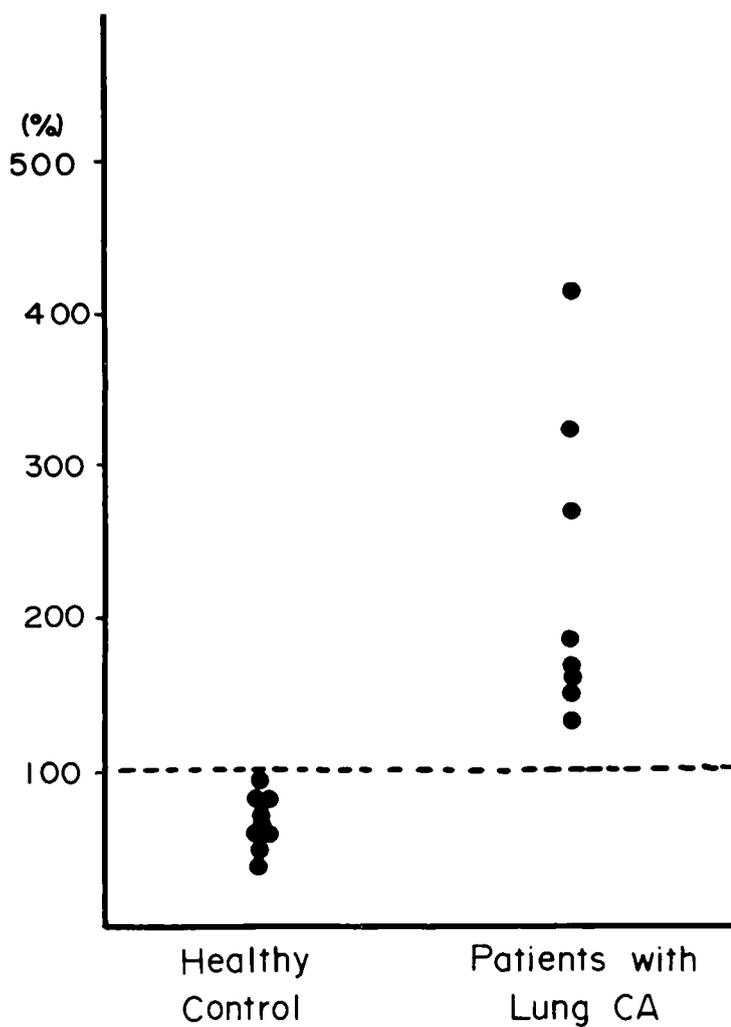


Fig.3. Changes of Nonadherent cells in repeated LAI assay.

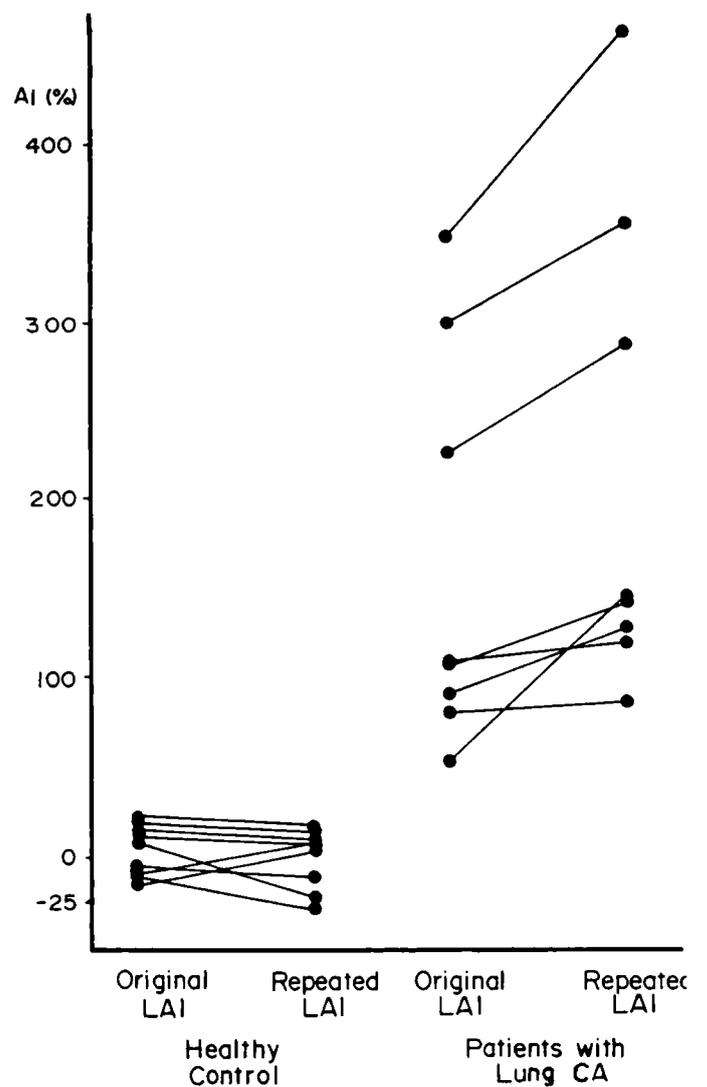


Fig.4. NAI in Original and Repeated LAI assay.

## DISCUSSION

There has been considerable interest in the development of methods for detecting and measuring specific immune response to tumors. Virtually all of these tests are suffering from lack of specificity and reproducibility.

The LAI assay is a reliable, relatively simple, reproducible, noninvasive and qualitative method for detection of tumor specific immunity and, has been used successfully to detect the cell mediated immune response in patients with a variety of tumors (Armistead and Gowland 1975; Flores *et al.* 1977; Grosser and Thomson 1976; Halliday *et al.* 1977; Lopez *et al.* 1978; Marti and Thomson 1976; Maluish 1979; Rutherford *et al.* 1977).

The original hemocytometer LAI assay was first devised by Halliday and Miller (1972). There have been two major modifications of the original hemocytometer or slide LAI method. These involve the use of tubes and wells in plates as the surfaces for cell adherence. Patrick Holt (1975) first developed the well method using microtest plates and visual counting of adherent cell. Holan *et al.* (1974) devised the tube LAI method. Using these 3 different techniques, over one hundred studies have been published by more than 20 different laboratories to date.

In the present study, tumor-specific immunity to organ type specific neoantigens of lung cancer, cervix cancer, stomach cancer and hepatoma was studied by the tube LAI assay.

Peripheral blood leukocyte (PBL) from patients with lung cancer exhibited LAI reactivity to extracts of lung CA, while the same LAI reactive leukocytes showed no reactivity to extracts of normal lung tissue. Similarly, LAI reactive leukocytes from patients with cervix cancer, stomach cancer and hepatoma reacted only to an extract of a cancer similar to the sensitizing tumor.

Moreover, none of LAI-positive patients with cervix cancer reacted to the hepatoma or stomach cancer antigens. None of the LAI-positive patients with stomach cancer reacted to hepatoma or cervix cancer antigen, and none of LAI-positive patients with hepatoma reacted to the cervix or stomach cancer antigen. Hence, these results indicated that the LAI-reactive PBL recognized on organ-type specific neoantigen.

With the observation's of Tataryn *et al.* (1979)

that PBL from patients with non-neoplastic disease of the colon, pancreas or stomach didn't react to the tumor specific antigens expressed in the colon, stomach or pancreatic cancers, it seems likely the tumor specific antigens present in colon, stomach and pancreatic cancer appear to be neoantigens that are unique for the organ of origin and do not exist in the normal tissues of the organ.

Tumor specific immunity exists at a very early stage in the development of cancer and therefore the LAI assay is potentially a very useful immunodiagnostic test in the early stages of cancer. However there is a disagreement on the nature of the LAI response in patients with large tumor burdens.

Results of a couple of studies indicated that with small tumor burdens antitumor immunity was present, but in patients with widespread metastases, it was not detectable and most leukocytes from patients with advanced cancer did not display LAI activity (Thomson *et al.* 1979). Some investigators suggest that the surface of the LAI-reactive cell in advanced cancer is already coated with tumor specific antigen (TSA) in vivo, so the leukocytes manifest the property of increased nonadherence to glass when incubated with either the specific or non-specific cancer antigens (Grosser and Thomson 1975, 1976; Lopez and Thomson 1977). In other words, the PBL of patients with limited cancer bind in vitro to TSA in the supernatant and are inhibited from adhering to glass. Whereas the PBL of advanced cancer patients if they are responding immunologically to the tumour will be coated in vivo with TSA and should also show the same phenomenon of antigen induced leukocyte adherence inhibition to glass without the necessity of exposure in vitro to the sensitizing antigen.

According to the Maluish (1979), in contrast, there was no quantitative relationship between initial adherence and degree of tumor spread. The tube LAI assay was highly dependent not only on the specific cell-mediated immune response but also on protein concentration in the medium. Decreased adherence was found with human and even with fetal calf serum.

The crude tissue extracts contain many protein besides tumor specific antigens. These crude tumor extracts which contain many pro-

teins other than tumor specific antigen, induce the nonspecific LAI response. Therefore, the LAI to lung cancer extract in patients with lung cancer may be the sum total of the nonspecific LAI by the proteins other than tumor specific antigen in the crude extract and the specific LAI by the tumor specific antigen. In this regard, highly purified tumor specific antigen is ideal to exclude the nonspecific LAI response. However it is practically impossible to obtain tumor specific antigen in highly purified form. One of the major reasons why many investigators cannot agree on the mechanism of LAI may be that they can not use highly purified tumor specific antigens.

If one uses the normal tissue extract as nonspecific antigen in which all components except tumor specific antigen are the same and express the results by the NAI, then the problem could be reduced.

A couple of investigators concluded that, when studying allogeneic leukocyte responses to tumor extracts, one must use normal tissue extract as the control. In the present study, normal lung extract was used as the nonspecific antigen to distinguish between the LAI response of patients with lung cancer and that of control donors.

The number of nonadherent cell increase by the addition of a small amount of nonspecific antigens. Therefore, it is probable that, if the extent of nonspecific LAI reaction to nonspecific antigen exceeds that to specific antigen, the results of the LAI assay will be negative even in patients in whom the monocytes have the potential to react with tumor specific antigen. Our preliminary experiments showed that a protein concentration of 200 ug/ml was optimal to demonstrate the difference in reactivity to the specific and nonspecific antigen in the tube LAI assay, although some variations were observed. Morizane *et al.*(1980) designed the repeated tube LAI assay in order to eliminate the leukocytes involved in nonspecific LAI before applying them to the assay. In the present study the tube LAI assay was repeated in patients with lung cancer according to the method of Morizane *et al.*(1980), after discarding the nonadherent cells in the initial tube LAI assay with normal lung tissue. We found the values of NAI in the repeated tube LAI assay were significantly higher than those obtained in the original tube NAI assay

in patients with lung cancer.

The results presented in the our study suggest that the tube NAI assay has a good potential as an immunodiagnostic test for lung cancer, cervix cancer, stomach cancer and hepatoma and that the repeated tube LAI assay can reduce the false negative results.

#### ACKNOWLEDGEMENT

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= 국문 초록 =

## 백혈구접착억제의 종양항원 특이성 및 반복백혈구접착억제 검사에 관한 연구

서울대학교 의과대학 소아과학교실

서정기 · 고광욱

각종 종양환자(폐암, 자궁암, 위암 및 간암)에서 백혈구 시험관 접착억제검사가 종양항원에 특이성을 가지고 있는가 알아보려고 종양환자로부터 얻은 말초혈액 백혈구를 가지고 시험관백혈구 접착억제검사를 시행하였다.

폐암환자의 백혈구는 폐암조직추출항원에 선택적으로 백혈구접착억제반응을 보였으나 정상폐조직추출항원에는 반응을 보이지 않았다. 마찬가지로 자궁암, 위암 및 간암환자의 백혈구도 종양항원에 특이한 백혈구접착억제반응을 보였다.

이러한 연구결과는 백혈구접착억제검사가 양성인 백혈구가 기관형에 특이한 종양항원을 인지한다는 것을 의미한다.

비종양항원에 의한 접착억제를 감소시키고 종양항원 특이성 접착억제를 효과적으로 하기 위하여 정상폐조직추출액으로 초회 백혈구접착억제시험을 한 후 다시 폐암조직추출액으로 남은 시험관접착세포에 대하여 시험관 백혈구접착억제검사를 시행한 결과 비접착지수(non adherence index)는 초회 시험관 백혈구접착억제 실험시의 비접착지수에 비해 현저히 높았다.