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In Vitro Toxicity of Gallium Arsenide in Alveolar Macrophages Evaluated by Magnetometry, Cytochemistry and Morphology

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OKADA, M., KARUBE, H., NIITSUYA, M., AIZAWA, Y., OKAYASU, I. and KOTANI, M. *In Vitro Toxicity of Gallium Arsenide in Alveolar Macrophages Evaluated by Magnetometry, Cytochemistry and Morphology.* Tohoku J. Exp. Med., 1999, **189** (4), 267-281 —— Gallium arsenide (GaAs), a chemical compound of gallium and arsenic, causes various toxic effects including pulmonary diseases in animals. Since the toxicity is not completely investigated, GaAs has been used in workplaces as the material of various semiconductor products. The present study was conducted to clarify the toxicity of GaAs particles in the alveolar macrophages of hamsters using magnetometry, enzyme release assays and morphological examinations. Alveolar macrophages obtained from hamsters by tracheobronchial lavage and adhered to the disks in the bottom of wells were exposed to ferrosoferric oxide and GaAs particles. Ferrosoferric oxide particles were magnetized externally and the remanent magnetic field was measured. Relaxation, a fast decline of the remanent magnetic fields radiated from the alveolar macrophages, was delayed and decay constants were decreased dose-dependently due to exposure to GaAs. Because the relaxation is thought to be associated with cytoskeleton, the exposure of GaAs may have impaired the motor function of them. Enzyme release assay and morphological findings indicated the damage to the macrophages. Thus the cytotoxicity causes cytostructural changes and cell death. According to DNA electrophoresis and the TUNEL method, necrotic changes occur more frequently than apoptotic changes. ———— magnetometry; toxicity; alveolar macrophages; gallium arsenide

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Recently, gallium arsenide (GaAs) has been widely used in the microelectronics industry as a semiconductor material for transistors, solar cells and lasers. GaAs consists of arsenic, a metalloid and gallium (trivalent), a transition element.

It is generally known that the toxicity of inorganic elements changes with their atomic valence and chemical structures. For example, trivalent arsenic (arsenite) is more toxic than pentavalent arsenic (arsenate). Furthermore inorganic arsenic is more toxic than organic forms (Vahter 1988). Once trivalent arsenic is absorbed within cells, arsenicals disrupt the enzymatic reactions vital to cellular metabolisms by interacting with sulfhydryl groups (Lewis 1997; Williams 1999). Because the affinity of a trivalent sulfhydryl-containing enzyme is stronger than pentavalent, the toxicity of a trivalent is contained for a longer period of time until excretion (Rosner and Carter 1987).

Epidemiologic research reports edited by International Agency for Research on Cancer (IARC) have shown that inorganic arsenic is a human carcinogen in the lungs and the dermis (IARC 1987). Although much evidence has been accumulated suggesting that arsenic has caused cytotoxicity in respiratory organs, the precise mechanism of arsenic toxicity to the antigen-presenting system is unknown (Harrison 1986; Edelman 1990).

The purpose of this study is to determine whether alveolar macrophages can tolerate GaAs particles. We submit a report here of an evaluation of the toxicity of GaAs particles in the alveolar macrophages, antigen-presenting cells, of hamsters. The toxicity of GaAs in alveolar macrophages of hamsters was evaluated not only by cytomagnetometry as a biophysical method, but also by intracellular enzyme release as biochemical methods, supravital staining, blood cell staining, and electron microscopy as morphological methods. We also performed DNA ladder and terminal deoxynucleotidal transferase mediated dUTP-biotin nick end labeling (TUNEL) (Sgonc and Wick 1994) as DNA fragment detection.

The magnetometric system is a unique method used for the evaluation of the cytotoxicity due to the exposure to chemicals (Cohen 1973; Cohen et al. 1979). Magnetometric studies show that remanent magnetic fields, which have been generated by external magnetization of magnetic iron oxide particles in alveolar macrophages, decrease rapidly as time passed. This phenomenon, relaxation, is principally attributed to the result of the turning of the phagocytized magnetic iron particles in the alveolar macrophages. We reported some cytotoxic chemicals which influenced relaxation (Aizawa et al. 1994a, b; Karube et al. 1998; Keira et al. 1998a, b; Mashimo et al. 1998; Okada et al. 1998; Sugiura et al. 1998).

Materials and Methods

Additional chemicals

We used ferrosoferric oxide (Fe_3O_4) as the index of cellular magnetometry, GaAs particles as a test material of the cellular toxicity and polystyrene micro-

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Elevated Plasma Level of Plasminogen Activator Inhibitor-1 (PAI-1) in Patients with Relapsing-Remitting Multiple Sclerosis

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Onodera, H., Nakashima, I., Fujihara, K., Nagata, T. and Itoyama, Y. *Elevated Plasma Level of Plasminogen Activator Inhibitor-1 (PAI-1) in Patients with Relapsing-Remitting Multiple Sclerosis.* Tohoku J. Exp. Med., 1999, **189** (4), 259-265 —— Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system and one of the earliest changes in inflammatory focus involves the activation of vascular endothelial cells. We determined the plasma level of plasminogen activator inhibitor-1 (PAI-1), a key regulator of fibrinolysis and cell migration, in patients with MS. The level of plasma PAI-1 was significantly higher in active MS cases when compared to stable MS and controls. Plasma concentrations of tissue plasminogen activator, transforming growth factor β-1, and lipoprotein-a remained normal in spite of disease activity. These results suggested that PAI-1 plasma levels are associated with MS disease activity and is a good marker for MS relapse. ———— fibrinolysis; multiple sclerosis; plasminogen activator inhibitor-1; relapse

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. One of the earliest and pivotal changes in inflammatory focus involves the activation of vascular endothelial cells and fibrin deposition (Wakefield et al. 1994). Endothelial cell activation antigens are expressed on isolated central nervous system microvessels in MS patients (Washington et al. 1994). However, endothelial injury and fibrinolytic processes involved in MS have received little attention in comparison with studies investigating the role of adhesion molecules. Plasminogen activator inhibitor-1 (PAI-1) is a potent inhibitor of fibrinolysis that functions in the regulation of the plasmin-based pericellular proteolytic cascade (Stoop et al. 1997). PAI-1 also serves to regulate cell migration by binding to matrix proteins such as vitronectin and heparin. PAI-1 is synthesized in endothelial cells and its release may be stimulated by the onset of inflammation. Transforming growth factor-β (TGF-β), which has been

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mT for 10 milliseconds by the coils. Then the glass tube was installed in the stage which was turned at a speed of 10 rpm above the probe of a fluxgate magnetometer and was kept at 37°C by a heater with a thermostat. The remanent magnetic field was measured as previously described (Keira et al. 1998b). We performed two way analysis of variance (ANOVA) for magnetic field strength and calculated the relaxation rate (λ; decay constant). That was obtained by using the equation of $B = B_0 \exp(-\lambda t)$ where B is the field strength at time t, B_0 the field strength at time $t = 0$ after magnetization. Then we performed Fisher's multiple comparison procedure for decay constants.

Intracellular enzyme release

To evaluate cellular enzyme release, lactate dehydrogenase (LDH) was measured. After the collection of the alveolar macrophages, 1 ml of serum-free medium (Macrophage-SFM, liquid, Life Technologies, Inc., Rockville, MD, USA) containing 10^6 cells was poured into each well with a cell disk at the bottom and was incubated 18 hours with exposure to chemicals in the 5% CO_2 incubator at 37°C. Then the medium in the well was taken out and centrifuged at 1800 rpm for 5 minutes. Then 50 μl of supernatant solution was applied to LDH-UV test kits (Wako Pure Chemical Industries, Ltd., Osaka) following Wroblewski-LaDue's method (Wroblewski and LaDue 1955) and was measured for determining the decreasing ratio at 340 nm during 2 minutes by use of a U-3000 type autospectrometer (HITACHI Ltd., Tokyo). For measuring the total LDH activation index, which was derived from both the intracellular and the extracellular matrices, Triton-X 100 was added to the control group. The LDH release rate (%) was calculated by the following equation;

([LDH activation index from GaAs exposed group]
−[LDH activation index from control group]) × 100
/([total LDH activation index]
−[LDH activation index from control group]).

We performed Scheffe's multiple comparison procedure for LDH release rates.

Morphological examinations

In light microscopic analysis, we used 0.03% of trypan blue as a supravital staining and Giemsa solution (Dacie and Lewis 1995) as a morphological cell staining. In ultramicroscopic analysis, we used a scaning electromicroscope (SEM) and a transmission electromicroscope (TEM). The macrophages that adhered to the polycationics-treated glass were washed with 0.1 M cacodylate buffer (pH 7.4), and prefixed with 1% of glutaraldehyde at 4°C for 3 hours. After removing the buffer, they were postfixed with 1% of osmium tetroxide at 4°C for 3 hours. Ultrathin sections were stained with 3.0% uranyl acetate and Reynolds' lead citrate. For the observation on an SEM (S-4500FE, HITACHI Ltd.), the

specimen was treated with iron spatter coating. For the observation on a TEM (H-600, HITACHI Ltd.), the specimen was dehydrated and embedded in resin.

DNA ladder method

The DNA ladder method was performed by using two steps: DNA extraction and agarose gelelectrophoresis.

After the adding of the chemicals as described previously, we cultured the macrophages overnight (18 hours) in the wells (Multi Well Plate, MS-8048R, SUMITOMO BAKELITE Co., Ltd.) which were used for suspending the cell culture. Then, we obtained about 1.0 ml of the cell suspension and centrifuged at 1800 rpm for 5 minutes. The supernatant was removed and 400 μl of DNA extraction buffer was poured into the sediment. After 20 minutes reaction at room temperature, 1 μl of 20 mg/ml Proteinase K (Wako Pure Chemical Industries Ltd.) and 20 μl of 10% sodium dodecyl sulfate (SDS, Wako Pure Chemical Industries Ltd.) were added and mixed gently. Afterward the solution was incubated for 30 minutes at 37°C, a solution of 400 μl of PCIA (phenol: chloroform : isoamylalcohol = 25 : 24 : 1) (Wako Pure Chemical Industries Ltd.) was poured into the solution described above and allowed to react for 20 minutes at room temperature. Then, the solution was centrifuged at 10 000 rpm for 10 minutes. Approximately 300 μl of supernatant solution was taken out and 30 μl of 3 M CH_3COONa (Wako Pure Chemical Industries Ltd.) and 300 μl of isopropyl alcohol (Wako Pure Chemical Industries Ltd.) was added to the supernatant for preservation at -80°C freezer.

Prior to electrophoresis, we thawed, centrifuged at 12 000 rpm for 10 minutes, removed the supernatant, added 200 μl of 70% ethanol (Wako Pure Chemical Industries Ltd.) and dried it naturally. After that, 10 μl of 10 mM Tris- 1 mM EDTA buffer (Tris HCl; Schwarzy-Mann Biotech Co., Cleveland, OH, USA, EDTA; Wako Pure Chemical Industries Ltd.) and 2 μl of 10 mg/ml RNase (Wako Pure Chemical Industries Ltd.) were added to the sediment. And the solution was incubated at 37°C for 1 hour. After the preparation of an agarose gel, specimens with bromophenol blue (Wako Pure Chemical Industries Ltd.) were applied to a mini horizontal electrophoresis unit (MUPID-3, Advance Co., Tokyo), stained for 15 minutes by ethidium bromide (Wako Pure Chemical Industries Ltd.) and photographed under UV transillumination.

TUNEL method

After adding the chemicals as previously described, we cultured the macrophages overnight (18 hours) in the wells (Nunc LAB-TEK 8 Chamber Slide, Nunc Inc., Naperville, IL, USA) which were used for light microscopic stain. Then, we removed the medium and fixed the specimen by using 5% paraformaldehyde for over 90 minutes. The specimens were applied to an in situ apoptosis detection kit (Apop Tag Plus-Peroxidase, Oncor Co., Gaithersburg, MD, USA) with some

modifications (Satoh and Tsuchiya 1999). The differences are the following. In order to pretreat cultured cells, we used protainase K (Wako Pure Chemical Industries Ltd.). As a secondary antibody, we used anti-digoxigenin-alkaline phosphatase (Boehringer Mannheim Co., Tokyo) instead of anti-digoxigenin-peroxidase. Therefore we skipped over quenching endogenous peroxidase. Then we used 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazorium chloride (DAKO JAPAN Co., Kyoto) in place of diaminobenzidine substrate for staining. We used tris buffer saline for PBS. For positive control specimens, 4 μm thick histologic sections of 10% buffered formalin-fixed and paraffin wax-embedded human tonsils were used.

Results

Cytomagnetometry

Fig. 1 shows the characteristics of the relaxation which we evaluated. The relaxation means a fast decline of the remanent magnetic field which was radiated from alveolar macrophages. The relaxation of the GaAs exposed group was delayed dose-dependently, whereas neither the polystyrene exposed group nor the control group showed any effect. The relaxation curve shows the declining curve which is the plotted remanent magnetic field normalizing to 100% at immediately after stopping the magnetization. We performed two way ANOVA and recognized the significance among groups ($p<0.001$). Then λ, the relaxation rate (decay constant), was obtained from the equation of $B=B_0\exp(-\lambda t)$. The data was summarized in Fig. 2. As the result of Fisher's multiple comparison procedure, the significance was recognized between the control group and the GaAs 4

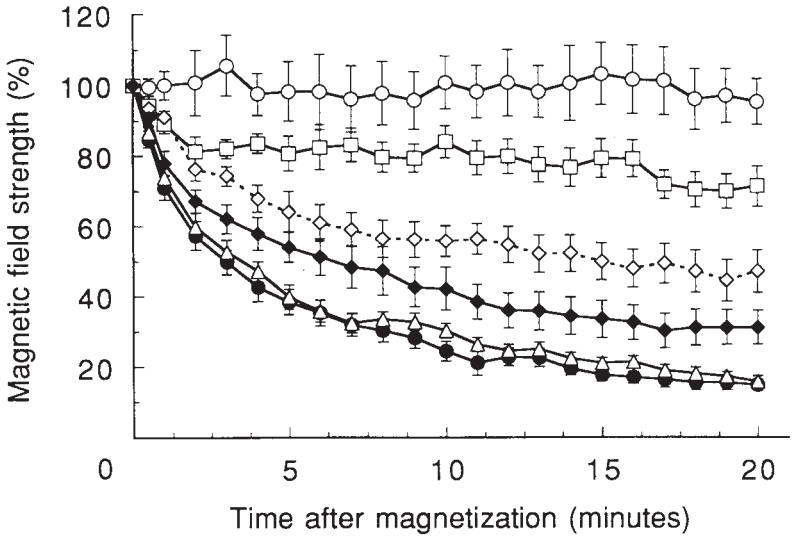

Fig. 1. The relaxation curves in the alveolar macrophages exposed in vitro to various doses of GaAs, polystyrene and PBS as control group. The vertical percentage presents the remanent magnetic field with S.E. from 7 hamsters in each group. The initial remanent magnetic field is plotted as 100%.
●, control group; △, polystyrene exposed group; ◆, 2 μg/ml; ◇, 4 μg/ml; ▯, 10 μg/ml; ○, 20 μg/ml of GaAs exposed group.

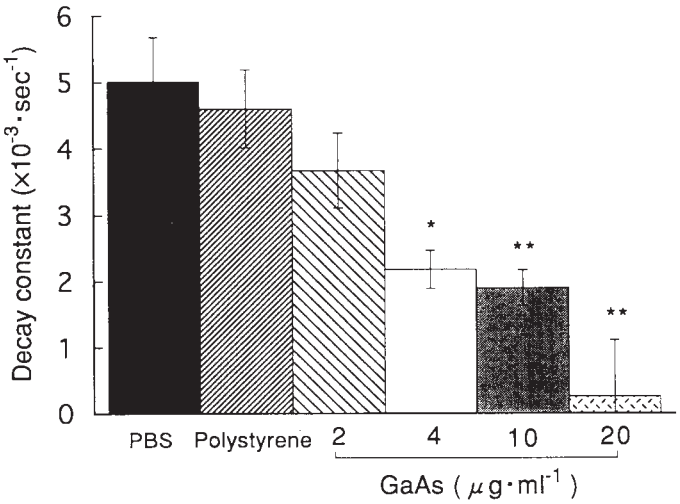

Fig. 2. The decay constant at two minutes after magnetization. Values present mean±S.E. obtained from 7 hamsters in each group. Significant difference from control group was shown by $*p<0.05$ and $**p<0.01$.

μg/ml exposed group ($p<0.05$). The significance was also recognized between the control group and the GaAs 10 μg/ml exposed group and also between the controls and the GaAs 20 μg/ml exposed groups ($p<0.01$).

Intracellular enzyme release

The data was summarized in Fig. 3. The considerable release of LDH from the alveolar macrophages exposed to GaAs was shown. Neither PBS nor polystyrene had any effects on the LDH release from the alveolar macrophages. As the result of Scheffe's multiple comparison procedure, the significance was recognized between polystyrene exposed group and each GaAs exposed group ($p<$

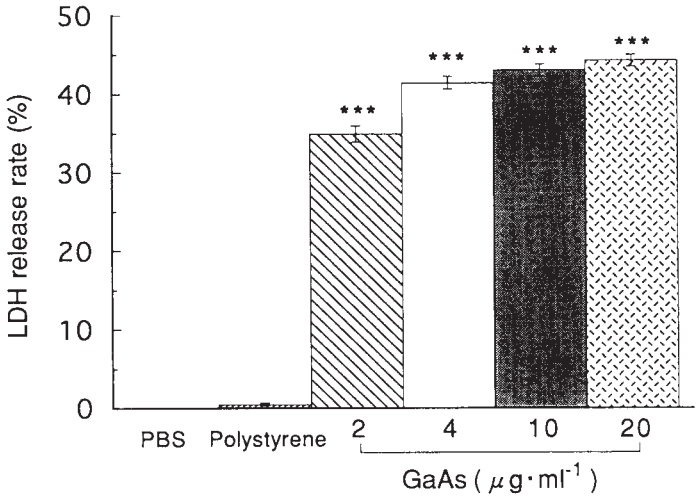

Fig. 3. The LDH release rate in the alveolar macrophages exposed in vitro to various doses of GaAs, polystyrene and PBS as control group. The vertical percentage presents mean±S.E. obtained from 9 hamsters in each group. Significant difference from polystyrene exposed group was shown by $***p<0.001$.

0.001).

Morphological methods

The data was summarized in Figs. 4 and 5. Trypan blue staining showed cell injuries to the surface membrane of the alveolar macrophages. The positive rates for 2, 4, 10, 20 μg/ml of GaAs were 12.4, 36.3, 44.6, 46.1% and those were increased dose-dependently (Figs. 4A and 4B). Giemsa staining showed enlarged cells, low nucleus cytoplasm ratio, collapsed and poor stained nuclei of GaAs exposed alveolar macrophages. In light microscopic findings, destructive changes of alveolar macrophages were shown in GaAs exposed group. SEM findings showed the disappearance of cytoplasmic projections (Figs. 5C and 5D). TEM findings showed the disappearance of cytoplasmic projections, that is to say, smoothing and rounding appearance changes of the cell surface, and rounding and indistinct changes of the nucleolus (Figs. 5E and 5F). In electron microscopic findings, organic degeneration of cell membrane, cytoplasm and nuclei were shown in GaAs exposed group. Neither the control group nor the polystyrene exposed group showed abnormal findings in contrast to the GaAs exposed group.

DNA Ladder method

The photograph of the electrophoresis is shown in Fig. 6. The photo shows that the fragmentations of nucleosome units (about 180 bp) are not observed in the DNA pattern of alveolar macrophages exposed to GaAs particles. Both the

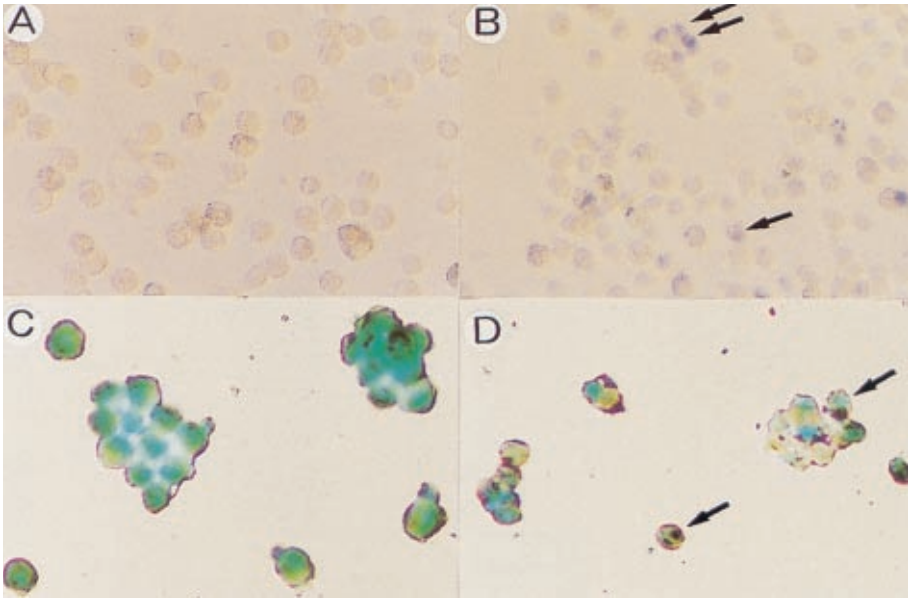

Fig. 4. The light microscopic findings in the alveolar macrophages. A: Control group with trypan blue staining. B: 20 μg/ml of GaAs exposed group with trypan blue staining. Arrows present positive cells. C: Control group on TUNEL staining. D: 20 μg/ml of GaAs exposed group on TUNEL method. Arrows present positive cells. (Original magnification: A & B $\times$200, C & D $\times$100).

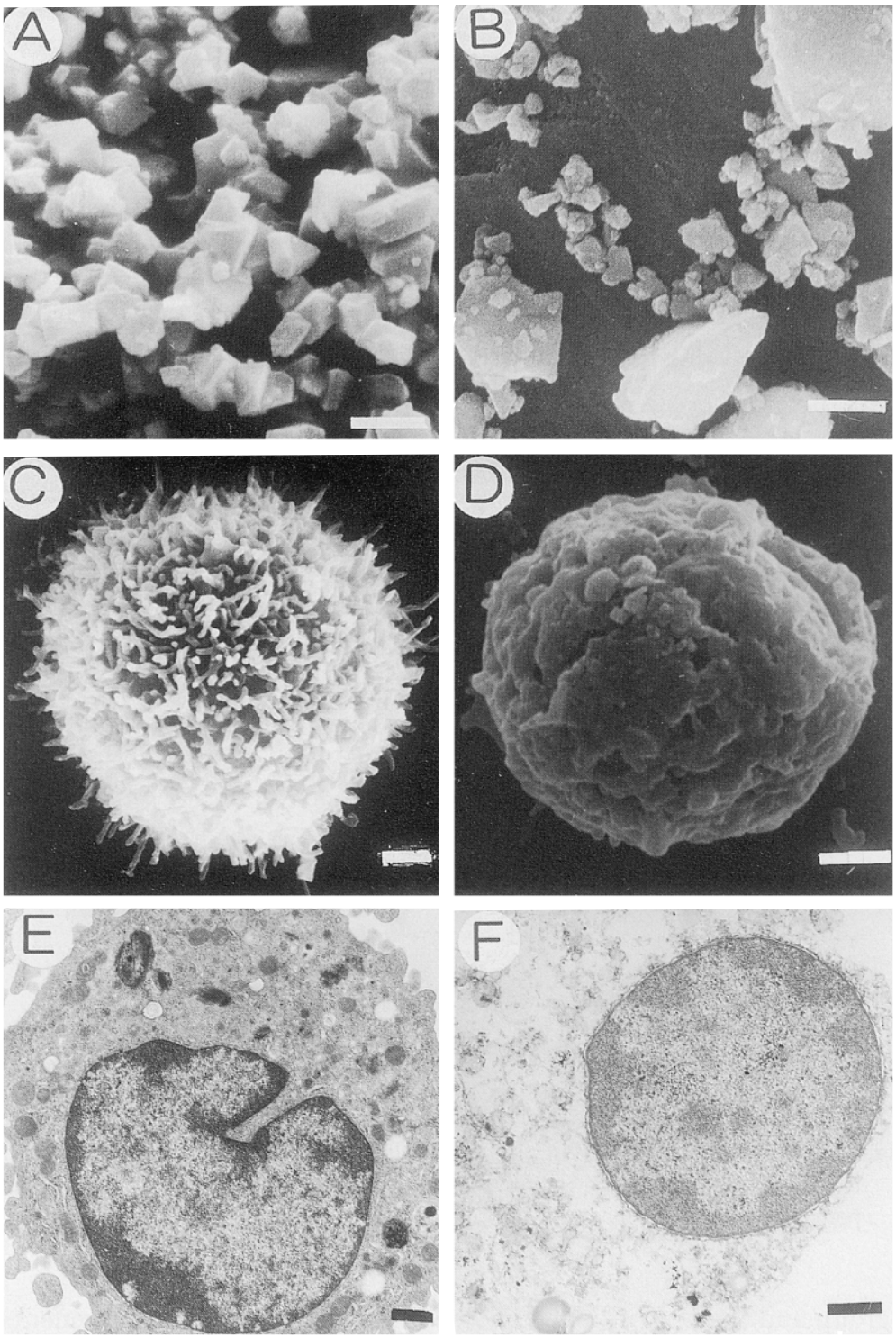

Fig. 5. The ultramicroscopic findings in the alveolar macrophages. A: Particles of Fe_3O_4 by scaning electromicroscope (SEM). B: Particles of GaAs by SEM. C: An alveolar macrophage in control group by SEM. D: An alveolar macrophage in 20 μg/ml of GaAs exposed group by SEM. E: An alveolar macrophage in control group by transmission electromicroscope (TEM). F: An alveolar macrophage in 20 μg/ml of GaAs exposed group by TEM. Double stainings with uranyl acetate and Reynolds' lead citrate. (Each bar shows 0.5 μm for A and B, 1.0 μm for C through F.).

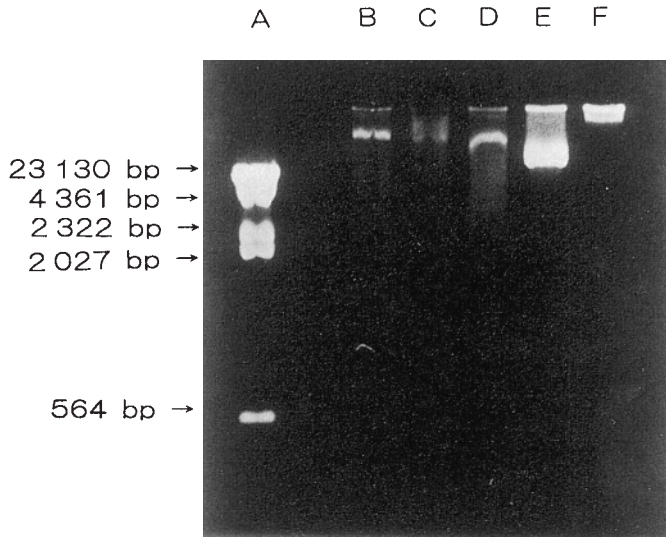

Fig. 6. The electrophoretic image by DNA ladder method. Lane A: DNA marker (λDNA/Hind III Fragments). Indicators with various base pairs (bp) were shown. Lane B, C and D: Extracted DNA from control group. Lane E and F: Extracted DNA from 20 μg/ml of GaAs exposed group.

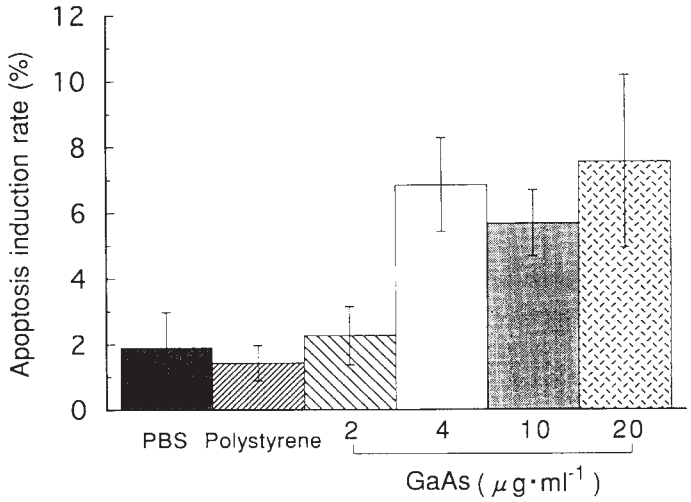

Fig. 7. The apoptosis induction rate in the alveolar macrophages exposed in vitro to various doses of GaAs, polystyrene and PBS as control group. The vertical percentage presents mean ± S.E. obtained from 13 hamsters in each group.

control group and the polystyrene exposed group show negligible fragmentation of nucleosome units.

TUNEL method

The data and the photograph of apoptotic staining were summarized in Figs. 4C, 4D and 7. Even in the group exposed to the highest dose of GaAs, the average positive rate of staining was less than 10%. Therefore the slight apoptotic changes in the GaAs exposed alveolar macrophages were shown. Both the control group and the polystyrene exposed group showed that the average rate of apoptotic changes was below 2.0%.

Discussion

Cytomagnetometry is a modified method of pneumomagnetometry which was invented by Cohen (1973). The magnetometric system is a unique method used for evaluation of the cellular toxicity or lung toxicity by chemical substances. The alveolar macrophages phagocitize iron oxide particles during 18 hours incubation. The iron oxide particles located in the phagozomes of macrophages are magnetized externally (Brain et al. 1988). After stopping external magnetization, the remanent magnetic field strength decreases rapidly as time passes. This phenomenon named relaxation is thought to occur due to the random rotation of phagosomes containing magnetized iron oxide particles in the alveolar macrophages (Valberg and Brain 1988). Cytoskeleton is thought to contribute to the random rotation of phagosomes (Nemoto et al. 1989). Although detailed mechanism is still to be elucidated, we presume that delayed relaxation is due to the cytoskeletal dysfunction induced by exposed chemical substances. Then some experiments which include in vivo and in vitro have been reported (Aizawa et al. 1994a, b; Karube et al. 1998; Keira et al. 1998a, b; Mashimo et al. 1998; Okada et al. 1998; Sugiura et al. 1998).

In the in vivo experiment, the relaxation are measured in the lungs of mammals which were intratracheally instilled with both iron oxide particles and a chemical substance. In rabbits, an administration of GaAs showed dose-dependent delay of relaxation (Aizawa et al. 1994a, b), while that of calcium carbonate showed normal relaxation (Mashimo et al. 1998). In human beings, cigarette smoke caused delay of relaxation (Meller et al. 1996).

There are some reports on in vitro experiments using alveolar macrophages of hamsters as follows: Calcium carbonate showed normal relaxation (Sugiura et al. 1998), silicon carbide whiskers caused moderate delays of relaxation (Karube et al. 1998), titanium dioxide whiskers caused dose-dependent delays of relaxation (Okada et al. 1998) and chrysotile fibers indicated moderate delays of relaxation (Keira et al. 1998a). In this study, the exposure ranged from 2 μg/ml to 20 μg/ml doses of GaAs indicated dose-dependent delay of relaxation and decrease of decay constants. Polystyrene microsphere suspension was added to evaluate the effect of phagocytosis of inert particles to alveolar macrophages. The exposure of this suspension did not influence the relaxation.

LDH exists in the cytoplasm of alveolar macrophages. Its release into the external fluid shows the alternation of cell membrane. Therefore, to measure the LDH activity which is released from the cytoplasm is one of evaluating methods for determining the cell toxicity induced by the chemical particles. In the present report, the exposure ranged from 2 μg/ml to 20 μg/ml doses of GaAs indicated dose-dependent increase of LDH release rate. This result indicates the change of permeability in the cell membrane. We used a serum free medium for measuring LDH instead of the medium used for magnetometry examinations,

because MEM with FBS contains LDH.

Morphological examinations show remarkable changes in the alveolar macrophages. In light microscopic findings, the dose-dependent degeneration of alveolar macrophages was shown in the GaAs exposed group. In electron microscopic examinations, the structural degeneration of cell membrane and intracytoplasmic organella was prominent in the GaAs exposed group. We conclude that those changes resulted from the GaAs exposure, because neither the control groups nor the polystyrene exposed groups showed abnormal findings.

In the GaAs exposed group, the DNA electrophoresis showed that the fragmentation of each nucleosome unit (about 180 bp) was not detected. Therefore, alveolar macrophages exposed to the GaAs were not effected by apoptotic changes. Both the control group and the polystyrene exposed group showed negligible fragmentation of nucleosome units.

A result of apoptotic staining by TUNEL method showed that the average rate of stained macrophages in the GaAs exposed group was less than 10%. Thus, slight apoptotic changes of alveolar macrophages exposed to the GaAs were shown. Both the control group and the polystyrene exposed group showed that the average rate of apoptotic changes was below 2.0%. It is reported that asbestos (Holian et al. 1997), silica (Hamilton et al. 1996; Iyer et al. 1996), TNF-α (Iyer and Holian 1997; Ortiz et al. 1998) and endotoxin (Bingisser et al. 1996) induced apoptotic changes in the alveolar macrophages. It has been reported that arsenic trioxide induced apoptotic changes of lymphocytes and myelocytes (Bazarbachi et al. 1999; Dai et al. 1999; Koken et al. 1999; Rousselot et al. 1999; Tamm et al. 1999). Arsenic trioxide has been used as a medication for adult T-cell leukemia and acute promyelocytic leukemia (Bazarbachi et al. 1999; Dai et al. 1999; Koken et al. 1999; Rousselot et al. 1999; Tamm et al. 1999).

Regarding the toxicity of GaAs, it should be elucidated which elements of GaAs mainly causes cytotoxicity and what kind of condition, dissolution or insolution, mainly induces cytotoxicity. Moreover the solubility of GaAs is an important factor for evaluating cytotoxicity (Pierson et al. 1989). According to the report on comparative pulmonary toxicity of GaAs, gallium (III) oxide or arsenic (III) oxide, GaAs had greater toxicity than arsenic or gallium (Webb et al. 1984, 1986). The dissolved GaAs was more toxic to the cells than the insoluble one (Webb et al. 1984, 1986; Rosner and Carter 1987).

In this study, during 18 hours exposure of GaAs to the alveolar macrophages caused the cytoskeletal dysfunction which was detected by cytomagnetometry, the injuries of cell membrane which was detected by enzyme release and morphological methods, and the necrotic changes in most cells which were detected by DNA ladder method and TUNEL method. For the reasons stated above, cytomagnetometry is thought to be an effective methods for evaluating the functional toxicity of GaAs to alveolar macrophages.

Acknowledgments

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