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Cell-mediated immunity against digestive
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Abstract

The present study was conducted to investigate the usefulness of the direct leucocyte migration agarose method for studying cell-mediated immunity in vitro. Comparative studies of the purified protein derivative (PPD) skin test and the leucocyte migration inhibition test (LMIT) in which PPD was used as test antigen indicated a significant qualitative and a weak quantitative correlation between these two tests. Furthermore a positive correlation was found between the LMIT and the macrophage migration inhibition test (MIT) using ultrasonicated autochthonous tumor antigen. Comparative studies of the LMIT, MIT, PPD skin and DNCB tests on the same patients showed that cases responding positively to the PPD skin and DNCB tests tended to respond positively to the LMIT and MIT. Patients with digestive organ cancers were examined by the LMIT. With the advance of cancer, decreased positive test rates were found. After gastric cancer operations the LMIT findings were divided into two groups: one type changed from positive to negative, and the other type changed from negative to positive. The former response was suggestive of a successful operation, and the latter response was suggestive of a non-curative operation. These results indicated that the direct leucocyte migration inhibition agarose test was useful investigating cell-mediated immunity.

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**CELL-MEDIATED IMMUNITY AGAINST DIGESTIVE
ORGAN CANCERS: LEUCOCYTE MIGRATION
INHIBITORY FACTOR ACTIVITY AS
AN IMMUNOLOGICAL PARAMETER**

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Abstract. The present study was conducted to investigate the usefulness of the direct leucocyte migration agarose method for studying cell-mediated immunity *in vitro*. Comparative studies of the purified protein derivative (PPD) skin test and the leucocyte migration inhibition test (LMIT) in which PPD was used as test antigen indicated a significant qualitative and a weak quantitative correlation between these two tests. Furthermore, a positive correlation was found between the LMIT and the macrophage migration inhibition test (MIT) using ultrasonicated autochthonous tumor antigen. Comparative studies of the LMIT, MIT, PPD skin and DNCB tests on the same patients showed that cases responding positively to the PPD skin and DNCB tests tended to respond positively to the LMIT and MIT. Patients with digestive organ cancers were examined by the LMIT. With the advance of cancer, decreased positive test rates were found. After gastric cancer operations the LMIT findings were divided into two groups: one type changed from positive to negative, and the other type changed from negative to positive. The former response was suggestive of a successful operation, and the latter response was suggestive of a non-curative operation. These results indicated that the direct leucocyte migration inhibition agarose test was useful for investigating cell-mediated immunity.

Experimental tumor transplantation studies have shown that tumor-bearing animals possess cell-mediated immunity against tumor cells. In man such *in vivo* methods are not possible and it is necessary to develop various *in vitro* parameters for evaluating cell-mediated immunity. Both specific and non-specific tests are necessary to meet such requirements. Specific tests include the lymphocyte direct cytotoxicity test, tumor colony inhibition test, estimation of lymphokine, especially the macrophage migration inhibitory factor (MIF) and the leucocyte migration inhibitory factor (LIF), and mixed lymphocyte-tumor reaction. Non-specific tests include the estimation of the lymphocyte transformation rate against mitogens, peripheral lymphocyte counts, and the ratio between B-cells and T-cells (1). It is usually not easy to obtain target tumor cell strains by tissue culture

and thus a cytotoxicity test can not normally be conducted. Therefore clinically cell-mediated immunity is being investigated by the macrophage migration inhibition test (MIT) and the lymphocyte transformation rate against mitogens.

It is well known that when sensitized T-cells are in contact with respective antigens, cell-free mediators (so-called lymphokines) are liberated (2). The most commonly known lymphokine at present is MIF. It was reported that MIF activity had a close mutual relationship with the delayed-type of hypersensitivity (3). Akiyama (4) obtained favorable results in diagnosing malignant tumors by MIT. However, it is not possible to measure the total immunological potential by MIT, so that other parameters are required to determine this capacity more precisely. The present study was conducted to determine whether the leucocyte migration inhibition test (LMIT) can serve as a parameter for cell-mediated immunity.

Sj borg and Bendixen (5) measured LIF activity by the capillary tube method and reported it to be an excellent method for studying cell-mediated immunity. Since then, many confirmatory studies have appeared using various antigens (6-9). This method, however, requires a large quantity of blood so that it is difficult to use as a routine test.

In 1971 Clausen (10) developed a leucocyte migration agarose test that was simple to manipulate and was highly reproducible with a small quantity of blood. Some reported (11-13) the usefulness of the agarose test with soluble antigen and tumor extract antigen.

In the present investigation LMIT and MIT were carried out simultaneously with patient peripheral blood to study the relationship between the two tests and the relationship between LMIT and the macroscopic stage of gastric cancer and colon cancer. Postoperative immunity was followed-up in some gastric cancer patients. Furthermore, comparisons were made between LMIT and MIT and between the purified protein derivative (PPD) and 2,4-dinitrochlorobenzene (DNCB).

MATERIALS AND METHODS

Subjects. The subjects of the study consisted of 58 cases of gastric cancer and 20 cases of colon cancer, operated in the First Department of Surgery, Okayama University Medical School. The gastric or colon cancer stages were classified in accordance with the recommendations of the Japanese Gastric Cancer Research Society (14). The gastric cancer patients were classified as follows: 9 cases in stage I to II, 16 cases in stage III and 15 cases in stage IV. The colon cancer patients were classified into 10 cases in stage I to II and 10 cases in stage III to IV.

Skin test. PPD skin and DNCB tests were conducted. PPD (Japan BCG Co.) was injected intradermally at 0.05 μ g/0.1ml at the middle of the flexion side

of the forearm, and the reaction was measured 48 hr later. When the reddened diameter area was less than 5 mm, the reaction was judged negative, and diameters over 10 mm were considered positive. For the DNCB test 0.05 ml of 2% acetone solution of DNCB (DNCB, Wako Co.) was smeared over a 2 cm skin area of the inner thigh, and the flare-up reaction was measured 14 days later. If there was no reaction, 0.05 ml of 0.1% DNCB solution was again smeared, and the final determination was made 48 hr later by the reddening and induration response.

Preparation of lymphocytes. Lymphocytes were isolated according to the method of Böyum (15). Two to three ml of heparinized peripheral blood were mixed with 6 ml of 0.9% physiological saline solution. The diluted blood (6–9 ml) was poured gently over a layer of 3 ml Ficoll-Conray mixture solution in a test tube. The tube was centrifuged at 2,000 rpm for 30 min, and the lymphocyte layer was aspirated. This lymphocyte layer was mixed with 0.9% saline and centrifuged at 2,000 rpm for 10 min. The cells were washed with 0.9% saline three times, and the lymphocytes were suspended in TC-199.

Preparation of macrophages. Fluid paraffin (20–30 ml) was injected into the peritoneal cavity of a guinea pig, and 4–5 days later the animal was sacrificed by bleeding after severing the cervical artery under ether anesthesia. The abdominal wall was opened, the cavity was rinsed with 100–150 ml saline solution and the rinsed fluid was collected. The fluid was centrifuged at 1,000 rpm for 5 min. The precipitated cells were washed three times and suspended in TC-199.

Preparation of antigens for LMIT and MIT. PPD was used as antigen at a concentration of 100 μ g/ml. Tumor antigen was prepared as follows. About one gram of tumor harvested aseptically at operation was minced and sonicated at 20 Kc for 15 min (7 ϕ tip, 150 mA) in about 5 ml of TC-199. The homogenate prepared was centrifuged at 0°C at 3,000 rpm for 30 min, and the supernatant was used as tumor antigen. Protein concentration was determined by Lowry's method (16). Non-specific migration inhibition to normal human leucocytes was measured by LMIT using the agarose method. The percent migration $\pm$ S.D. was: 92.0 $\pm$ 7.0 at 50 μ g of antigen/ml; 92.3 $\pm$ 3.3 at 100 μ g/ml; 87.0 $\pm$ 1.2 at 200 μ g/ml; 84.3 $\pm$ 4.1 at 400 μ g/ml; and 73.3 $\pm$ 10.8 at 800 μ g/ml (Fig. 1). Antigen at 400 μ g/ml was used as the optimal concentration. The optimal concentration of antigen solution for MIT was set at 50–100 μ g of protein/ml of TC-199. This concentration was determined previously by preliminary testing.

Macrophage migration inhibition test (direct method). This test was carried out according to the method of George and Vaughan (17). Peritoneal exudative cells and lymphocytes were mixed in a ratio of 4:1; the mixture was sealed in a capillary tube (micropipet 75 mm $\times$ 1.5 mm, Terumo Co., Tokyo), and cells were precipitated to the bottom of the micropipet by centrifugation at 800 rpm for 3 min. After cutting the micropipet at 1–2 mm distance from the end of the precipitated cell layer, the capillary was fixed on the bottom of a Mackness type micro-Petri dish. The capillary specimens were divided into two groups. To one group was added TC-199 containing antigen and 20% bovine serum, and

to the other group was added TC-199 containing 20% bovine serum as a control. The micro-Petri dish was sealed with a cover glass, incubated at 37°C for 24 hr in a 5% CO₂-incubator, and the migration area was measured. Four capillaries were used in one group to measure the average migration area, and the migration index was calculated by the following formula :

$$\text{Macrophage migration index} = \frac{\text{Average migration area of antigen-added group}}{\text{Average migration area of antigen-free control group}} \times 100(\%)$$

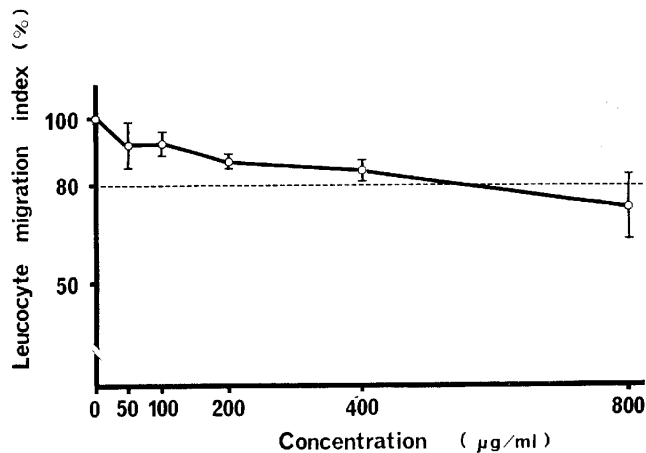


Fig. 1. Optimal concentration of tumor antigen (Mean $\pm$ S. D.).

Preparation of agarose plate. A 2% solution of Bacto-agar (Difco, U.S.A.) was prepared with sterile water and autoclaved. When the solution cooled to 47°C, TC-199 (10 time strength), horse serum, NaHCO₃ and sterile water were added to make a final concentration of 1% Bacto-agar, 10% horse serum, 400 μg NaHCO₃/ml and single strength TC-199. The final pH of the solution was 7.3. Six ml of the 1% solution of Bacto-agar were poured into a disposable plastic Petri dish (50 × 15 mm, Wako Pure Chemicals), and allowed to harden. The dish was then placed in a refrigerator for 30 min to sufficiently harden the agar in the agar wells. Six holes 3 mm in diameter were made in the agar plate with a stainless steel punch, and the agar plugs were plucked out by a hypodermic needle.

Preparation of leucocytes. Ten ml of heparinized peripheral blood were poured into a test tube and left standing at 37°C for one hour to allow the red cells to sediment. The buffy coat layer was collected and was centrifuged at 1,000 rpm for 5 min. The precipitated leucocytes were washed three times with physiological saline solution. When the erythrocyte contamination was marked, the red cells were disrupted by treatment with hypotonic saline solution (0.35%). The viability of leucocytes after the treatment was over 97% by the trypan-blue exclusion test. Leucocytes were suspended in TC-199. The composition of cells

in the buffy coat was 60–94% (68% average) polymorphonuclear cells and 6–40% (32% average) mononuclear cells. Leucocytes at 2×10^7 were usually obtained from 10 ml of peripheral blood.

Direct leucocyte migration inhibition test (agarose method). Leucocytes prepared as described above were divided equally into two test tubes. Tumor antigen was added in one tube, and another tube was used as the control. The cell number in each test tube was adjusted to 2×10^8 /ml. Both tubes were pre-incubated at 37°C for 30 min, and then 10 μ l of cell suspension (2×10^6 cells) was distributed into each well by a microsyringe (Hamilton Co.). Each specimen was tested independently three times. After incubating the agar plate in the 5% CO₂-incubator at 37°C for 18 hr, the plate was fixed with ethanol-formalin solution, and removed from the Petri dish. The plate was stained with May-Grünwald-Giemsa solution. The mean diameter (2 r) of the migration area was measured by a micrometer (Mini Master, Tajima Industry Co.) and the migration area was calculated as: πr^2 minus the area of well.

$$\text{Leucocyte migration index} = \frac{\text{Average migration area of antigen-added group}}{\text{Average migration area of antigen-free group}} \times 100 (\%)$$

For determination of significant differences, the Student's t-test or Chi-square test was used.

RESULTS

PPD skin test and LIF activity. Both the PPD skin test and the LMIT using 100 μ g/ml of PPD antigen were conducted on the same 20 patients (Fig. 2). There was a highly significant difference between the LIF positive-skin test positive group and the LIF negative-skin test negative group ($P < 0.01$). The correlation between the degree of the LIF activity inhibition and the strength of the skin test was -0.52 .

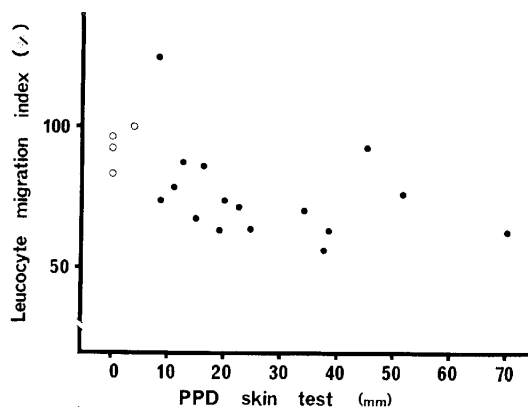


Fig. 2. Correlation of leucocyte migration index and the PPD skin test. ○, skin test negative; ●, skin test positive.

Comparison of LIF and MIF activity in gastric cancer patients and colon cancer patients. LMIT and MIT were simultaneously conducted 90 times in 47 gastric cancer patients and 20 times in 16 colon cancer patients to determine the correlation between these two activities (Fig. 3). The index r was found to be .62 in gastric cancer cases and .70 in colon cancer cases.

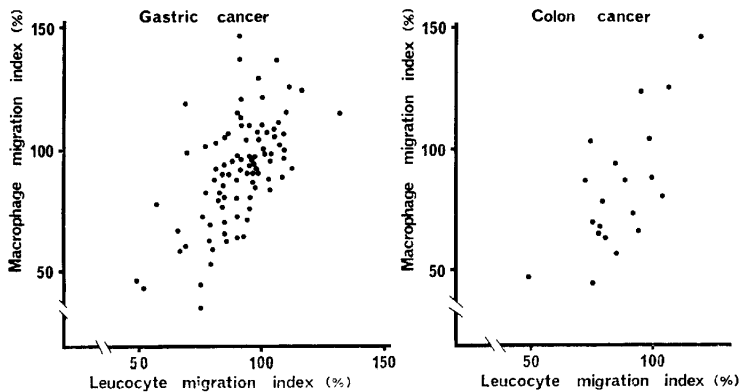


Fig. 3. Correlation of LIF activity and MIF activity on patients with gastric cancer and colon cancer.

LIF activity in various stages of gastric and colon cancer. In gastric cancer, the LIF positive rates were (Fig. 4): stage I plus stage II, 44.4% (4 of 9 cases); stage III, 37.5% (6 of 16 cases); and stage IV, 13.3% (2 of 15 cases). Although these differences were not statistically significant, a trend was apparent for more advanced patients to show negative LIF activity. In colon cancer, the LIF positive rates were: stage I plus stage II, 7 of 10 cases and stage III plus stage IV, 3 of 10

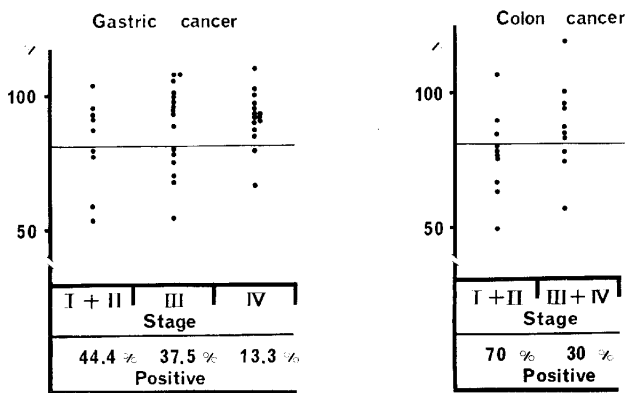


Fig. 4. LIF activity of patients with gastric cancer and colon cancer.

cases. This difference was not statistically significant but the same trend toward negative LIF was present with an advance in cancer.

Correlation between PPD skin test and DNCB test. Both the PPD skin test and DNCB test were conducted on 46 cancer patients. A significant difference was not present in the responses to these tests (Table 1).

TABLE 1. RELATIONSHIP BETWEEN PPD SKIN TEST AND DNCB TEST

	PPD skin test		Total
	Negative	Positive	
DNCB test			
Negative	5	9	14
Positive	7	25	32
Total	12	34	46

Correlation between LIF activity, MIF activity and DNCB test. No case responded positively to LMIT when responding negatively to the DNCB test (0 of 6 cases), but 55.5% of cases (6 of 11 cases) responded positive to both the DNCB test and LMIT (Fig. 5). A significant difference ($p < 0.05$) was present between

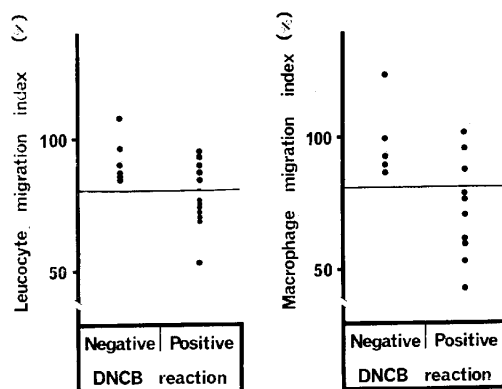


Fig. 5. Relationship between DNCB test versus LIF activity and MIF activity.

the DNCB positive-LIF positive group and the DNCB negative-LIF positive group. In cases negative to the DNCB test, the MIF activity was not positive (0 of 5 cases), and in those positive to the DNCB test, the MIF positive rate was 70% (7 of 10 cases). This difference between the DNCB positive-MIF positive group and DNCB negative-MIF positive group was statistically significant ($p < 0.05$).

Correlation between LIF activity, MIF activity and PPD skin test. In patients negative to the PPD skin test, no case showed positive LIF activity (0 of 5 cases), and in those positive to the PPD skin test, the positive rate of LIF activity was 46% (6 of 13 cases) (Fig. 6). This difference between the PPD positive-LIF

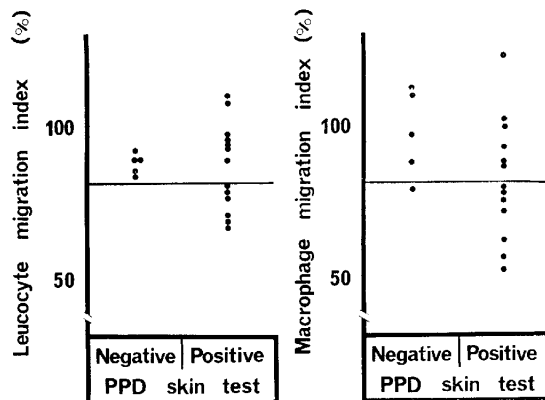


Fig. 6. Relationship between PPD skin test versus LIF activity and MIF activity.

positive group and the PPD negative-LIF positive group was not significant. On the other hand, 1 of 5 cases showed positive MIF activity and negative PPD skin test. The MIF positive rate in those positive on the PPD skin test was 54% (7 of 13 cases). This difference between the PPD positive-MIF positive group and the PPD negative-MIF positive group was not significant.

Changes in LIF activity before and after operation in gastric cancer patients. In 11 gastric cancer patients (stage III and IV), LIF activity was measured before and after the operation (Fig. 7). There were three cases of positive activity

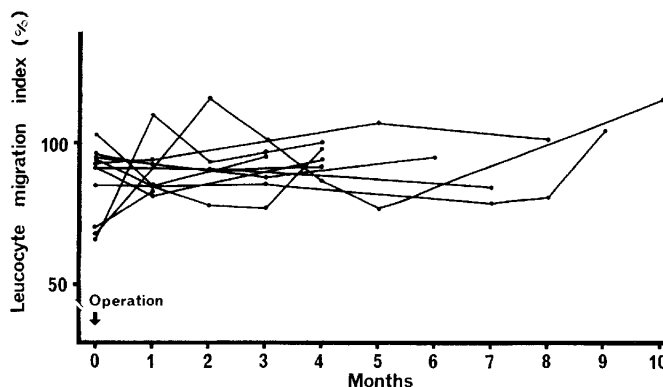


Fig. 7. Follow-up of LIF activity in gastric cancer patients.

before the operation that turned negative after the operation, and all three had successful operations. On the other hand, there were eight surgery cases that turned from negative to positive or pseudopositive or that showed no changes, and five of them had severe metastasis.

DISCUSSION

LMIT was first described by Sjøborg and Bendixen (5) in 1967, though the assay procedure was not appropriate for routine examination. Federlin *et al.* (18) developed a micro-capillary method, and Carpenter *et al.* (19) and Clausen (10) developed an agarose plate test. The presence or absence of inhibition of LIF activity correlated very well with the PPD skin test, but the degree of LIF activity inhibition did not correlated so well with the strength of the skin test. Clausen (20) and Astor *et al.* (11) stated that with the PPD as well as streptokinase-streptodornase and candida, the skin test results correlated not so quantitatively as qualitatively with the LMIT results.

Rich and Lewis (21) discovered that cell migration from tissue specimens of spleen and lymph-node of guinea pig sensitized with BCG was inhibited in the presence of tuberculin. George and Vaughan (17) observed that the migration of peritoneal cells prepared from BCG sensitized guinea pig and packed in a capillary tube was inhibited with PPD. Thor *et al.* (22) and Rocklin *et al.* (23) found that MIF liberated from human lymphocytes also inhibited the migration of heterogenous guinea-pig macrophages. We now have a well-established method for measuring MIF activity. It was reported that MIT was useful for specific assay of tumor immunity in animals (24-26). Moreover, Akiyama (4) reported that MIT was useful for diagnosis and prognosis of malignant tumor. We studied the correlation between LIF and MIF activity in cancer patients to determine whether LMIT could be used to investigate tumor immunity. A positive correlation was shown between LIF activity and MIF activity in patients with gastric cancer or colon cancer, with few exceptions. LMIT was thus thought to closely reflect tumor immunity. Rocklin (27) reported that LIF had a molecular weight of 69,000, and that it was non-dialyzable, heat stable at 56°C for 30 min and inhibited the migration of human and guinea-pig polymorphonuclear leucocytes, but that it did not inhibit the migration of human monocytes and guinea-pig macrophages. It differed from MIF which has a molecular weight of 35,000 to 55,000, and MIF inhibited the migration of human monocytes and guinea-pig macrophages (28). It remains unclear whether LIF and MIF are identical biochemically, though clinically both apparently show the same biologic function.

We observed that the positive rate of LIF activity decreased along with the advance of cancer. The LIF activity was reported to decrease with the advance in stage in malignant melanoma (29), mammary cancer (30, 31) and colon cancer

(32). In gastric cancer there has been no report on LMIT, but studies on MIT have been made by Orita *et al.* (33, 34), and it was reported that MIF activity decreased along with the progress of cancer.

We observed some cases showing enhanced migration. Weisbart *et al.* (35) reported a new lymphokine in β -fraction, migration enhancement factor, which accelerated the migration of leucocytes. The precise properties of such lymphokine require further studies in the future. Wolberg (36) suggested that the non-specific toxicity of the tumor extracts might play a role in the inhibition of leucocyte migration. However, we could not find non-specific cytotoxicity against normal leucocytes at a concentration of 400 μ g of protein/ml. A useful antigen was prepared containing specific tumor antigen by the ultrasonication method. However, methods for extracting tumor specific transplantation antigen are not yet perfected, and further improvements are required.

PPD skin test and DNCB test gave low responses as the cancer advanced. Hughes and Mackay (37) reported with PPD skin tests that the positive rate was 52% in cancer patients and 81% in control patients. On the other hand, Waldorf *et al.* (38) stated that the PPD skin test in aged person was 58% positive. Thus, in judging the test values it is necessary to take into consideration the patient's age. DNCB does not exist in nature so that there is no danger of natural sensitization. Eilber and Morton (39) stated that 93% (27 of 29 cases) of cancer patients who failed to react to DNCB were inoperable or developed early recurrence, whereas 92% (50 of 54 cases) of patients who reacted to DNCB were free of disease for 6 months.

Positive correlations were found between LIF activity, MIF activity and DNCB test. In DNCB positive cases LIF and MIF activities were significantly positive. In positive PPD skin test cases, the LIF and MIF activities tended to be positive. These results suggest indirectly that the LIF and MIF activities decreased with the advance of cancer. The skin test with tumor extract antigen has not been used in Japan, but in other countries it was reported that about 30% of patients with solid cancers showed positive responses, and the results coincided roughly with the clinical findings (40-42). This method has a promising future as a research tool for measuring specific cell-mediated immunity *in vivo*.

We could roughly divide gastric cancer patients before and after surgery into two groups by LIF activity: one group changed from positive to negative and the other group changed from negative to positive. Patients in the former group showed favorable response to surgery, and most patients in the latter group were not improved by surgery. Akiyama (4) reported similar results with various cancer patients. Patients turning from positive to negative after the operation had successful operations, but patients turning from negative to positive did not have successful operations. Other investigators stated that patients with positive

LIF activity turning negative after the operation had successful operations for colon cancer (43) and for breast cancer (44). We are now continuing to collect more data on additional subjects and these further results will be reported in the future.

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