한국환경위생학회지, 제26권 제4호(2000)
Kor. J. Env. Hlth. Soc., Vol. 26, No. 4, pp 115~121(2000)

In Vitro Magnetometry, LDH Activity and Apoptosisas Indices of Cytotoxicity in Alveolar Macrophages Exposed to Cadmium Chloride

Young Chae Cho†

Department of Preventive Medicine and Public Health, College of Medicine, Chungnam National University

(Received 13 November 2000 ; Accepted 19 December 2000)

카드뮴에 폭로된 폐포대식세포의 세포독성 평가를 위한 세포자계측정, LDH활성도 및 Apoptosis

조 영 채†

충남대학교 의과대학 예방의학교실

ABSTRACT

To evaluate the cytotoxicity of cadmium compounds, this study was conducted to measure the in vitro magnetometry, LDH release and cellular apoptosis using alveolar macrophages of hamsters. A series of magnetometric measurements in cadmium-added groups showed a significant dose-dependent decay of the relaxation curves. The LDH release rates showed a dose-dependently increasing tendency as the dose gradually increased. The positive rates of apoptosis were significantly higher in cadmium-added groups than the control groups. Conclusively, the cytotoxicity increased in a dose dependent way as the concentration of cadmium added increased, which was reflected in the decay of relaxation curve in magnetometry, and increased LDH release rate and positive rate of apoptosis.

Keywords : Cytotoxicity, Cadmium, Magnetometry, LDH release assay, Apoptosis

요 약

본 연구는 여러 산업장에서 사용하고 있는 인체 유해중금속인 카드뮴화합물에 대한 세포독성을 평가하기 위해 햄스터의 폐포 대식세포를 사용하여 *in vitro* 자계측정을 시도하고, LDH 활성치 및 세포의 apoptosis 등을 측정, 검토하였다. 세포자계측정 결과 카드뮴 1 μg, 25 μg 및 50 μg첨가군 모두 대조군(PBS첨가군)에 비해 완화곡선이 유의하게 지연되었다. 카드뮴 첨가 군간에서는 1 μg첨가군보다 25 μg과 50 μg첨가군에서 완화곡선이 유의하게 지연되었으나 25 μg과 50 μg첨가 군간에는 차이가 없어 25 μg이상에서는 용량 의존적인 완화곡선이 상실되는 것을 알 수 있었다. 자화 후 2분간의 완화계수는 대조군에 비해 카드뮴 25 μg과 50 μg첨가군에서 유의하게 낮아지는 용량의존성이 높은 경향이었으나 1 μg첨가군에서는 유의성이 인정되지 않았다. LDH활성치는 카드뮴 1 μg첨가군에서 10.71%, 25 μg첨가군에서 27.21%, 50 μg첨가군에서 42.98%로 카드뮴 첨가농도에 따라 용량 의존적으로 점차 높아지는 경향이었다. 세포의 apoptosis 양성 유발율은 카드뮴 1 μg첨가군 15.3%, 25 μg첨가군 69.2%, 50 μg첨가군 69.7%로 대조군의 9.1%보다 높았으며 특히 25 μg과 50 μg첨가군은 대조군보다 통계적으로 유의한 차이를 보였다. 위와 같은 결과는 세포의 자계측정, LDH활성치 및 apoptosis 모두 카드뮴 첨가농도의 증가에 따라 용량 의존적으로 카드뮴 독성이 증가하는 것을 확인할 수 있었으며, 특히 카드뮴 농도가 25 μg이상일 때 독성 영향이 유의하였음을 시사하였다.

I. Introduction

With increasing developments in the industry of Korea, a wide variety of toxic chemicals are being used, but the evaluation of their toxicity has not been sufficiently clarified. Thus, it is acknowledged that the evaluation of safety standards of various chemicals used in workplaces need to be required.

Most of the chemicals to which we are exposed enter the interior of the body through the airways, and these are very likely to be processed by the alveolar macrophages

†Corresponding author : Department of Preventive Medicine and Public Health, College of Medicine, Chungnam National University
Tel: 042-580-8265, Fax: 042-583-7561
E-mail: choyc@cnu.ac.kr

with phagocytic capabilities. Thus, the evaluation of their effect on the alveolar macrophages is estimated to be one of effective ways to screen for environmental hazards of chemical substances. The cadmium is a noxious heavy metal which invades the interior of the worker's body through airways in the form of dust and its compounds, and causes various symptoms resulting from acute and chronic poisoning.

The currently attention-getting magnetometry initiated by Cohen(1973)[1] is the method to evaluate the influence of chemicals on the body making use of alveolar macrophages. It allows the magnetic field to be serially measured and plotted over time after external magnetization of magnetic particles retained in the lungs.

The phenomenon that the remanent magnetic field decreases rapidly just at the end of magnetization is designated as "relaxation" and this is caused by random rotation of phagocytosed iron oxide particles which have aligned in response to an external magnetic field in macrophages. Gehr *et al.*(1983)[2], Valberg(1984)[3], and Brain *et al.*(1984)[4] have found that after intratracheal instillation of iron oxide suspension, consistent magnetization of oxide particles in the lungs was achieved by application of a 500 G magnetic field and was followed by relaxation, a rapid decrease of remanent magnetic field. Valberg *et al.*(1987)[5] stated that relaxation is observed as phagosomes which have engulfed phagocytosed iron oxide particles by encircling, are randomly rotated by cytoskeletions. In Japan, Kotani *et al.*(1981)[6] first applied the magnetometry to the evaluation of the amount of dust retained in the lungs of dust workers and Nakadate *et al.*(1996)[7] evaluated the relation between the magnetometry and pulmonary function of welders.

While the enzyme release assays or morphological examinations have classically been used for evaluation of cytotoxicity, the magnetometry, an epochal method in the evaluation of alveolar cytotoxicity was developed by Aizawa *et al.*(1996)[8]. To screen for the effects of noxious chemicals on the body, they exposed alveolar macrophages obtained from lavage fluid of a smaller animal's airways, to iron oxide as the *in vitro* index of cellular magnetometry and the chemical as the sample of cytotoxicity, and serially measured the remanent magnetic field after external magnetization. They, thereby, used the degree of relaxation as the index for evaluating cytotoxicity and validated its effectiveness.

The magnetometirc evaluation of cytotoxicity in alveolar macrophages have been widely tried for other chemicals such as welding fumes (Yagami *et al.*, 1987)[9], iron dust retained in the lungs (Brain *et al.*, 1988)[10], GaAs compound (Karube *et al.*, 1996)[11], limestone powder (Okada *et al.*, 1996)[12], and substitue fiber for asbestos (Karube *et al.*, 1997)[13], but to date a study focusing on a heavy metal has not been tried. Thus, to evaluate the celluar toxicity of alveolar macrophages, this study was designed to make magnetometry of cadmium compound, one of noxious heavy metals in various workplaces and to compare the results with LDH release rates as biochemical parameters and cellular changes including apoptosis.

II. Materials and Methods

1. Materials

Ferrosoferric oxide (Fe_3O_4) was used as the index of cellular magnetometry (Toda Industry Inc., Japan) and Cadmium chloride ($CdCl_2$) as a sample of the cellular toxicity (Junsei Chemical Co., Japan), which was finely pulverized in an agate ball mill and the powder was sieved manually through a 38 μm mesh microsieve. These materials werewell mixed with pH 7.4 phosphate buffered saline (PBS) by a ultrasound generator for 20 minutes and their supernatants were removed after 10 minutes of centrifugation at 1,800 rpm. They were mixed up together with additional PBS and final materials were obtained.

2. Collection of alveolar macrophages

Male Syrian golden hamsters weighing about 100 g were anesthetized by intraabdominal nembutal injections (3 m*l*/kg). The animals were bled to death by incision of aorta. The lungs were collapsed by incision of diaphragm. After revealing the trachea, 3 m*l* of pH 7.4 PBS with 0.1% EDTA filtered by Millipore filter, was instilled to each hamster through the intratracheally inserted silicon catheter and the lavage fluid was withdrawn. Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co.) with 10% fetal bovine serum (FBS, Gibco) was poured into each well with a cell disk at the bottom. Approximately the number of alveolar macrophges were 6 millions per animal as a result of calculation by a blood cell calculator usingsome part of the above materialafter stained by

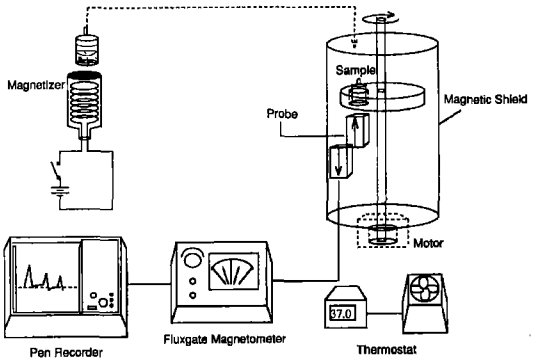

Fig. 1. Magnetometric apparatus. Macrophages adhering to the disk at the bottom of the glass tube are magnetized at 70 mT for 10 milliseconds by the magnetizer. After the cessation of external magnetization, the stage rotates by the motor. The remanent magnetic field strength(RMF) is measured every six seconds by the fluxgate magnetometer with the probe and recorded by the pen-recorder. The temperature inside the shield is kept at 37°C by the heater.

trypan blue.

3. Cellular magnetometry

One m*l* of minimum essential medium with 10% FBS containing 10^6 cells was poured into each well (Nunc Co., Denmark) with a cell disk at the bottom. 60 g of Fe_3O_4 as the index of cellular magnetometry were added to all wells of both experimental and control group in the magnetometry. Each 50 μ*l* of 1, 25 and 50 μg/m*l* of $CdCl_2$ were added as experimental group and 50 μ*l* of pH 7.4 PBS was added as control group. Then they were incubated overnight in the 5% CO_2 incubator at 37°C and moved to a glass tube containing one m*l* of minimum essential medium with 10% FBS. Macrophages adhered to the disk at the bottom of the glass tube were magnetized at 70 mT for 10 msec by the magnetizer (Magnetoscope, Institute Dr. Foerster, Germany). After the cessation of external magnetization, the sample stage within the magnetic shield of the magnetometric apparatus is designed to rotate by the motor. The remanent magnetic field strength was measured by a magnetometer every six seconds for 20 minutes and was simultaneously recorded by the pen recorder. The temperature inside the shield is kept at 37°C by the heater (Fig. 1). A two-minute period of relaxation was fitted to the exponential function $B = B_0 \exp(-\lambda t)$ where B is the remanent field strength at time t, Bo the field strength at time t = 0, and λ the relaxation rate (decay constant) for 2 min, and t the time at the end of magnetization (Brain *et al.*, 1984)[4].

4. LDHassay

One m*l* of minimum essential medium with 10% FBS containing 10^6 cells was poured into each well (Nunc Co., Denmark) with a cell disk at the bottom and they were incubated overnight in the 5% CO_2 incubator at 37°C. The medium in the well was taken out and they were washed two times with pH 7.2 washing fluid. One m*l* of minmal essential medium with 2% bovine serum albumin was poured into each well. After exposure of alveolar macrophages to chemicals as previously, the wells were incubated for 3 hours in the 5% CO_2 incubator at 37°C, and they were centrifuged at 1400 rpm for 10 minutes and 50 μ*l* of their supernatant solution was applied to LDH-UV test kits (Wako Pure Chemical Inc., Japan) to measure a decrease in absorption for two minutes by spectrophotometer (Hitach Co. U-3000, Japan). To measure total LDH activity 50 μ*l* of Triton X-100 were added to control group with only PBS added and they were installed at room temperature to allow cell degeneration. Following Wroblewski-LaDue's method (1955)[14], LDH activity of alveolar macrophages were obtained by the following equation.

LDH release rate (%) = [LDH activity of each experimental group - LDH activity of control group]/[total LDH activity - LDH activity of control group] × 100

5. Apoptosis

The cells with positive signals of apoptosis in cultured cells were assessed by TUNEL (TdT-mediated dUTP-biotin nick end labeling) using Apop Tag Plus In Situ Apoptosis Detection Kit (Oncor Co., MD, USA). After washing with PBS solution cultured alveolar macrophages exposed to chemicals as previously and those cultured in control group, they were fixed with 4% paraformaldehyde for 30 minutes, and they were washed again with PBS solution. They were processed with 0.1% Triton X-100 in ice for 2 minutes and installed for 60 minutes in the incubator at 37 after addition of 50 μ*l* of TUNEL mixture. After taking out TUNEL solution, they were deposited in TBE (Tris/Borate/EDTA) buffer(1 ×) for 5 minutes to cease the reaction, and washed with PBS solution three

times. 50 μl of converter-AP (anti-fluorescein antibody, conjugated with alkaline phosphatase) was poured into each well and they were installed for 20 minutes in the incubator at 37°C. Additionally 100 ml of substrate solution including Buffer III(100 mM tris HCl + 100 mM NaCl + 100 mM $MgCl_2$) and 200 ml of NBT/X-phosphate (BCIP) was poured and installed for 10 minutes at room temperature, and subsequently washed three times. They were counter-stained with methyl green for light microscopy(×200). The cells were determined to be positive when their nuclei were stained to be brown, and the positive rate was calculated according to the number of apoptosis per 100 cells.

6. Statistical analysis

The results were expressed as mean±S.E. from seven animals in each group. The statistical differences among group means were determined using one-way or two-way analysis of variance, and Scheffe's method.

III. Results and Discussions

1. Magnetometry

The serial measurements of the remanent magnetic field for 20 minutes after the cessation of the external magnetization revealed that relaxation curves of the experimental group were significantly delayed compared to the controls on the basis of setting the remanent magnetic field strength just at the end of magnetization as 100% ($p<0.001$). Among the experimental group with

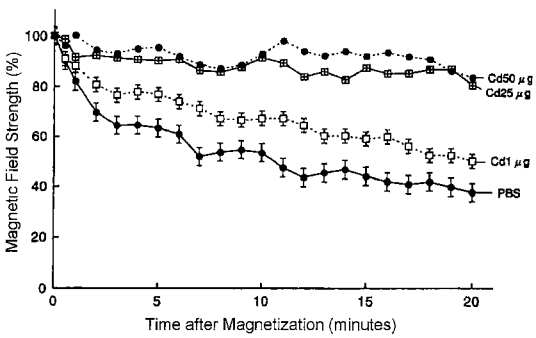

Fig. 2. Relaxation curves in the alveolar macrophages exposed *in vitro* to various doses of $CdCl_2$ and PBS as control. The normalized percent means of the remanent magnetic field strength(RMF) from seven hamsters are plotted as the initial RMF as 100%.

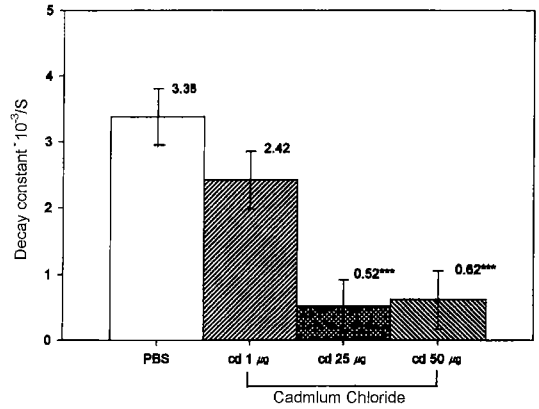

Fig. 3. Decay constant for the first two minutes after magnetization. Decay constant(λ) is an initial relaxation rate calculated by the equation $B=B_0 \exp(-\lambda t)$. Asterisks indicate the significant differences from PBS group as follows; ***: $P<0.001$.

$CdCl_2$ added, 25 μg- and 50 μg-exposed group showed a delay of relaxation compared with 1 μg-exposed group but a dose-dependent delay of relaxation was not observed between 25 μg-and 50 μg-exposed groups (Fig. 2).

A significant decrease in the decay constant for the first two minutes of relaxation was observed in 25 μg- and 50 μg-exposed groups compared with the controls ($p<0.001$), but not observed between the 1-exposed group and the controls ($p>0.05$)(Fig. 3).

The magnetometry has been said to be able to be used as one of experimental methods to screen for cytotoxicity of noxious chemicals by observing the appearance or absence of relaxation, which allows us not just to ascertain whether the cell is dead or not, but to confirm whether alveolar macrophages maintains their unique ability of phagocytosis or not. But any direct comparison can't be made on the results of the present study with those of other existing reports to date because of few studies performed on magentometirc evaluation of cytotoxicity macrophages by cadmium compound. But trial of the magnetometric method for other various chemicals such as welding fume(Nakadate *et al*, 1996)[7], iron dust(Brain *et al*, 1988)[10], asbestos(Cohen *et al*, 1981; Stroink *et al*, 1981)[15,16], silica(Aizawa *et al*, 1991)[17], and gallium arsenide(Aizawa *et al*, 1993)[18] has demonstrated a dose-dependent delay of relaxation after the cessation of external magnetization. This study results also showed that relaxation decreased with a dose-dependent tendency

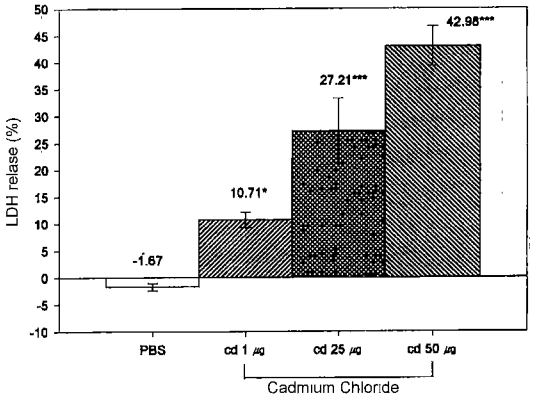

Fig. 4. Mean lactic dehydrogenase(LDH) release rates in the alveolar macrophages exposed in vitro to various doses of $CdCl_2$, and PBS as control. The LDH releases rate (%) was calculated by the following equation: [LDH from each experimental group − LDH from control (PBS) group]/[total LDH-LDH from control group]× 100.

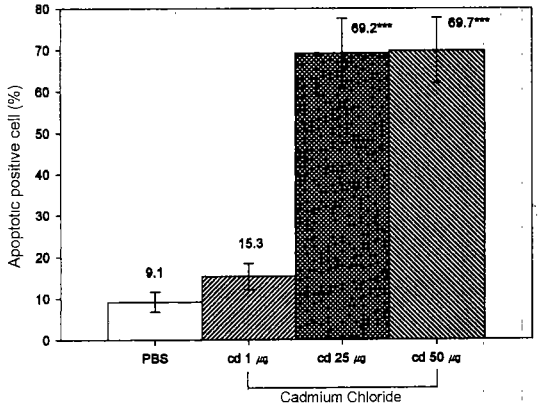

Fig. 5. Mean positive rates of apoptosis in the alveolar macrophages exposed in vitro to various doses of $CdCl_2$, and PBS as control. Data are means± S.E.(n=7). ***p<0.001: compared with the apoptotic cell count in control group.

in the experimental groups in which cadmium was added compared with the control group. But there was no significant difference in the relaxation decay between 25 μg- and 50 μg-exposed group. There was a significant decrease in the decay constant for the first two minutes of relaxation in the experimental group compared with the controls, but a significant difference was not found between 25 μg- and 50 μg-added group. Thus the addition of greater than 25 μg of cadmium did not further result in a dose-dependent decay of relaxation presumably because they have already been in the far advanced state of cellular degeneration and destruction.

2. LDH activity

Each of the experimental groups with various doses of $CdCl_2$ added showed the increase in LDH activity while the PBS-added control group 0%. The LDH activities in 1 μg-, 25 μg- and 50 μg-added group were 10.71%, 27.21%, and 42.98%, respectively and showed a dose-dependent increasing tendency as the dose gradually increased (Fig. 4).

The measurement of LDH release in the cytoplasms of alveolar macrophages can be a good indication of cytotoxicity (Raymond *et al*, 1982)[19]. The destruction of cell membranes and subsequent changes in their permeability caused by noxious chemicals including cadmium account for LDH release and a decay of relaxation curve by the magnetometry. The more of LDH release means the greater cytotoxicity. The LDH release and a relaxation decrease reflecting the cellular destruction and dysfunction of cytoskeleton, have also been observed in the study with GaAs compound as a sample of cytotoxicity(Aizawa *et al*, 1993)[18]. Waseem *et al*(1993)[20] reported that the addition of $CdCl_2$ in doses ranging from 0.04 mM to 1.0 mM in alveolar macrophages of in vitro experiment, markedly increased LDH release compared with that obtained by the addition of the same amount of Nickel chloride, and Koizumi *et al*(1996)[21] noticed the degeneration of plasma membranes of rat hepatocytes under theconcentration of 50 microM of cadmium. Further studies on the cytotoxcity need to be performed based on the various concentrations of cadmium.

3. Cellular apoptosis

The positive rates of apoptosis in 1 μg, 25 μg and 50 μg-added groups were 15.3%, 62.9% and 69.7%, respectively, and each of which was higher than that of the control group (9.1%) with a statistically significant difference except for 1 μg-added group ($p<0.001$)(Fig. 5).

The ideal method for evaluating cellular apoptosis is the observation of DNA laddering using electrophoresis but this has the limitation in the observation of apoptosis localized to the specific cells of the tissue (Tian *et al.*, 1991)[22]. On the other hand, the recently developed TUNEL method could overcome this limitation and made

possible the observation of apoptosis even in the relatively early stage. In this study, TUNEL staining method confirming DNA segmentation by DNase has been used.

The positive rates of apoptosis in 1 μg-, 25 μg-and 50 μg-exposed groups were 15.3%, 69.2% and 69.7%, respectively, in contrast to 9.1% in the control group, and there was a dose-dependent increasing tendency in the positive rates of apoptosis. Especially 25 μg- and 50 μg-exposed groups showed significantly high positive rates of apoptosis compared with the control.

Conclusively, the cadmium-exposed alveolar macrophages showed statistically significant values in the magnetometry, LDH release and positive rate of apoptosis compared with the control group. The cytotoxicity increased in a dose-dependent way as the concentration of added cadmium increased and a decay of relaxation curve after external magnetization was observed especially over 25 μg/m*l* of cadmium concentration.

IV. Conclusions

To evaluate the cytotoxicity of Cadmium compounds, one of noxious chemicals used in various workplaces, magnetometry, LDH release, and cellular apoptosis of *in vitro* alveolar macrophages of hamsters were measured. By magnetometric evaluation, the relaxation curve decayed in each of the exposed groups (1 μg-, 25 μg- and 50 μg of cadmium -exposed) in contrast to the control group. Among the exposed groups, 25 μg- and 50 μg-exposed group showed a significant delay of relaxation curve compared with the 1 μg-exposed but there was no intergroup difference between 25 μg- and 50 μg-exposed group, which indicated the loss of a dose-dependent delay of relaxation curves in the concentration of above 25 μg. A significant decrease in the decay constant for the first two minutes of relaxation with a high dose-dependent tendency was observed in 25 μg- and 50 μg-exposed groups compared with the controls but a significant decrease was not observed between the 1-added group and the control. The LDH release rates in 1 μg-, 25 μg- and 50 μg-added group were 10.71%, 27.21%, and 42.98%, respectively and showed a dose-dependent increasing tendency as the dose gradually increased. The positive rates of apoptosis in 1 μg-, 25 μg- and 50 μg-exposed group were 15.3%, 69.2% and 69.7%, respectively, in contrast to 9.1% in the control group, and especially 25 μg- and 50 μg-exposed groups showed a significantly high positive rate of apoptosis compared with the control.

Conclusively, the cytotoxicity increased in a dose dependent way as the concentration of cadmium added increased, which was reflected in the increase in magnetometry, LDH release rate, positive rate of apoptosis, and it was suggested that cytotoxicity was very significant in the concentration of above 25 μg.

Acknowledgements

The author would like to express their appreciation to Yoshiharu Aizawa MD, professor and chairman, department of preventive medicine and public health, Kitasato University, and his research team who worked so hard to make this test a success.

References

1) Cohen D.: Ferromagnetic contamination in the lungs and other organs of the human body. *Science* **180**, 745-748, 1973.
2) Gehr P, Brain JD, Nemoto I, Bloom SB: Behavior of magnetic particles in hamster lungs: estimates of clearance and cytoplasmic motility. *J Appl Physiol*, **55**, 1196-1202, 1983.
3) Aizawa Y, Takata T, Karube H, Nakamura K, Kotani M: Effects of GaAs and Ga_2O_3 on magnetometric behavior of iron oxide particles in rabbit lungs. *Appl Organometallic Chem.*, **8**, 207-213, 1994.
4) Brain JD, Bloom SB, Valberg PA, Gehr P: Correlation between the behavior of magnetic iron oxide particles in the lungs of rabbits and phagocytosis. *Exp Lung Res.*, **6**, 115-131, 1984.
5) Valberg PA, Butler JP: Magnetic particle motions within living cells: physical theory and techniques. *Biophys J.*, **52**, 537-550, 1987.
6) Kotani M, Uthikawa Y, Hurugawa H: Evaluation of the amount of dust retained in the lung by magnetometry. *JJTOM,* **29**, 792-798, 1981.
7) Nakadate t, Aizawa Y, Yagami M, Kotani M, Ishiwata K: Cumulative low-level exposure to welding fumes and pulmonary function. Book of abstracts of the 25th International Congress on Occupational Health : p281, 1996.
8) Aizawa Y, Karube H, Niitsuya M, Sinohara S, Keira T, Kotani M: Magnetometric evaluation of cytotoxicity of macrophage: dissociation with LDH release. Book of abstracts of the 25th International Congress on Occupational Health : p281, 1996.
9) Yagami T, Kotani M, Aizawa Y, Takata T, Chiyotani

K: Trial of new magnetization method in magnetopneumographic examination in welders. Abstracts of the 6th International Conference on Biomagnetism, p.184-185, 1987.

10) Brain JD, Bloom T, Hu P, Gehr P, Valberg PA: Magnetic iron dust as a probe of particle cytotoxicity in the lungs. *Ann Occup Hyg.*, **32**, 783-793, 1988.
11) Karube H, Aizawa Y, Keira T, Niitsuya S, Mashimo K, Kotani, M: *In vivo* and vitro magnaetometric evaluation for toxicity to alveolar macrophages of GaAs. Book of abstracts of the 25th International Congress on Occupational Health : p182, 1996.
12) Okada M, Keira T, Aizawa Y, Karube H, Niitsuya M, Sugiura Y, Mashimo K, Kotani, M: Effect of limestone on the lungs of rabbits. *JJTOM*, **44(10)**, 682-687, 1996.
13) Karube H, Okada M, Furukawa Y, Mahimo K, Sugiura Y, Aizawa Y: Index of toxicity of substitute fibers for asbestosis on magnetometry. *MAG*-**97-42**, 73-77, 1997.
14) Wroblewski F, La Due J: Lactic dehydrogenase activity in blood. *Proc Soc Exp Bio Bid.*, **90**, 210-213, 1955.
15) Cohen D, Growther TS, Gibbs GW, Becklake MR: Magnetic lung measurements in relation to occupational exposure in asbestos miners and millers of Quebec. *Environ Research*, **26**, 535-550, 1981.
16) Stroink G, Dahn D, Holland J: Magnetopneumographic estimations of lung dust loads and distribution in asbestos miners and millers. *Am Rev Respir Dis* **123**, 144-156, 1981.
17) Aizawa Y, Takata T, hashimoto K, Tominaga M, Tasumi H, Inokuchi N, Kotani M, Chiyotani K: Effects of different dose of silica on magnetometric behavior of iron particles in rabbit lungs. *JJTOM*, **39**, 18-23, 1991.
18) Aizawa Y, Takata T, Karube H, Tatsumi H, Inokuchi N, Kotani M, Chiyotani K: Magnetometric evaluation of the effects of gallium arsenide on the clearance and relaxation of iron particles. *Industrial Health,* **31**, 143-153, 1993.
19) Raymond PW: Occupational lung disorders. Butterworths, p113-133, 1982.
20) Wassem M, Bajpai R, Kaw JL: Reaction of pulmonary macrophages exposed to nickel and cadmium in vitro. *J of Env Patho, Toxi & Onco* **12(1)**, 47-54, 1993.
21) Koizumi T, Shirakura H, Kumagai H, Tatsumoto H, Suzuki KT: Mechanism of cadmium-induced cytotoxicity in rat hepatocytes: cadmium-induced active oxygen-related permeability changes of the plasma membrane. *Toxicology,* **114(2)**, 125-134, 1996.
22) Tian Q, Streuti M, Saito H, Schlossmann SF, Anderson P: A poluadenylate binding protein localized to the granule of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell,* **67**, 627-639, 1991.