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Innate-immune Therapy for Lung Carcinoma Based on Tissue-macrophage Activation with Lipopolysaccharide

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Abstract. *Background: Over the last decade, tumor-specific antigens have been discovered, but so far it has not been possible to use them as part of an effective acquired immunotherapy. This failure may be due to the fact that the expression of the MHC class 1 is low and in lung cancer cells is heterogeneous. Therefore, it may be advantageous to develop techniques that activate the antitumor mechanism of the innate immune system. An experimental model was developed for testing lung cancer therapies that are based on the stimulation of macrophages, which then activate innate immunity. Materials and Methods: A549, a human lung adenocarcinoma cell line, was co-cultured with a rat macrophage cell line (NR8383), or a human macrophage cell line (THP 1) at the ratios of 1:1 or 1:5. The experiments were performed with lipopolysaccharide (LPS) or in its absence. The cytotoxicity rate to A549 cells was estimated over time using a dye-uptake method and the amount of lactate dehydrogenase released was measured. The amount of nitric oxide (NO) induced in the medium was assayed, because it may be a candidate as a useful cytotoxic factor. Results: High cytotoxicity was observed to A549 cells when co-cultured with NR8383 cells in the presence of LPS. This effect was not observed in the absence of LPS. Similar results, although to a lesser extent, were observed when A549 cells were co-cultured with THP-1 cells. A high concentration of NO was measured in the co-culture medium of A549 cells and NR8383 cells when LPS was present. Conclusion: The induction of cell death in lung cancer cells occurred after contact with macrophages that had been activated by LPS. The NO that was produced by macrophages in response to LPS was responsible for some of this effect.*

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Worldwide, lung cancer is the major cause of death by malignant neoplasms and was responsible for 15.4% of the cancer deaths in 2002. Therapy for lung cancer consists of surgical therapy, chemotherapy, radiation therapy and immunotherapy. However, none of these therapies are highly effective, and new innovative therapies are still required. Of the different types of lung cancers, small cell cancer is known to show the best responsiveness to chemotherapy. However non-small cell lung cancer, which makes up 80% of the lung cancer cases in Japan, shows resistance to chemotherapy and only surgical therapy is effective (1, 2). Over the last decade, there has been active research into tumor-specific antigens, which are the target of acquired immunity, and many antigens have been discovered (MAGE and BAGE families, gp100, SART-1, Tyrosinase, MUC-1, HER-2/new, *etc.*) (3-5). However, effective tumor-specific antigens have not yet been found for lung cancer. This is probably because the expression of MHC class 1 is low and the expression of specific antigens is not regular (6). Because innate immunity and acquired immunity utilize different mechanisms for expressing antitumor effects, activating innate immunity may be useful in reinforcing the effect of acquired immunity.

Monocytes, macrophages, neutrophils and NK cells are the cells that implement innate immune functions (7-10). Macrophages exist in all animals and in every kind of tissue. They are preserved phylogenetically and are in control of the innate immune process (11, 12). They possess essential functions to initiate acquired immunity such as antigen presentation, production of cytotoxic-active oxygen or nitric

oxide (NO), induction of differentiation of dendritic cells, activation of T cells and secretion of the cytokines that regulate acquired immunity (13). Furthermore, abundant macrophages invade the local area of a cancer, and are known to either exclude cancer cells or promote the growth of cancer cells (14, 15). Therefore, macrophages are considered to be suitable targets to activate the innate immunity for the purpose of cancer immunotherapy.

As an example of cancer therapy using innate immunity, Fidler *et al*. treated metastatic lung cancer by activating macrophages using liposomes (16-18). They reported the antitumor effect of a macrophage-activating reagent incorporated into muramyl dipeptide by intravenous administration. However, the report dealt with metastatic lung cancer, and they reported that stimulated tumor-associated macrophage (TAM) invaded cancers. The targeting of lung cancer with alveolar macrophages (19) was not investigated.

The goal of this study was to establish a therapy for lung cancer that utilizes macrophages. To achieve this, it is necessary to find ways to activate alveolar macrophages so that they kill lung cancer cells. We have shown that the tissue specificity of macrophages greatly influences the expression of functions. For example, a comparison of tumor necrosis factor (TNF) production by alveolar, intestinal and peritoneal macrophages showed that intestinal macrophage produce relatively little TNF (20). Also, it was reported that the recognition of lipopolysaccharide (LPS) by Kupffer cells (macrophages residing in the liver) does not depend on CD14, which is different from the response of peritoneal macrophages (21). Macrophages differentiate in the bone marrow to a certain stage and are then released into the peripheral blood. They infiltrate every body tissue after circulation in the blood. Depending on the tissue, the macrophages differentiate and develop different characters and become tissue-specific macrophages (13). Therefore, it is essential to consider tissue specificity when developing a therapy for lung cancer that depends on the action of macrophages (22).

NR8383 is a cell line derived from normal rat alveolar macrophages, which has the property of proliferation during culture (23, 24). These cells have maintained the functions of normal alveolar macrophages with respect to phagocytotic activity and production of TNF (with or without LPS). The goal of this study was to establish a model for testing innate immunotherapies for lung cancer. In these experiments, cytotoxic effects during the co-culture of human lung cancer cells (A549) and normal rat alveolar macrophages (NR8383) in the presence of LPS were examined.

Materials and Methods

Culture of cells. A rat alveolar macrophage cell line, NR8383, was purchased from the American Type Culture Collection (Manassas, VA, USA; ATCC No. 2192) and was maintained in Ham F-12K medium (Invitrogen, Tokyo, Japan) supplemented with 15% FCS (Invitrogen) and 60 μg/ml ampicillin, at 37˚C in a 5% CO_2 atmosphere. A human monocytic leukemia cell line, THP-1, was purchased from the Japanese Collection of Research Bioresources (Japan, JCRB0112) and was grown in RPMI 1640 medium supplemented with 10% FCS, at 37˚C in a 5% CO_2 atmosphere. A human lung adenocarcinoma cell line, A549, was donated by TAIHO Pharmaceutical Co., Ltd. (Tokushima, Japan). These cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FCS and 100 μg/ml kanamycin, at 37˚C in a 5% CO_2 atmosphere.

Co-culture of macrophages and carcinoma cells. THP-1 cells were differentiated by treatment with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma, P-8139) at 37˚C for 24 hours. The cells were collected and centrifuged at 250 x g for 5 minutes. The pelleted cells were washed with phosphate-buffered saline (PBS) at room temperature. After washing three times, the cells were resuspended in RPMI 1640 medium with 5% FCS. A549 cells were plated in 48-well plates at a density of $1x10^4$ cells/well and incubated in Ham F-12K medium or RPMI 1640 medium with 5% FCS, for 4-6 hours at 37˚C. After the A549 cells had adhered to the plate, NR8383 cells or differentiated THP-1 cells were added to each well at a ratio of 1:1 or 5:1 (macrophages: A549 cells), with or without 1 μg/ml of *Escherichia coli* LPS (LPSe) or *Pantoea agglomerans* LPS (LPSp), and were then incubated for 1, 3 and 5 days at 37˚C.

Evaluation of cytostasis. After co-culture, each well was washed with sterile saline to remove the macrophages and dead A549 cells. The surviving A549 cells were stained with 0.1% crystal violet/MeOH at room temperature for 10 minutes. The plates were read on a microplate reader at an absorbance of 570 nm (BIO-RAD, Model 550, Tokyo, Japan). Cytostasis was calculated as a percentage. The absorbance of surviving A549 cells in the absence of macrophages (control absorbance) was set at 100%, and the experimental absorbance was divided by the control absorbance.

Evaluation of macrophage cytolysis. To evaluate macrophage cytolysis, lactate dehydrogenase (LDH) activity was measured in the culture supernatants (LDH is a stable cytosolic enzyme, which is released upon cell lysis). The amount of LDH that had been released into the culture supernatants was measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay kits (Promega) (25, 26). LDH activity was determined with reference to the standard curve using bovine heart LDH. Cytolysis was measured as the amount of LDH released from the A549 cells. The amount released spontaneously by macrophages was subtracted from the experimental release values.

Measurement of NO production. The NO concentration was measured by a microplate assay method with Griess reagent (1% sulfanilamide, 0.3% naphthylethylene diamine dihydrochloride, 7.5% H3PO4) (27, 28). Briefly, culture supernatants (100 μl) were mixed with 100 μl of the Griess reagent. The nitrite concentration in the culture supernatant was measured at an absorbance of 550 nm 10 minutes after mixing. The nitrite concentration was determined with reference to the standard curve using sodium nitrite.

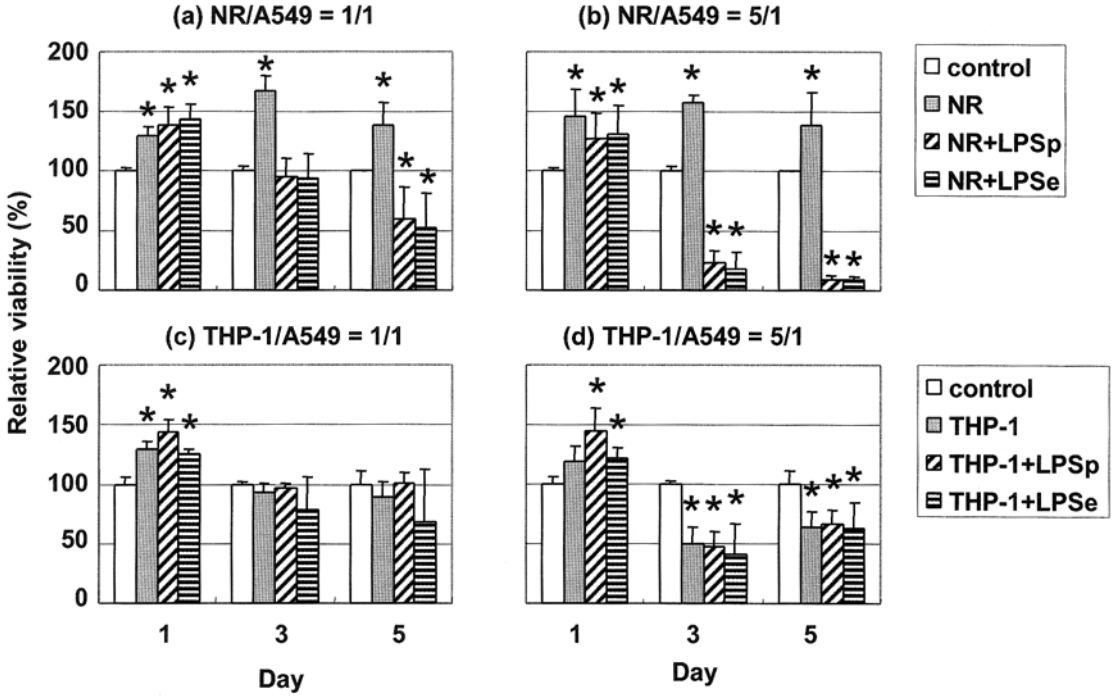

Figure 1. *Comparison of cytostatic effect during the co-culture of macrophages and lung adenocarcinoma. A549 cells were co-cultured with NR8383 cells (a, b) or THP-1 cells (c, d) in the presence or absence of LPS for 1, 3 and 5 days. The macrophage-to-A549 cell ratio was 1:1 (a, c) or 5:1 (b, d). Cytostasis was measured as relative viability (control absorbance was set at 100%) and was calculated as the experimental absorbance divided by the control absorbance. The data are expressed as means±SD of two independent experiments, each conducted in duplicate. *Significantly different ($p<0.005$) from control.*

Results

Decrease in the viability of lung cancer cells when co-cultured with macrophages. Figure 1 shows the death rate of A549 cells with time when co-cultured with macrophages (NR8383 cells or THP-1 cells) at the ratios of 1:1 or 1:5 (with and without LPS). Two types of LPS were used (LPS derived from *Eschericlia coli* (LPSe) and from *Pantoea agglomerans* (LPSp)). Neither type of LPS has a suppressive effect on the growth of A549 cells or NR8383 cells (data not shown). The results are given in terms of relative viability (%), which is the percentage of cells that remained viable when compared to single cultures of A549 cells. When A549 cells were co-cultured with NR8383 cells (alveolar macrophages) at a ratio of 1:1, the relative viability of A549 cells did not decrease unless LPS was present (Fig 1(a)). Instead, the relative viability was enhanced relative to single cultures. In co-cultures with LPSe or LPSp, a decrease in the relative viability of A549 cells was observed from day 3.

A similar result was also observed for the co-culture of A549 cells and NR8383 cells at a ratio of 1:5 (Fig 1(b)). The relative viability of the A549 cells after 5 days of co-culture with NR8383 cells at a ratio of 1:5 with LPSp or LPSe was 2.6 % or 1.8 %, respectively. The decrease in the relative viability of A549 cells was not significantly different between LPSp and LPSe. These results suggest that the suppression of cellular proliferation or induction of cell death of lung cancer cells occurs because the cells have been in contact with macrophages that have been activated by LPS.

The same experiment was done with THP-1, a human monocyte cell line, instead of NR8383 cells. At a ratio of 1:1, neither A549 cells nor THP-1 cells had a significant effect during co-culture. However, at a ratio of 1:5, the relative viability of the A549 cells with THP-1 cells and LPS showed a decline after day 3. The relative viability of the A549 cells after 5 days of co-culture with THP-1 cells at a ratio of 1:5 with LPSp or LPSe was 37.8 % or 35.9 %, respectively. The relative viability was higher than co-culture with NR8383 cells under the same conditions. This difference may be caused by the difference in the responsiveness to LPS by THP-1 cells when compared to NR8383 cells.

Induction of cell death in lung cancer cells when co-cultured with macrophages. Figure 1 shows the relative viability (percent of viable cells to total number of cells) for A549 cells when cultured alone and without LPS. Therefore, it is not clear whether the NR8383 cells or the THP-1 cells

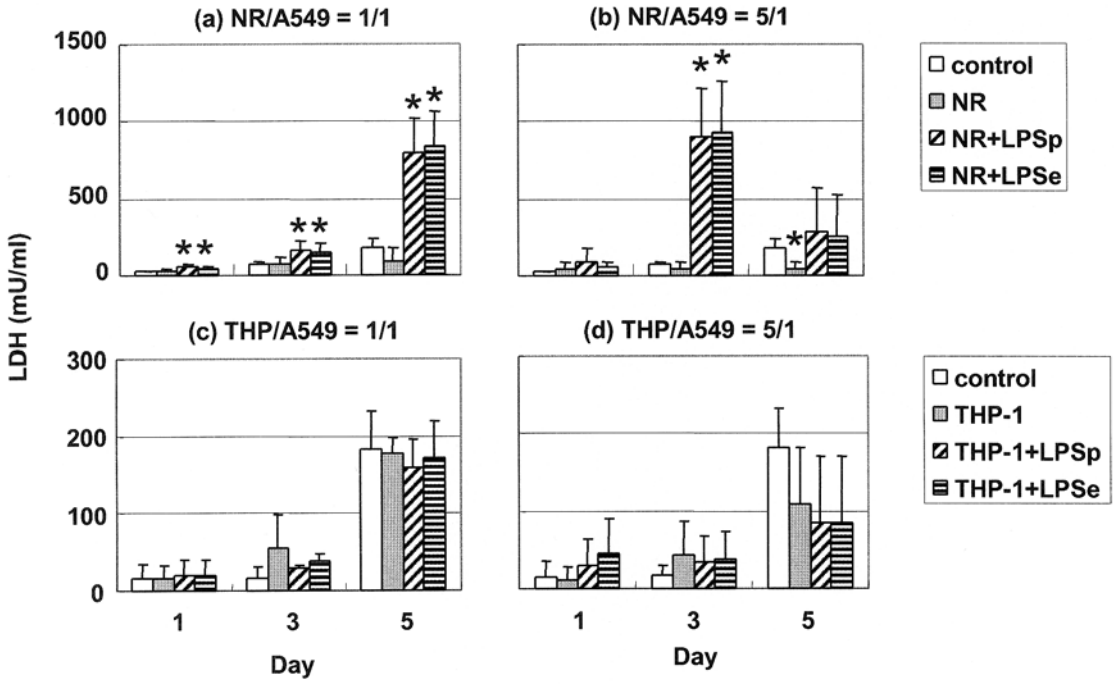

Figure 2. *Comparison of cytolytic effect during the co-culture of macrophages and lung adenocarcinoma. A549 cells were co-cultured with NR8383 cells (a, b) or THP-1 cells (c, d) in the presence or absence of LPS for 1, 3 and 5 days. The macrophage-to-A549 cell ratio was 1:1 (a, c) or 5:1 (b, d). Cytolysis was measured as the amount of LDH released from A549 cells. The macrophage spontaneous release was subtracted from the experimental release. Data are expressed as means±SD of two independent experiments, each conducted in duplicate. *Significantly different ($p<0.005$) from control.*

suppressed cellular proliferation or induced cell death in the A549 cells when in the presence of LPS.

To study the induction of cell death during the co-culture of A549 cells with macrophages, the release of the intracellular enzyme, LDH, was measured. The amount of LDH released from A549 cells was adjusted by subtracting the amount of LDH released during the single culture of macrophages (with or without LPS) from the total amount of LDH in the co-culture medium.

As shown in Figure 2 (a), during the co-culture of A549 cells with NR8383 cells (ratio 1:1), no release of LDH was observed in the absence of LPS. However, there was a significant release of LDH during co-cultures that included LPSe or LPSp (day 1 to day 5). There was a very high release of LDH in the co-culture of A549 cells and the NR8383 cells (ratio 1:5) on day 3. Excessive replication of NR8383 cells caused mortalities, and decreased the amount of LDH released from A549 cells on day 5. These results indicate that cell death was induced in A549 cells during co-culture (in the presence of LPS).

The same experiment was performed with THP-1 cells instead of NR8383 cells (Figure 2(c)(d)). The amount of LDH released from A549 cells during the co-culture with THP-1 cells (with LPS) was shown to be lower than during co-culture with NR8383 cells. This result corresponds to those in Figure 1.

NO production during co-culture with macrophages. As clearly shown by the results in Figures 1 and 2, cell death did not occur simply as a result of the co-culture of A549 cells with macrophages. Also, LPS did not induce cell death directly in A549 cells. Therefore, it is assumed that the cell deaths induced in the A549 cells during co-culture with macrophages and with LPS was caused by factors derived from macrophages that had been activated by LPS stimulation. LPS causes macrophages to secret cytotoxic factors, of which the most well-known are TNF and NO (29). As A549 cells are known to be non-responsive to TNF, only the amount of NO secreted in the co-cultures, was determined.

As shown in Figure 3 (a)(b), an increase in the amount of NO is dependent on time and the cell count of NR8383 cells in the co-culture medium of A549 cells and NR8383 cells with LPS. As A549 cells do not produce NO in response to LPS stimulation, this NO was secreted from NR8383 cells that had been stimulated by LPS. By contrast, THP-1 cells were shown to produce almost no NO after LPS stimulation.

These results show that the deaths may be due, in part, to the amount of NO secreted by NR8383 cells. However, the death of A549 cells was also observed during co-culture with THP-1 cells, even though the THP-1 cells do not produce NO. This suggests that an unknown cytotoxic factor

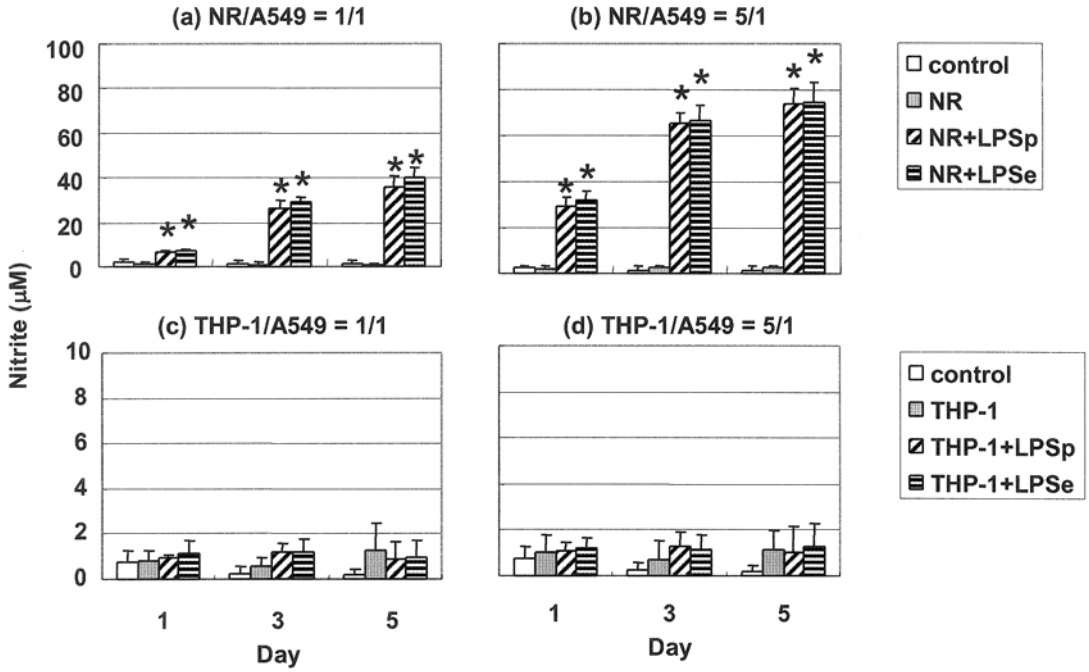

Figure 3. *Comparison of released NO concentration during co-culture of macrophages and lung adenocarcinoma. A549 cells were co-cultured with NR8383 cells (a, b) or THP-1 cells (c, d) in the presence or absence of LPS for 1, 3 and 5 days. The macrophages-to-A549 cell ratio was 1:1 (a, c) or 5:1 (b, d). The concentration of the released NO in the supernatants were measured by Griess reagent. Data are expressed as means±SD of two independent experiments, each conducted in duplicate. *Significantly different ($p<0.005$) from control.*

was secreted after the macrophages had been stimulated with LPS.

Discussion

In this study, we tested an *in vitro* model of lung cancer and an anticancer immunotherapy that relied on the activation of localized innate immunity. Specifically, the cytotoxicity to lung carcinoma cells (A549 cell line) by alveolar macrophage cells (NR8383 cell line) in the presence of LPS was studied. The results showed a high level of cytotoxicity when A549 cells were co-cultured with NR8383 cells in the presence of LPS; no cytotoxicity was observed without LPS. Similar results were obtained for the co-culture of A549 cells with a human monocytes (THP-1 cell line), although the effect was smaller than that observed in the co-culture with NR8383 cells. Our results indicate that NR8383 cells showed stronger cytotoxicity to A549 cells than THP-1 cells (in the presence of LPS) (Figure 1). NR8383 cells are derived from rats and are xenotropic to A549 cells (derived from humans), so their cytotoxicity cannot be simply compared with that of THP-1 cells, which have been derived from the same species. However, generally, alveolar macrophages are known to show high responsiveness to LPS (30, 31), and this is probably the reason for the lower cytotoxicity of the THP-1 cells, which are derived from monocytes. It appears that macrophages derived from lung tissue showed higher cytotoxicity to lung cancer cells than macrophages derived from blood.

Cell death (not just suppression of proliferation) was induced in A549 cells when co-cultured with NR8383 cells or THP-1 cells in the presence of LPS (Figure 2). This result clearly demonstrates that a cytotoxic factor was secreted by the macrophages when stimulated by LPS. TNF is a major cytokine secreted by macrophages after stimulation by LPS (32), with cytotoxicity to tumor cells (26, 33). In contrast, the A549 cells employed in our experiments were non-responsive to TNF. (Apoptosis was not induced in the absence of actinomycin D (data not shown)). Therefore, the death of A549 cells when co-cultured with macrophages and LPS was not caused by TNF. A different cytotoxic factor, similar to TNF, must have been induced in response to the LPS stimulation. NO is one possibility (34-36). NO is synthesized from arginine by NO synthetase (NOS) (37). As NOS is induced with the activation of NF-κB, stimulation of LPS through TLR4 leads to NO production (38). Our results showed the presence of a high concentration of NO in the co-culture medium of A549 cells and NR8383 cells when LPS was present (Figure 3). Since the amount of this NO correlated with the cytotoxicity to A549 cells, some of the A549 cell deaths can be attributed to the presence of NO. In contrast, very little NO was detected in the co-culture medium of A549 cells and THP-1 cells.

Generally, human-derived macrophages show a low response to LPS, as measured by the production of NO (39). This is caused by the structural absence of the co-factor tetrahydrobiopterin (BH_4), which is necessary to produce NO (39). The lower cytotoxicity of THP-1 cells to A549 cells when compared to NR8383 cells may be explained, in part, by the low NO production. The fact that cell death was induced in A549 cells in co-culture with THP-1 cells, even though NO was not produced, indicates that there are other cytotoxic factors besides NO. The possibilities include Fas ligands or membrane-bound TNF (40, 41). As this was an *in vitro* study, only the direct effect of cytotoxicity to cancer cells was observed. However, *in vivo*, co-existing factors that influence other immune cells probably play important roles.

We believe we have successfully established a model in which cytotoxicity to lung cancer cells is exhibited by a normal rat alveolar macrophage cell line, NR8383. This model allows basic studies on how alveolar macrophage cells can be stimulated to kill lung cancer cells. The results obtained by this model can be easily adapted for *in vivo* studies. We also established a basic model for a therapy that relies on activating macrophages, and more studies on developing activation techniques are planned.

Current immunotherapy techniques for cancer focus on the reinforcement of acquired immunity such as the search for tumor antigens, induction of dendritic cells presenting tumor antigens and the proliferation of T cells that recognize tumor antigens. However, even more effective immunotherapies may be developed by simultaneously promoting the activation of innate immune cells.

We used LPS because this molecule possesses the highest macrophage-activating activity known, and it is an immune stimulator with the longest history of use in cancer therapy. Coley, a pioneer of the immunotherapy of cancer, used two types of bacteria (*Streptococci* sp., a Gram-positive bacteria and *Serratia marcescens*, a Gram-negative bacteria) and developed a cancer vaccine (called the Coley's vaccine), which showed an effect in the treatment of sarcoma (42, 43). Since then, over the last century, LPS has been used in cancer therapy.

It has been shown that the receptor for LPS is a Toll-like receptor (TLR4). It recognizes foreign bodies, such as bacteria, and induces pre-inflammatory cytokines that activate an innate-immunity response (44, 45). The signal mediated by TLR induces the expression of pre-inflammatory molecules and they contribute to the activation of acquired immunity, in which T cells are the main cells (45). Over the last decade, a large number of tumor-associated antigens have been identified (3-5). As a tumor cell expresses specific antigens on its surface, it is clear that activation of signal transduction from TLR can play an important role in tumor therapy as well as in the prevention of infection. From the above-mentioned points, we believe that a therapy activating alveolar macrophages with LPS can complete and increase the effect of acquired immunity by inducing the activation of the innate immune system.

Testing is currently taking place in designing TLR ligands and using them for intractable diseases including cancer. The CpG motif, abundant in the genome of prokaryotes, binds to TLR 9 and activates innate immunity (46, 47). At present, a CpG oligodeoxynucleotide (CpG ODN) is in phase III clinical trials for non-small cell lung cancer, melanoma and cutaneous T-cell lymphoma. It is also anticipated that it can be a therapeutic agent for atopitic dermatits (48, 49). Also in development as antitumor agents are TLR 7 (or a component belonging to the imidazoquinoline family ligand) and TLR 8 (50, 51). Though LPS has long been a problem clinically because it acts as an endotoxin, it may become the most effective reagent for immunotherapy as a TLR 4 ligand (52). LPS is a cell-wall component of Gram-negative bacteria (53). The structure contains a glycolipid with a molecular weight of about 2,000 (lipid A), a core sugar moiety next to KDO, and an O antigen. LPS varies somewhat according to the species of bacteria and has different molecular weights (53). For example, the average molecular weight of the LPS (derived from *Pantoea agglomerans*) used in our experiments was about 5,000 Da and was smaller than the LPS derived from *Escherichia coli*, at 20,000 Da (54). Since the binding to TLR 4 is mainly dependent on lipid A (55), no difference was observed in the induction of cytotoxicity to A549 cells in our experiments. However, as differences in the sugar moiety and molecular weight might influence the degree of absorption through the skin or mucosa, low molecular weight LPS may be more advantageous than large molecular weight LPS when manufactured as a medicine.

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