Superoxide Assay-Leukocyte Adherence Inhibition Test and a Soluble Factor Which Stimulates the Adherence of Macrophages

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ABSTRACT
A modified method for the leukocyte adherence inhibition test is described. Peritoneal cells from immune guinea pigs or peripheral mononuclear cells from cancer patients were incubated with immunizing antigen or tumor extract in a 4-mm-wide glass microcell for spectrophotometer analysis. Instead of visual cell counting, the cells adherent to the bottom of the microcell were stimulated with cytochalasin E and wheat germ agglutinin, and the amount of the superoxide \( \text{O}_2^- \) generated from the adherent macrophages or monocytes was assayed. Antigen-specific adherence inhibition of peritoneal cells of the immunized guinea pigs was detected 2 weeks after immunization. In contrast, cell adherence was stimulated after 3 weeks. It was shown that a soluble factor was responsible for the adherence stimulation and that nonadherent cells were necessary for its production. Peripheral mononuclear cells of 70% of the tumor-bearing lung cancer patients reacted to the lung tumor extract (9 adherence inhibitions and 7 adherence stimulations per 23 patients). Twenty-five % (3 of 12) of tumor-free patients showed positive reactions, all with adherence stimulation. Of the 12 healthy donors, 3 cases of pneumonia, one case of angiosarcoma of the left flank, one case of hemangio-pericytoma of the mediastinum, and 8 cases of breast cancer, none reacted with the lung tumor extract (9 adherence inhibitions and 7 adherence stimulations per 23 patients). Twenty-five % (3 of 12) of tumor-free patients showed positive reactions, all with adherence stimulation.

INTRODUCTION
Since the LAI\(^3\) test was first reported by Halliday and Miller (4), the assay has been performed using a variety of different techniques, including the use of hemocytometer, tubes, capillary, and microplates (4, 5, 9, 19). All of these methods, however, involve the steps of visual cell counting which introduce variability into the procedure and demand technical dexterity. Pierce et al. (16) and Russo et al. (17) modified the test for measurement of adherent cells with radioisotope, but those methods are limited in use because they require a radioisotope. Recently, Leveson et al. (7) and Goldrosen et al. (3) modified the method of Holt by using an automated differentiating scanning system for cell counts and reported excellent results for early diagnosis of cancer patients. We have developed a new method for the LAI test which does not require visual cell counting and is technically simple. Experiments on guinea pigs demonstrated the usefulness of the method for detection of cell-mediated immunity. We assayed the superoxide anions generated from peritoneal macrophages of guinea pigs and human peripheral monocytes. Macrophages, monocytes, and polymorphonuclear leukocytes release superoxide anions accompanying phagocytosis-associated metabolic changes. These metabolic changes are also induced without the ingestion of any particles by some membrane-perturbing agents such as CYE, D, phorbol myristate acetate, and WGA (11). The combined treatment of these cells with CYE and WGA markedly stimulates the production and the release of superoxide anions, and it is possible to detect superoxide anions released from as few as \( 1 \times 10^5 \) human peripheral monocytes (13).

Recently, Noonan reported the adherence stimulation of peripheral mononuclear cells of cancer patients by tumor antigen after chemoinmunotherapy (15). She also demonstrated a soluble factor which stimulated the adherence of leukocytes in the supernatant of leukocytes cultured with tumor extract. We also detected a biphasic phenomenon of LAI and leukocyte adherence stimulation in the experiments on immune guinea pigs and lung cancer patients. The above evidence may show that the phenomenon of adherence stimulation represents cell-mediated immunity as does adherence inhibition.

MATERIALS AND METHODS
Reagents. CYE and cytochrome c were obtained from Sigma Chemical Co. (St. Louis, Mo.), and WGA was from Pharmacia (Uppsala, Sweden). CYE was dissolved in dimethyl sulfoxide. The PPD was a kind gift from Dr. T. Koga, Department of Biochemistry, Faculty of Dentistry, Kyushu University.

Cell Preparation. Normal peritoneal cells were collected from Hartley guinea pigs weighing 400 to 500 g. Immune peritoneal cells were from guinea pigs immunized with 1 mg of egg albumin in complete Freund's adjuvant into 2 hind foot pads and 4 sites on the back. The guinea pigs were anesthetized with ether and given injections of 100 ml HBSS. The solution was withdrawn and collected in glass tubes in an ice bath. The tubes were centrifuged at 300 \( \times \) g for 6 min. The cell pellet was washed twice with 10 ml HBSS and finally diluted with Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) containing heat-inactivated 10% FCS to a concentration of 2 \( \times \) 10\(^5\) cells/ml. Purification of macrophages from peritoneal cells was performed as described in a previous paper (20). Briefly, the peritoneal cells were incubated at 37\(^\circ\) in 90-mm (diameter) glass dishes in HBSS containing heat-inactivated 10% FCS. After 45 min of incubation, the dishes were shaken well, and nonadherent cells were discarded. The dishes were washed twice with Krebs-Ringer phosphate buffer.
and refilled with Ca²⁺-free buffer. Then, the adherent cells were collected by repeated pumping with a pipet. At least 99% of the adherent cells thus obtained phagocytosed sheep RBC coated with a subagglutinating dose of rabbit anti-sheep RBC antibody. The suspension of lymph node cells was prepared by teasing lymph nodes and filtering the cells through a 200 mesh stainless steel sieve. The cell suspension was centrifuged at 300 × g for 10 min. The cell pellet was washed twice with 10 ml of HBSS and finally diluted with Medium 199 containing 10% heat-inactivated FCS, 50 units penicillin, and streptomycin (50 μg/ml) to a concentration of 1 × 10⁶ cells/ml.

Human peripheral blood mononuclear cells were prepared from 10 ml of heparinized venous blood according to the method of Boyum (1). Ten ml of blood were diluted 1:2 in HBSS, carefully layered onto Ficoll-Hypaque solution, and centrifuged at 500 × g for 30 min at 20°. The mononuclear cell layer at the (Ficoll-Hypaque)-blood interface was collected and centrifuged at 300 × g for 10 min. The cells were washed twice with 10 ml of HBSS and finally diluted with Medium 199 at a concentration of 2 × 10⁶ cells/ml. Thus, the obtained cells contained less than 1% polymorphonuclear leukocytes as determined by microscopic examination of Giemsa-stained cells.

**Antigen Preparation.** The tumor extract used for human experiments was prepared by the 3 M KCl extraction method as described by Meltzer et al. (10). Tissue samples were collected fresh from 5 patients with lung cancer undergoing surgical resection of the tumors (2 adenocarcinomas and 3 squamous cell carcinomas). Each sample was washed with HBSS, weighed, and placed in a blender containing 5 ml of 3 M KCl per g of sample. The extract was blended for 30 sec and placed on a magnetic stirrer for 24 hr at 4°. The extract was then centrifuged at 20,000 × g for 1 hr, and the supernatant was dialyzed with Medium 199 for 6 hr and then concentrated in a Visking tube with negative pressure. Some aggregated material was then removed by centrifugation at 30,000 × g for 30 min. Protein concentration of the extract was determined by the method of Lowry et al. (8), and the solutions were pooled and diluted to 2 mg of protein per ml and stored at -20°. Normal tissue extract was similarly prepared from the normal lung tissues.

**Preparation of MIF-rich and Control Supernatant.** Lymph node cells from guinea pigs immunized with 1 mg of Mycobacterium tuberculosis 3 weeks previously were cultured with or without PPD (20 μg/ml). After 24 hr of incubation, the supernatants were collected. The migration inhibition test was performed by the indirect capillary method described by David (2).

**LAI Technique.** Aliquots of 0.5 ml of peritoneal cell suspension (2 × 10⁶ cells/ml) from guinea pigs or mononuclear cells from human venous blood (2 × 10⁶ cells/ml) were placed in a microcell. Antigen (0.2 ml) either from tumor tissue extract or from normal tissue extract was added to each microcell. The mixture was brought to a final volume of 0.9 ml by the addition of an appropriate volume of Medium 199. As shown in Chart 1, the microcells were incubated in a horizontal position at 37° in a humidified atmosphere with 5% CO₂ in air. After 2 hr of incubation, the microcells were inverted for 10 min, and the medium was aspirated by Pasteur pipet. The microcells were washed twice with 1.3 ml of HEPES buffer to remove the nonadherent cells. The adherent cells were exposed to 1 ml of the medium (HEPES buffer, containing 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 100 μM ferricytochrome c) and assayed by their capacity to generate superoxide (O₂⁻). The assay was performed as described by Nakagawara et al. (13). Briefly, the microcell was preincubated at 37° for 5 min and CYE (5 μg/ml) was added. After incubation for 10 min, WGA (50 μg/ml) was added, and the reduction of ferricytochrome c in the medium was measured continuously by a 2-wave-length spectrophotometer (Hitachi 556; Hitachi Ltd, Tokyo, Japan) at 550 to 540 nm. The amount of reduced cytochrome c was calculated using a molar absorption coefficient of 19,000. An adherence index was calculated as follows:

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\text{Adherence index} = \left( \frac{\text{Generated } O_2^- \text{ from adherent cells cultured with } \text{tumor extract}}{\text{Generated } O_2^- \text{ from adherent cells cultured with normal tissue extract}} - 1 \right) \times 100
\]

for human experiments. In experiments on guinea pigs, aliquots of immune antigen or cultured supernatant of lymph node cells were used instead of tumor extract. The same volume of medium was used as control in place of normal tissue extract. Student’s t test was used to obtain the degree of significance of difference between the reactivities of normal and immune guinea pigs.

**Patients and Control Donors.** Patients included 35 cases of lung cancer, one angiosarcoma of the left flank, one heman-giolippericytoma of the mediastinum, 10 breast cancers, 8 active lung tuberculosis cases, and 3 pneumonias, all admitted or previously admitted to the Kyushu Cancer Center Hospital. Twenty-three (15 adenocarcinomas, 7 squamous cell carcinomas, and one small cell carcinoma) of 35 lung cancer patients were tumor bearing, and 12 (5 adenocarcinomas, 5 squamous cell carcinomas, one small cell carcinoma, and one large cell anaplastic carcinoma) were clinically tumor free more than 3 months after surgical resection. Diagnosis of all the cancer patients was confirmed by histological examination of the tumors. Healthy donors were staff in our laboratory or in other departments of the Kyushu Cancer Center Hospital.

**RESULTS**

**Development of Cell-mediated Immunity to Immune Antigen.** Guinea pigs were sacrificed at 0, 7, 14, 21, and 28 days after immunization with egg albumin in complete Freund’s adjuvant. The adherence index of peritoneal cells incubated with egg albumin was calculated using the O₂⁻ assay system. Significant adherence inhibition was detected 2 weeks after immunization. This inhibition was caused specifically by immune antigen (egg albumin) but not by bovine serum albumin. The reproducibility was also ascertained by 3 individual exper-
Experiments, all with significant adherence inhibition. At 3 weeks after immunization, the adherence of peritoneal cells was strongly enhanced, and this enhancement was observed also with specific immune antigen. Response to booster immunization was tested after reimmunization 2 weeks after the primary dose. Adherence inhibition appeared at 1 week after reimmunization, and then adherence stimulation followed after 2 weeks (Chart 2a).

The effect of antigen dose was tested for adherence stimulation and inhibition. Peritoneal cells obtained 1 week after reimmunization were used for the detection of adherence inhibition, and those obtained 4 weeks after primary immunization were used for the detection of adherence stimulation. The 100-ng to 10-μg antigen concentrations tested were almost equally effective in inducing both reactions (Chart 2b).

Effect of MIF-rich Supernatant on the Adherence of Peritoneal Cells. To study the mechanisms of adherence stimulation, we studied the effect of the supernatant obtained after incubation of immune lymph node cells with antigen in vitro on the adherence of peritoneal cells from nonimmunized guinea pigs. The supernatant was obtained from the guinea pigs 3 weeks after immunization with 1 mg of M. tuberculosis when the adherence stimulation appeared most strongly. This supernatant had the capacity to inhibit the migration of macrophages by more than 50% (data not shown). The supernatant was added to the normal peritoneal cell suspension in a microcell and incubated for 2 hr. As shown in Chart 3a, the supernatant stimulated the adherence of normal peritoneal cells. The control supernatant which was obtained from the cultured lymph node cells without antigen had no effect on the adherence of the cells, nor did it show any MIF activity. The effect of the supernatant on adherence stimulation was time dependent. It appeared after 2 hr of incubation, and over 100% of the adherence index was increased.

Chart 2. a, Development of cell-mediated immunity to antigen. Guinea pigs were sacrificed 0 to 4 weeks after primary immunization (— — —) or 1 to 3 weeks after reimmunization (— — —) with egg albumin in complete Freund's adjuvant containing 1 mg M. tuberculosis. Peritoneal cells were incubated with egg albumin (100 μg/ml) (C3) or PPD (C4), and the effect of antigens on the adherence of cells was assessed by the O2·− assay-LAI test. Points, mean of 3 experiments; bars, S.E. The significance of difference between normal and immune guinea pigs is: *, p < 0.05; **, p < 0.01. b, effect of antigen doses on adherence of immune peritoneal cells. Peritoneal cells prepared from guinea pigs 4 weeks after primary immunization or 1 week after reimmunization with egg albumin (alb.) were incubated with varying concentration of antigen, and the effect of the antigen doses on adherence of the cells was assessed. Points, mean of 3 experiments; bars, S.E.; **, p < 0.01.

Chart 3. a, Effect of MIF-rich supernatant on the adherence of peritoneal cells. Normal peritoneal cells (1.5 × 10⁶ cells/850 μl) were incubated with MIF-rich supernatant (50 μl) for 2 hr, and the effect of the supernatant on adherence of the cells was assessed. As a control, the effect of the supernatant of lymph node cells (L.N.C.) cultured without PPD was also tested. Points, mean of 3 experiments; bars, S.E. p compared with respective control value (medium plus PPD); b, time course of adherence of peritoneal cells incubated with MIF-rich supernatant. Points, mean of 3 experiments; bars, S.E.
adherence stimulation was detected after 6 hr (Chart 3b).

**Effect of Immune Antigen and MIF-rich Supernatant on Generation of O₂⁻.** Peritoneal cells from guinea pigs immunized with complete Freund's adjuvant 3 weeks previously were incubated with PPD (10 µg/ml) or MIF-rich supernatant of cultured lymph node cells for 2 hr at 37° in glass tubes. After repeated washing with HEPES buffer, 3 to 12 x 10⁶ cells were placed in the microcell and O₂⁻ generation was assayed. As shown in Chart 4, O₂⁻ generation increased in proportion to the cell concentration. Neither the immune antigen nor the supernatant had any direct effects on O₂⁻ generation of peritoneal cells. O₂⁻ generation of normal guinea pig peritoneal cells and human peripheral mononuclear cells was also in proportion to the cell concentration.

**Cell Population Which Influences Adherence Stimulation.** To ascertain what type of cell participates in the production of the soluble factor that stimulates the adherence of peritoneal cells, nonadherent cells were removed from the immune peritoneal cells. As shown in Chart 5, the adherence stimulation almost completely disappeared after the nonadherent cells were removed. The result shows the requirement of the nonadherent cells in the production of adherence-stimulating soluble factor.

**Results with Lung Cancer Patients.** Reactivity of mononuclear cells from 35 lung cancer patients before or after surgical treatment was tested against a lung tumor extract by using the O₂⁻ assay system. Antigen was prepared as described in "Materials and Methods." Dose response of this reaction was studied prior to the test by using the tumor extract containing 50 to 600 µg protein per ml. The optimal concentration of the extract was 400 µg/ml, and no reactivity was detected below 100 µg/ml. Since this antigen dose slightly stimulates the adherence of mononuclear cells from control donors, the normal range of adherence index was limited to between -10 and +20. Blood samples of patients were coded with numbers, and the test was performed without the operator's knowing the diagnosis of the blood donors. As shown in Chart 6, the 12 healthy donors, 3 pneumonia patients, and 10 patients with cancer of other organs did not react to the lung tumor extract, and only one of 7 lung tuberculosis patients reacted to the extract with positive adherence stimulation. Sixteen of 23 tumor-bearing lung cancer patients reacted to the extract with 9 showing adherence inhibition and 7 showing adherence stimulation. Only 3 of 12 patients who were clinically tumor free after the operation were reactive, all with adherence stimulation.

**DISCUSSION**

The LAI test was originally described by Halliday and Miller (4) and used as a rapid and simple test of cell-mediated immunity. However, some technical problems, remain including visual cell counting which introduces some variability into the procedure and demands technical dexterity (4). To overcome these problems, the computerized tube LAI assay and automated microassay were described (7, 18). However, these methods are applicable only to laboratories equipped with an automatic cell counter. Here we demonstrated a new method for the LAI test which does not need visual cell counting and is technically simple. After incubation of cells in a microcell for spectrophotometric analysis, nonadherent cells were easily removed by inverting the microcell for 10 min and washing with a buffer. Instead of cell counting, superoxide (O₂⁻) release

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**References**

from adherent cells upon stimulation with lectin was assayed. Nakagawara developed a new technique for O$_2^-$ assay by using CYE and WGA as stimulants for generation of O$_2^-$ from monocytes, macrophages, and polymorphonuclear leukocytes (12, 13). Using this technique, it was possible to detect the O$_2^-$ generated from as few as 1 x 10$^6$ human monocytes. As shown in Chart 4 and reported by Nakagawara (13), the amount of the released O$_2^-$ paralleled the cell number.

The reproducibility and immunological specificity of our methods were ascertained by experiments with guinea pigs. Adherence of peritoneal cells incubated with immune antigen was inhibited at 2 weeks after immunization. In contrast, adherence was stimulated after 3 weeks. Holt et al. (6) reported the phenomenon of adherence stimulation of murine peritoneal cells incubated with immune antigen at 35 days after immunization. The data of Leveson also suggested a biphasic phenomenon of adherence inhibition at 15 days and stimulation at 21 days in experiments with tumor-inoculated mice (7). Recently, Noonan et al. (15) reported that a soluble factor is responsible for adherence stimulation. We also detected leukocyte adherence-stimulating activity in the supernatant of lymph node cells cultured with immune antigen. The supernatant containing MIF revealed adherence-stimulating activity when appropriately diluted, and the stimulating effect was manifested as early as 2 hr of incubation. These results agree with that of Nathan et al. (14), who also detected macrophage adherence-stimulating factor in culture supernatants of immune guinea pig lymph node cells. One difference between our experiment and theirs is that we detected the activity of the factor as early as 2 hr of incubation, whereas they reported that over 24 hr of incubation was necessary before the appearance of the activity. This may be due to varying sensitivity of the methods or difference in the macrophage source used. We used uninduced peritoneal cells, but they used peritoneal exudate cells induced by liquid paraffin. It is not known whether leukocyte adherence-stimulating factor and MIF are the same molecule or not. Macrophage adherence stimulation diminished when the nonadherent cells were removed from peritoneal cells. From these results, it appears that leukocyte adherence stimulation is mediated by soluble factor and that the nonadherent cells are responsible for the production of the factor.

Lung cancer patients were tested with the new method. The phenomena of LAI and stimulation were observed in tumor-bearing patients, but only leukocyte adherence stimulation was detected in postoperative tumor-free patients.

In conclusion, we may say that not only LAI but also leukocyte adherence stimulation are meaningful phenomena for detection of cell-mediated immunity in the cancer patient. The clinical significance of cellular response measured by our different assay remains unsettled, but the new LAI test shown here should prove to be useful in studying tumor immunity.

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References

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