Clinical Evaluation of Immune Response in Patients with Lung Cancer

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To clarify a relationship between the degrees of cancer extension and immune response, 46 patients with lung cancer were eligible for entry into this study. These patients composed of 20 cases in adenocarcinoma, 14 in squamous cell carcinoma and 12 in undifferentiated carcinoma according to the classification of histologic types, and 6 cases in Stage I disease, 14 in Stage II, 12 in Stage III and 14 in Stage IV according to the classification of cancer stages.

1) Phagocytic activity in the reticuloendothelial system were evaluated with the use of Congo-red clearance test. The activity level in squamous cell carcinoma was superior to those in undifferentiated carcinoma and adenocarcinoma.

2) By the procedure of skin transplantation between the resected lung cancer and non-resected lung cancer patients, who were previously selected by matching of major histocompatibility using the normal lymphocyte transfer test, the responses of cellular immunity were evaluated in comparison with the living time intervals of the skin grafts transplanted in 42 cases with lung cancer, 30 cases underwent pulmonary resection for lung cancer and the remaining 12 cases were unresectable for advanced disease. The duration of living time of the skin graft was shorter in the resected lung cancer patients and rejection against the skin grafts is more enhanced. The responses to DNCB and PPD antigens by skin test were converted to be negative in accordance with the advances in the disease stages. The response to DNCB was more likely to correlate with the disease stages.

3) The results of cytotoxic tests of the lymphocytes circulate in the peripheral blood against lung cancer cells derived from surgical specimens indicated far depressive responses in advanced cases. According to the histologic types of lung cancer, in undifferentiated carcinoma it tended to be strongly inhibited, followed by adenocarcinoma and squamous cell carcinoma. Coordination of a volunteer, permitting the performance of transplantation
test of a mixture of the lymphocytes with his own lung cancer cells, evidenced directly inhibitory action of the lymphocyte against the growth of lung cancer cells.

4) Blastoid responses of surgically dissected lymph nodes were tested by the Methyl Green–Pyronin stain method. In Stage I patients, the stained grades using Methyl Green Pyronin were clearly depressed and closely correlated with advancing diseases. The reduction capacity of a $1 \times 10^7$ lung cancer cell suspension by Indophenol was also examined, adding $1 \times 10^7$ ml of the patients’ serum with incubation for 24 hours. With advancing disease, it was enhanced. In undifferentiated carcinoma, it was also prominent.

Changes in the levels of immunoglobulins of IgG, IgM and IgA were not significant between the classifications of the cancer stages and histologic types.

To clarify the existence of auto-antibody the immunoadherence tests were performed but these results were not closely parallel to the cancer stages and histologic types.

In conclusion, the results of various tests reflecting the capacity of cellular immunity coincided with the cancer stages and histologic types but those of the immunoadherence test and immunoglobulin levels, reflecting humoral immunity were not in concord with the classifications of cancer stages and histologic types.

INTRODUCTION

The immune response is now a well known term, with which newly growing tumor in the body is recognized as being not-self. In cancer diseases, it has experimentally and clinically been noted that tumor–growth might be inhibited by immune responses of tumor–burden host\textsuperscript{13}. In contrast, the high frequency of malignant diseases in immuno-suppressive status is thought to be a consequence of weak host defensive ability against tumor growth\textsuperscript{15}.

The aim of this study is to clarify a variety of immune responses in patients with lung cancer in accordance with the cancer stages, histologic findings and prognosis following surgery.

MATERIAL AND METHOD

Forty-six patients admitted at the First Department of Surgery, Nagasaki University Hospital for the surgical treatment of lung cancer from January, 1973 to December 1974 were available for study. Of 46 patients, the histologic types had been confirmed by surgical specimens in 30 and by cytological examination of the sputum and/or biopsy specimens in 16. Adenocarcinoma was seen in 20, squamous cell carcinoma in 14 and undifferentiated carcinoma in 12, and according to the classification of cancer stages by the Japanese General Rules for the lung cancer, Stage I disease was 6 cases, Stage II 14, Stage III 12 and Stage IV 14. The distribution of sex in this study was a 6.6:1 ratio of men to women. The immune responses of patients with lung cancer were assessed using the following methods.
1) Congo red clearance test: To assess the functions of the reticuloendothelial systems in these 42 patients with lung cancer, Congo red clearance test was used. Ten ml of blood sample were taken from the cubital vein in the morning and 12ml of 1% Congo red (Merk 1339, Gruber) was injected at the same route. The blood samples at the opposite cubital vein were drawn at interval of 4min and 60min. The concentrations of Congo red in serum were measured with the use of electrospectrometer (Beckmann-Elmer) and the Congo red index (CI) was calculated as the following formula

\[
\text{Congo red index (C.I)} = \frac{\text{Concentration at the 20min}}{\text{Concentration at the 4 min}} \times 100
\]

2) PDD reaction
A dosage of 0.1ml PPD used for conventional diagnosis of tuberculosis (Japan BCG Co.) was subcutaneously injected at the forearm and it was determined at the time of 48 hours after PPD inoculation. Strong positive reaction indicates indurations with formations of double redness, blister and necrosis of the tested skins. Positive one shows an erythema of more than 10mm in diameter. Suspicious positive one indicates an erythema of 5mm to 10mm in size and negative one corresponds to an erythema of less than 5mm in diameter.

2) DNCB reaction
Round pieces of the felts, 16mm in diameter, containing 0.02ml of 1% 2-4 DiNitroChloroBenzen (DNCB) aceton solution were used for desensitization during a period of 48 hours. Following this pretreated procedure, 0.02ml of 0.1% DNCB aceton solution was also applied 2 weeks later with the same manner to the opposite forearm. The response to DNCB following 48 hours were expressed as negative (no response), suspicious positive (erythema), positive (erythema and adema) and strong positive (erythema combined with blister).

3) Homologous skin transplantation.
The immune responses of patients with lung cancer were tested by means of homologous skin transplantation procedure. Major histocompatibility was previously matched each other using the method of the normal lymphocyte transfer test. The reactions to the normal lymphocyte transfer tests were examined with a $5 \times 10^6$ lymphocyte inoculation from the donor of non-cancer patients to the recipient of lung cancer one.

When the major histocompatibility was matched between patients with lung cancer and with other diseases undergoing surgery, a 1x1cm skin grafts were taken from the operative wounds in non-cancer patients at surgery. These skin grafts were transplanted to the patients with lung cancer immediately after taking the skin graft and positioned at the site of lower abdominal wall with conventional suturing method. Twenty one patients, 6 in non-resected cases and 15 in resected one, consented to enter this protocol to study the immune responses to transplantation antigens. The survival times of skin grafts transplanted were compared between 6 non-resected and 15 resected patients.

4) Cytotoxic test of lymphocytes against cancer cells.
For cytotoxicity test of the lymphocyte by dye exclusion method, the suspensions of lung cancer cells were prepared with the following steps. Lung cancer mass was aseptically excised at surgery, minced by the fine scissors immersing in the 199 tissue culture solution, filtrated with glass cotton to exclude fibrocollagen tissues from cancer cells and low viable cancer cells were also removed by means of a 3 hour incubation at room temperature. The cancer cells were resuspended 10 to 20 time with the 199 tissue culture solution in volume and these procedures were repeated 3 times and the cell counts were adjusted to a concentration of $8 \times 10^5$/ml.

Then the lymphocytes drawn from the peripheral vein and isolated by Conray 400 Ficoll method\(^3\) were also prepared for adjusting to a concentration of $8 \times 10^7$/ml, and the lymphocyte suspensions were diluted at 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 in order. and 0.2ml of cancer cell suspension and 2µl/0.2ml of Phytohemagglutinin–M (Difco) were also added to each tube containing the diluted lymphocyte suspension solution and cultured at 37°C for 30 minutes, centrifuged at 600 rpm for 10 min, thereafter, the target cancer cells were resuspended with 1.0ml of the 199 solution. Then 0.1ml of 0.1% Trypan Blue solution was added and % of the dead cell was calculated by the following formula.

$$\text{% dead cell} = \left(1 - \frac{\text{cell counts when lymphocytes are added}}{\text{cell counts when lymphocytes are not added}}\right) \times 100$$

5) Neutralization test of activity in cancer cells with lymphocyte.

In one candidate eligible for testing a neutralization of cancer cells with different lymphocytes obtained from either his own or the other non–cancer patients, A mixture of $1 \times 10^7$/ml lung cancer cells with $1 \times 10^7$/ml autologous or homologous lymphocytes derived from either own or the other patients were subcutaneously inoculated at the site of the thigh. The diameters of inoculated cell indurations were measured every 2 days until the indurations might be disappeared.

6) Methyl–Green Pyronin stain of surgically dissected lymph nodes.

Thirty-eight lymph nodes surgically dissected were stained using Methyl–Green Pyronin dye to evaluate the degree of RNA synthesis in cell cycles of the lymphocytes in the regional lymph nodes. Dark blue stain was expressed as a strong positive (++++), light blue, positive (+) and no staining, negative (−).


To determine an optimal dosis of Indophenol deoxidized, a preliminary test was attempted.

Three to 10g of lung cancer mass were aseptically resected and washed out several times with KGUB++ and 0.3g of lung cancer mass was weighed. It was placed to the small test tube containing 3ml of Dulbecco medium, mixed with 1ml of patient’s serum, incubated at 38–39°C for 24 hours, supplemented with 1ml of the horse serum. Finally, it was proved that 1ml of 1 : 3000 Indophenol was adequate to enhance a reductive activity in lung cancer mass.

The degrees of deoxidation of 0.3g lung cancer tissues as an indicator of Indophenol
color changes were compared using the same manner with a preliminary test.

No deoxidative activity was expressed as negative (−), when showing the inherent blue color of Indophenol and strong deoxidation was shown as strong positive (++) when showing a transparent decolorization which implicated a loss of the inherent Indophenol color.

8) Changes in the levels of immunoglobulins.

The levels of immunoglobulins of IgA, IgM and IgG were measured with the use of immuno-plate (Hyland Div. Travenol. Laboratories, INC, Los Angeles, Calif. 90039, USA) Each value was obtained from the diameters of precipitating rings on the plates, which were converted to a known concentration values using the plotted standard curve.

9) Immunoadherence test

Erythrocytes derived from several persons of O blood type were washed 3 times with KGVB++ solution, adding 3 times as much as Alsever solution in volume for prevention from hemolysis.

To make complement from guina pig inactive, 20ml of 150 to 180 CH50/ml complement obtained from a guina pig was added to 1 ml of the erythrocyte preserved as mentioned above and centrifuged at 2000rpm for 10min at 0°C and suspension was further centrifuged at 16000 to 30000rpm for 10 min. The serum from lung cancer patients was diluted with a range of 1/2, 1/4, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/251 using KGVB++ and 0.2ml of 8×10⁹/ml cancer cell suspension and 0.2ml of absorbed complement were supplemented and incubated at 37°C for 55 min. IA titers were microscopically determined according to the classification of the four grades and the IA50 was expressed as maximal dilution rate of more than a 50% IA value.

RESULT

Congo red clearance test

Phagocytic activity of the reticuloendothelial system examined by Congo red clearance test was compared according to the lung cancer stages as shown in Fig 1. With advancing disease stage, phagocytic activity for Congo red clearance was significantly depressed. (p<0.01). In particular, phagocytic activity in Stage Ⅳ disease was mostly reduced. Based on the histologic type, in undifferentiated carcinoma and adenocarcinoma it declined rather than in squamous cell. (p<0.01)

Delayed hypersensitivity test

PPD reaction: The results of PPD reactions in 44 patients with lung cancer weresummerized in Table 1. The responses to PPD were not inhibited in Stage Ⅰ, Ⅱ and Ⅲ diseases.

In Stage Ⅳ disease, however, the negative responses were common. (p<0.01)

DNCCB reaction: As shown in Table 1, the responses to DNBCB were depressed in
Fig. 1 Non-Specific Phagocytic Activity in Clinical Stage and Histological Classification

Table 1. Studies on Depayed Type Allergy

<table>
<thead>
<tr>
<th>Stage</th>
<th>I</th>
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<th>III</th>
<th>IV</th>
<th>PPD</th>
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<td>12</td>
<td>12</td>
<td>6</td>
<td>14</td>
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<td>10</td>
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<td>0</td>
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<td>7</td>
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(p<0.01)

according with cancer staging. In Stage III and IV diseases, these responses were significantly depressed. (p<0.01)

Homologous skin transplantation

Major transplantation histocompatibility was matched using the normal lymphocyte transfer test. The skin graft survival times were tabulated in Table 2. In 12 patients

Table 2. Survival Time of Skin graft.

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<tr>
<td>No. Case</td>
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<td>Survival Time</td>
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<td>Mean Survival</td>
<td>19.5 Days</td>
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<td>SD</td>
<td>9.3</td>
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(p<0.01)
with non-resected lung cancer due to far advancing diseases, the survival times averaged 19.5±9.3 days, ranging from 7 to 35 days.

Meanwhile, in 30 patients with resected lung cancer, the mean survival time was 8.7 ±1.1 days, ranging from 7 to 10 days. It was definitively shorter. The enhanced rejections such as acute rejection and white graft formation did not develop in resected lung cancer group. The skin graft survival times between resected and non-resected patients were significantly different. (p<0.01) The maximal survival time was 35 day in non-resected group, reflecting provoked weak immune response of the host.

Cytotoxic test

Cytotoxic activities of the lymphocytes to the lung cancer cells were expressed as % titer as shown in Fig. 2. With advancing cancer stage the levels of cytotoxic titer were reduced. In Stage IV disease, these were significantly depressed. (p<0.05) According to the histologic types, cytotoxic activities were greatly depressed in undifferentiated carcinoma despite less inhibition seen in squamous cell carcinoma rather than in adenocarcinoma. (p<0.01)

Neutralization of activity in cancer cell with lymphocyte

Changes in the sizes of inoculated cell indurations in only one candidate were shown in Fig 3, distinguishing from three types of different cell inoculations of $1 \times 10^7$ cancer cells alone, $1 \times 10^7$ cancer cells with $1 \times 10^7$ homologous lymphocytes taken from the other lung cancer patients and $1 \times 10^7$ cancer cells with $1 \times 10^7$ autologous lymphocytes from his own.

When mixed with lymphocytes, cancer cell induration failed to develop. Only cancer cell inoculation alone allowed cancer cells to continue to proliferate during a period of 3 to 4 weeks. The cancer stage of this candidate was a category of Stage IV disease and he died on the 42th day of this test, preserving the inoculated cancer cell induration.

Methyl-Green Pylonin stain for regional lymph nodes surgically dissected

RNA synthesis in the lymph nodes dissected at surgery were evaluated with the use of Methyl-Green Pyronin stain method. The results were shown in Table 3.

![Fig. 2 Cytotoxic Titer in Clinical Stage and Histoogical Classification](image)
According to advances in the disease stages, the degrees of staining with Methyl-Green Pyronin in the lymph nodes were depressed. In Stage IV disease, 57% were negative response in spite of 41% in Stage III disease and none in either Stage II or Stage I diseases. (p<0.01)

Reduction activity of lung cancer tissue

Reduction activity of lung cancer tissues were evaluated as a maximum dose of Indophenol deoxidized. Reductive activity was enhanced in accordance with advances in the disease stages (Table 4). Based on findings of the histologic types, undifferentiated carcinoma greatly enhanced rather than squamous cell carcinoma and adenocarcinoma (p <0.01) as shown in Table 5.

Changes in the levels of immunoglobulins

The levels of IgA, IgG and IgM in 46 patients with lung cancer were indicated in Fig 4. The IgG levels were similarly lowered but the levels of IgM and IgA were not constant and varied with a wide range. According to the histologic types, IgG levels

<table>
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<td>No. Case</td>
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<td>6</td>
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<tr>
<td>Response</td>
<td>+</td>
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significant difference between clinical stages and the degree of M-G pyronin stain (p<0.01)
Table 4. Detection of Dehydrogenase Activity in Clinical Stage (INK Method)

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<th>Stage</th>
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<tr>
<td>No. Case</td>
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Significant difference between clinical stages and reductive activity of cancer tumors (p<0.01)

Table 5. Detection of Dehydrogenses Activity in Histological Classification (INK Method)

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<td>No. Case</td>
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Significant difference between histologic types and reductive activity of cancer tumors (p<0.01)

Fig. 4 Quantitative Immunoglobulin Levels in Clinical Stage.
Fig. 5 Quantitative Immunoglobulin Levels in Histological Classification.

Statistically not significant.

Fig. 6 I-A Titer in Clinical Stage.

Statistically significant between each groups. (P<0.05)
were clearly low in squamous cell carcinoma and undifferentiated carcinoma as shown in Fig 5. As compared with those in adenocarcinoma, these changes were not statistically significant.

Immunoadherence test

The immunoadherence test were used for detecting the small amount of antibody and antigen in 44 patients with lung cancer. According to advancing cancer stages, the IA titers measured were significantly lowered (p<0.01) as shown in Fig 6. From the standpoint of histologic types, in undifferentiated carcinoma these titers were proved to be low. In squamous cell carcinoma and adenocarcinoma the levels of IA titers did not decline, maintaining a similar tendency as shown in Fig 7. These differences were not statistically significant.

![Immunoadherence test graph]

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Fig. 7 | -A Titer in Histological Classification.

DISCUSSION

An ability of tumor-bearing host to resist to tumor growth has long been recognized. Bush in 1868 noted that erysipelas affecting the cancer patients had enabled cancer tumors to reduce their sizes.

In 1966, Everson d Cole reported that the tumor bulks proven histologically as a cancer in 176 patients had become spontaneously regressed and these regressions had been thought to be caused by various factors such as the effects of fever by infection, endocrine organ function, palliative operation and auto-immune disease. Jinnouchi d Mori experienced the 7 patients in whom cancer tumors, 4 primary and 3 metastatic, had spontaneously regressed. These reports suggest that immune response of the host to
cancer tumor plays an important role in preventing the tumor growth.

The question arises whether which of two factors, the ability of inherent cancer cell proliferation and the intensity of host immune response, is strong enough to allow the tumor bulk to vigorously grow in cancer-advanced cases.

The report regarding the immune response to specific antigens, however, is quite a few due to difficulty of detection of specific antigen. Shibuzaki[10] indicates that the immune response to cancer disease seems to be a non-specific reaction and it closely correlates with the follow-up results. It is also well known that the function of the reticuloendothelial system also contributes to inhibition of tumor growth[11][12]. Old[13], however, identified that the growth rates of spontaneously induced cancer tumors are not in association with host immunity, whereas those of transplantable tumors are strongly influenced. In patients with lung cancer, Congo red clearance ability of the reticuloendothelial system was depressed in accordance with advances in the cancer stages. These results were in concord with findings reported by Hatori[14]. It is emphasized that the immune responses of tumor-burden host are partly participating in the functional intensity of the reticuloendothelial system.

From the standpoint of delayed sensitivity[15]-[17], skin test reactions to PPD and DNCB were assessed. Recent data imply that the responses of skin tests well correlate with their prognosis. Physician should be aware of common condition showing physiological depression of skin test responses in the aged. Complex mechanisms to represent the positive skin reactions were explained by Hisano[18] et al. These comprise three processes, recognition of antigens, sensitization and final step of producing local reactions. Then the skin test reactions are beneficial in easily and adequately evaluating the immune capacities of tumor-bearing host.

Waldorf[19] and Eilber[20] et al stressed that afferent limb of the immune arc widely recognized as an immunological mechanism had become susceptible rather than efferent one in advanced cancer patients. From these results, DNCB antigen, not so commonly exposed, is preferable to PPD one to evaluate the immune responses. In this study, the response to PPD was significantly depressed (p<0.01) in advanced patients of Stage İ and İV diseases. The close correlation between the degrees of skin test responses and the disease stages was more clearly emerged in the response to DNCB.

It is advisable that new antigens, which is not more frequently exposed, must be used for determination of the degrees of the immune responses. To clearly assess the degrees of the cellular immune responses in patients with lung cancer, comparative study of survival times of transplanted skin grafts in patients with various stages of lung cancer was introduced in this study. The survival times of skin grafts transplanted were made prolonged in non-resected lung cancer patients as compared to those in resected lung cancer patients. Advances in the cancer stage led to elongation of survival time of the transplanted skin graft. In not so heavily advanced patients, the transplanted skin grafts survived with a wide range and did not represent a constant living time.

Cytotoxic test was also valuable to inquire about the immune responses of tumor-
bearing host. Effector T-cells seem to contribute cytotoxic activity with an aid of lym-photoxin\textsuperscript{21}. This study substantiates that a wide cancer extension results in an inhibition of cytotoxic activity against cancer cells. It is more likely to relate to the speed of cancer extension.

In addition, it has become apparent that a mixture of spleen cells and cancer cells produces the tumor regression even if given homologously and heterologously as reported by Yoshida\textsuperscript{22} and Southam\textsuperscript{23} et al using MH 134 tumor to mice and by Mikulska\textsuperscript{24} et al using Benzpyren-induced tumor to rats and also it is obvious that the lymphocyte itself can exert on the cytolysis of cancer cells. Southam et al\textsuperscript{23} reported that about half of the 41 patients with advanced lung cancer, not eligible for surgery, showed a suppression of tumor cell growth by mixing the lymphocytes. Eleven of the 19 cases with suppressive responses to the tumor cell growth were afflicted with confined cancer lesions whereas 18 out of the 22 cases with non-suppressive responses to the tumor cell growth had a cancer spread of distant metastases. In this study, the role of the lymphocyte was clearly revealed in advanced lung cancer patients of Stage IV, demonstrating that the proliferation of inoculated cancer cells with the lymphocyte was not inhibited.

Immunoblast, converted immunologically competent cell to performing cells, plays a key role in achieving the strong immune response. To clarify the existence of the immunoblast, the Methyl-Green Pyronin stain method was used for the lymph nodes surgically resected, assessing the regional lymph node responses to cancer extension. Ogawa\textsuperscript{25} indicated that the immune responses of the lymphocytes in the lymph nodes to PHA were 2 times stronger than those in the peripheral veins.

Data obtained from this study also clarified that the lymph nodes adjacent to the tumor showed more vigorous immune response to cancer spread rather than those distant from the tumor. Shirakusa\textsuperscript{26} also reported the enhanced responses of the neighbouring lymph nodes as well as lung tissues around the tumor.

Viability of cancer cells were tested by the evaluation of reduction using Indophenol. Nishioka\textsuperscript{27} reported that this test had become more sensitized when using the tumor bulk rather than isolated tumor cells. In this study, 0.3g of the tumor bulk was used. The results clearly indicate that biological activity of the tumor has become pronounced in accordance with advances in the cancer stages.

The levels of immunoglobulins were evaluated in patients with lung cancer by many reportors. With respect to IgG, high levels are reported by Huglies\textsuperscript{28}, Rowinska-Zakrewsk\textsuperscript{29}, Kumasaka\textsuperscript{30} et al, no significant difference byshima\textsuperscript{31}, Yamazaki\textsuperscript{22}, Tsuji\textsuperscript{33}.

As for IgM, low levels or no difference from the normal level are indicated by Kumasaka\textsuperscript{30} Shima\textsuperscript{31} Tsuji\textsuperscript{33} et al.

As for IgA, there are many reports of the increased levels by Mitsuhashi\textsuperscript{3}, Shima\textsuperscript{31} Shirakusa\textsuperscript{26} and no significant change by Tsuji\textsuperscript{33}. Associated infection with lung cancer is influential on changes in the immunoglobulin levels, particularly in IgG level. In this study, there is the tendency for IgG value to increase but it is not so
significant. Detection of tumor specific antigen has not completely been achieved so far. Nishioka indicates that the immunoadherence test is sensitive to detection of a $0.005\text{gN}$ concentration of antibody. Availability of this test is evidenced using the tumor of glioblastoma and astrocytoma by Shimizu & Nishioka et al whereas Kikuchi notes that spontaneously induced cancer does not enhance the IA titer and a repeated immunization with tumor cell is required for enhancing the IA titer. We must bear in mind that the results of the immunoadherence tests are not only directed on the immune responses to very small amount of antibody against the tumor but on the response to antibody derived from the smooth muscle. The differences of the IA titer levels between the cancer stages were statistically significant ($p<0.01$) whereas those between the histologic types were not so.

It was confirmed from this results that the immune responses related to cellular immunity had clinically close correlation with the classification of cancer stage. Immunoglobulin levels related to humoral immunity, however, did not reveal an exact relationship between classification of cancer stages and histologic types.

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