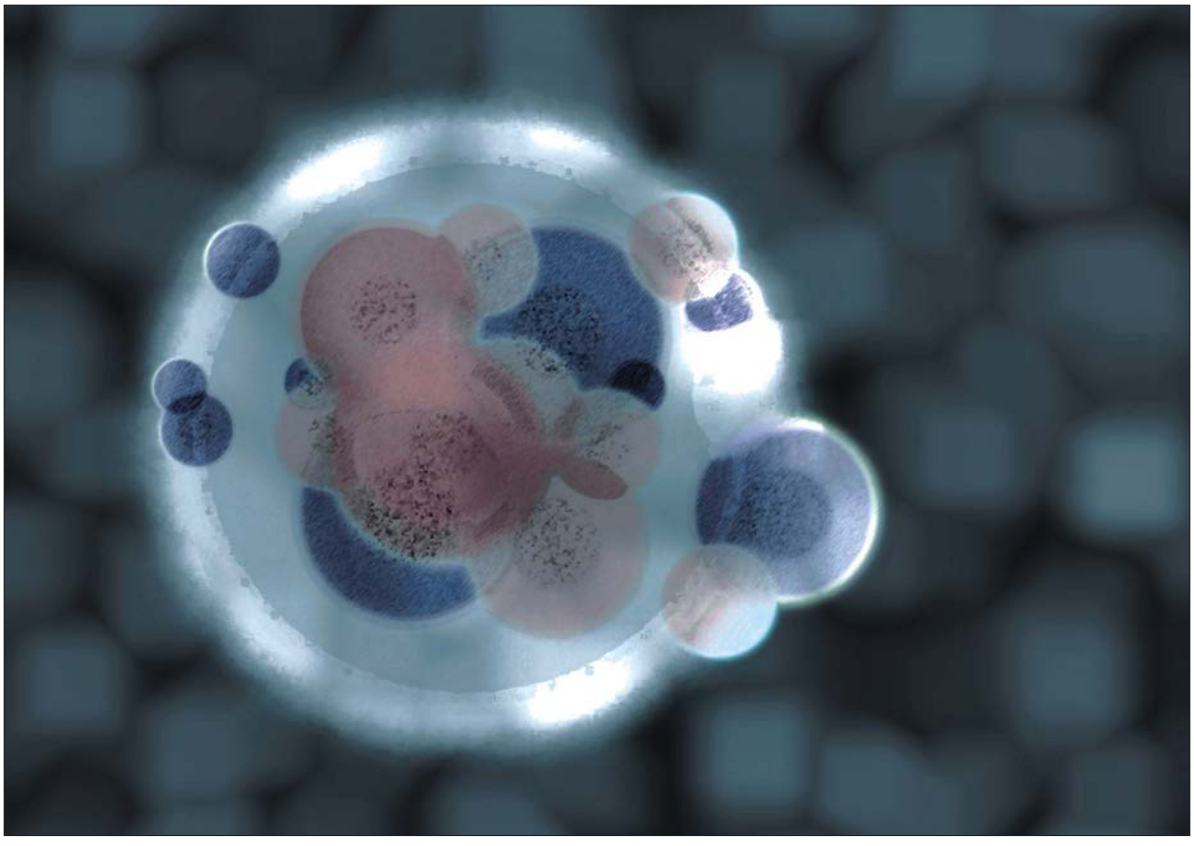

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 4 (4): 532-536, 2004
ISSN 1727-3048

Mutagenesis of Bacteria by Fibrous or Clay Minerals

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Abstract: In the present cells of *Escherichia coli*, *Agrobacterium radiobacter*, or *Thiobacillus intermedius* were exposed to chrysotile asbestos, kaolinite, or bentonite by culture in medium containing these minerals. Chromosomal DNA was extracted from exposed cells, then amplified by the RAPD method and band patterns were compared. DNA of bacterial cells exposed to these minerals, was amplified, in whereas that in control cells was not, or that amplified in control cells disappeared. Not only asbestos, but also kaolinite and bentonite, which are principal soil components, induce bacterial mutation. Growth was inhibited by 66%, when *A. radiobacter* was cultured in LB medium containing 20 mg mL^{-1} of asbestos for 24 h. Growth inhibition by asbestos was reduced by adding 420 U mL^{-1} of catalase or 500 U mL^{-1} of superoxide dismutase to the culture medium.

Key words: Mutagenesis, chrysotile, kaolinite, bentonite, RAPD, scavenger

INTRODUCTION

Mutagens such as radioisotopes, X rays, ultraviolet rays and chemical compounds are found in nature. Fine particulate heavy metals in mutagens cause cancer and neuropathy in animals after chronic exposure[1-3]. Asbestos, which is used in building or as friction fibrous materials, is a mineral mutagen[4]. Chronic aspiration of asbestos causes mesothelioma seen in asbestos miners[4]. Asbestos fibers floating in the atmosphere is very harmful to human health. Among several types of asbestos, chrysotile [$Mg_6Si_4O_{10}(OH)_8$] is a serpentine fiber that accounts for >90% of industrial asbestos and crocidolite [$Na_2(Fe^{3+})_3Si_8O_{22}(OH)_2$] is a rod-like, durable amphibole fiber that is associated with a greater risk of pleural mesothelioma[5].

The toxicity of heavy metal particles were studied such as cadmium toward bacteria including *Escherichia coli* JM109, *Agrobacterium radiobacter* IFO12665b1 and *Thiobacillus intermedius* 13-1, as well as the mechanism of toxicity caused by heavy metal particle[6,7]. The mutagenesis of bacteria by the asbestos has not yet been examined. The present study uses the random amplified polymorphic DNA (RAPD) method[8] to determine whether or not mutagenesis occurs in *E. coli* B, *A. radiobacter* and *T. intermedius* after exposure to asbestos. It was also examined whether or not kaolinite [$Al_2Si_2O_5(OH)_4$] and bentonite $(Na,Ca)_{1/3}(Al, Mg)_2[(OH)_2Si_4O_{10}]nH_2O$ (a clay mineral with a structure similar to that of asbestos) are mutagens.

Within the past few years, several laboratories have focused on active oxygen species as causative agents of asbestosis. Increased amounts of superoxide anion (O_2^-) have been produced after rodent macrophages were exposed *in vitro* to long asbestos fibers[2]. The observation that exogenous administration of scavengers of active oxygen species prevents asbestos-induced cell death to cultures of cells[1] suggested that active oxygen species are intimately related to asbestos toxicity. Moreover, the present study investigated whether chrysotile asbestos fibers attached to bacterial cells *in vitro* generate active oxygen species.

MATERIALS AND METHODS

Microorganisms and reagents: *Agrobacterium radiobacter* IFO12665b1 and *Escherichia coli* B was obtained from the Institute for Fermentation, Osaka, Japan. *Thiobacillus intermedius* 13-1 was isolated from corroded concrete by Morinaga *et al.*[9]. Chrysotile asbestos was obtained from Yoneyama Chemical Co. Ltd. (Osaka). Taq DNA polymerase was obtained from Toyobo Co. Ltd. (Osaka). Catalase derived from bovine liver was purchased from SIGMA, St. Louis, MO, USA. Superoxide dismutase (Mn type) derived from *Bacillus* sp. was purchased from Wako Chemical Co. Ltd. Osaka.

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Culture conditions: *E. coli* B, *A. radiobacter* and *T. intermedius* were precultured in Luria-Bertani (LB) medium at 30°C on a rotatory shaker at 150 rpm for 24 h. Pre-cultured bacterial cells of each type at the stationary growth phase (500 μl) were added to 50 mL of LB liquid medium in Erlenmeyer flasks containing 20 mg mL^{-1} of chrysotile asbestos, kaolinite, or bentonite, which had been washed in distilled water and autoclaved. The cells were then cultured at 30°C on a rotatory shaker at 150 rpm for 24 h. All subsequent experiments were performed under exactly the same conditions.

DNA isolation: Each bacterium was cultured in LB with or without 20 mg mL^{-1} of asbestos, kaolinite, or bentonite at 30°C for 24 h. Bacterial cells exposed or not exposed to these minerals were harvested by centrifugation at 3,000×g for 10 min and were then washed twice in distilled water. Genomic DNA of bacteria was prepared using a DNA Extraction Kit (ISOPLANT, Nippon Gene Co. Ltd., Toyama).

PCR: The dodecamer oligonucleotides, A25 (5'-CTCAGCGATACG-3', Nippon gene Co. Ltd, Toyama), which produce polymorphic and reproducible RAPD bands, were used as the primers. Reaction mixtures containing 1×PCR buffer (Toyobo Co. Ltd.), 1.5 mM $MgCl_2$, 200 μM dNTP, 0.5 μM primer, 80 ng template DNA and 2.5 U Taq DNA polymerase (Toyobo Co. Ltd.) were overlaid in tubes. Amplification proceeded in a Perkin-Elmer 2400 thermal cycler programmed as follows: 95°C for 3 min, then 40 cycles of 93°C for 1 min, 50°C for 2 min, 72°C for 2 min and finally 72°C for 5 min. The amplified mixtures were maintained at 4°C, then separated by electrophoresis in 1% agarose gels. The gels were stained with ethidium bromide and the presence or absence of bands was recorded. The amplification was repeated at least twice and only reproducible data were retained.

Growth inhibition: *A. radiobacter* was precultured in Luria-Bertani (LB) medium at 30°C on a rotatory shaker at 150 rpm for 24 h. Pre-cultured cells in the stationary growth phase (500 μl) were added to 50 mL of LB liquid medium in the Erlenmeyer flask containing 2-20 mg mL^{-1} of chrysotile asbestos, which had been washed in distilled water and autoclaved and then cultured at 30°C on a rotatory shaker at 150 rpm for 36 h. During culturing, growth was evaluated by measuring the optical density of the upper phase of the culture medium at 550 nm. All subsequent experiments were performed under exactly the same conditions.

RESULTS

Comparison of amplified DNA among bacterial cells exposed to asbestos: DNA was extracted from the bacteria exposed to asbestos and amplified by PCR using the primer A25. Figure 1 shows the band profile of amplified DNA in a 1% agarose gel. Although 2.0 and 0.9 kbp *E. coli* B DNA was amplified by the control, 3.0 and 1.3 kbp DNA, which was not present in the control, appeared in the exposed cells (Fig. 1A). DNA of 1.0 and 2.5 kbp was amplified by the *A. radiobacter* control. On the other hand, 1.4 kbp DNA was amplified in *A. radiobacter* cells exposed to asbestos. The signal emitted by the 1.4 kbp DNA from *A. radiobacter* exposure to asbestos was very intense, whereas that from control cells was very weak. A 1.0 kbp DNA band in the control cells disappeared after exposure to asbestos (Fig. 1B). A 0.5 kbp of DNA in *T. intermedius*, which was not present in the control, appeared after exposure to asbestos (Fig. 1C). It is expected that mutation occurred in

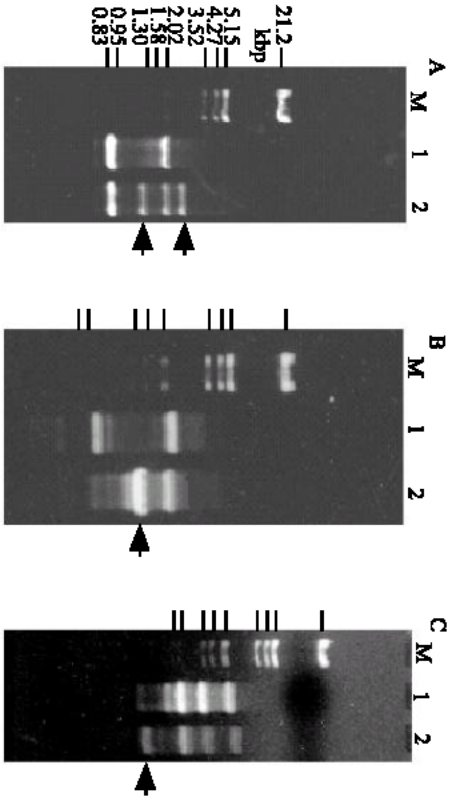

Fig. 1: Amplified genomic DNA of *E. coli* B (A), *A. radiobacter* (B) and *T. intermedius* © exposed to chrysotile asbestos or not (control). Lanes: 1, control; 2, exposed cells; M, molecular marker. Arrow shows DNA bands absent in control cells

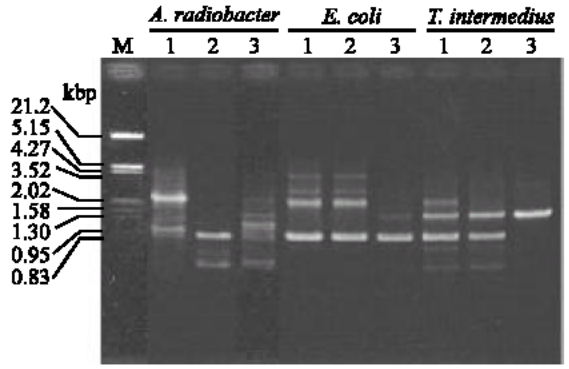

Fig. 2: Amplified genomic DNA of *E. coli* B, *A. radiobacter* and *T. intermedius* exposed to kaolinite or bentonite. Lanes: 1, control; 2, cells exposed to kaolinite; 3, cells exposed to bentonite; M, molecular marker

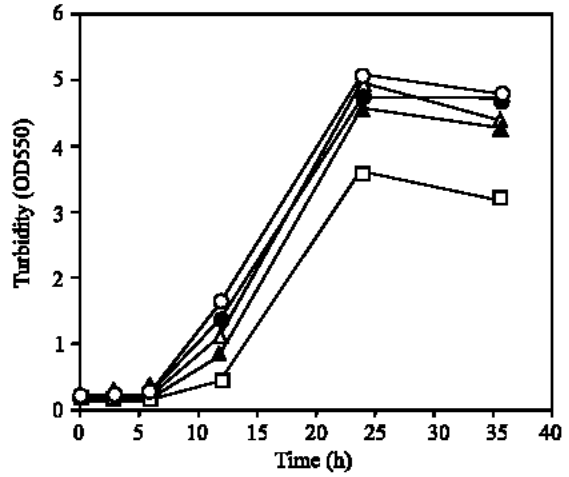

Fig. 3: Growth of *A. radiobacter* in LB liquid medium in the presence of 2 mg mL^{-1} (•), 6 mg mL^{-1} (△), 10 mg mL^{-1} (▲) or 20 mg mL^{-1} (□) of chrysotile asbestos or absence of chrysotile asbestos as the control (○). The results are expressed as mean values of triplicate experiments

chromosomal DNA, resulting the region, where a primer A25 annealed, generated by asbestos newly, or the region, where originally a primer annealed, mutated by asbestos.

Comparison of amplified DNA from bacterial cells exposed to kaolinite or bentonite: DNA was extracted from cells exposed to kaolinite or bentonite and amplified by PCR using the primer A25. Figure 2 shows the band profile of amplified DNAs. Amplified *E. coli* B DNA after exposure to kaolinite was identical to the control. However, a 2.0 kbp DNA disappeared from *E. coli* B cells

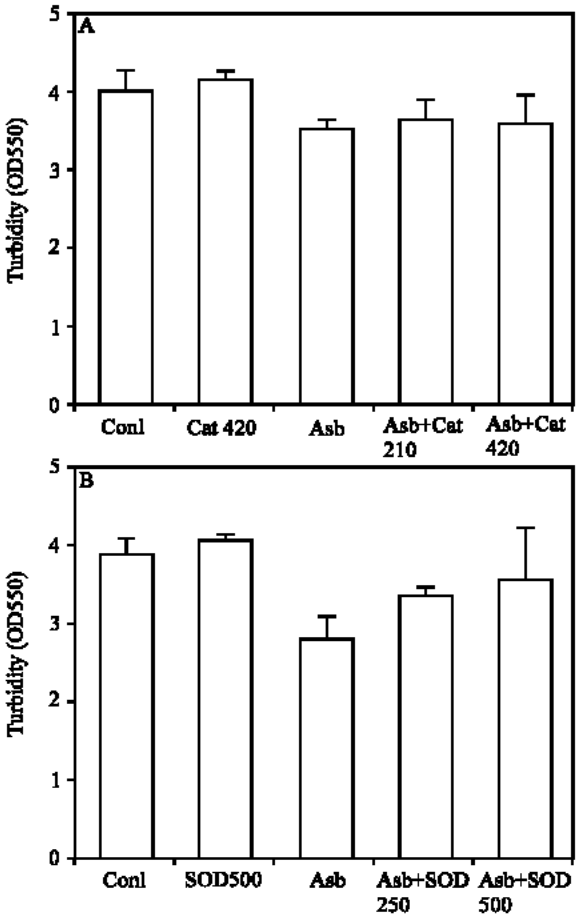

Fig. 4: Growth at 30°C for 24 h was determined by measurement of turbidity at OD550. *A. radiobacter* (A) was cultured in LB medium in the absence of chrysotile asbestos (Conl) or in the presence of 420 U mL^{-1} of catalase (Cat 420) alone and cultured in LB medium including 20 mg mL^{-1} of asbestos (Asb) with 210 U mL^{-1} of catalase (Asb + Cat210) or 420 U mL^{-1} of catalase (Asb + Cat 420). *A. radiobacter* (B) was also cultured in LB medium in the absence of asbestos (Conl) or in the presence of 500 U of SOD alone (SOD500) and cultured in LB medium including 20 mg mL^{-1} of asbestos (Asb) with 250 U mL^{-1} of superoxide dismutase (Asb+SOD250) or 500 U mL^{-1} of SOD (Asb+SOD500). The results are expressed as the mean±SD of three separate incubations

exposed to bentonite. Amplified 2.5 kbp DNA disappeared from *A. radiobacter* exposed to kaolinite and bentonite, respectively. DNA under 0.83 kbp that was not amplified in the control cells was amplified after exposure to kaolinite or bentonite. Amplified 2.0 kbp DNA disappeared from *T. intermedius* after exposure to

kaolinite, whereas 1.0 and 2.0 kbp disappeared after exposure to bentonite.

Growth inhibition: Growth of *A. radiobacter* was increasingly inhibited as asbestos amounts increased, during cultivation. In particular, growth was inhibited strongly in the medium containing 20 mg mL^{-1} of asbestos and was 22% of the control after 12 h cultivation. On the other hand, growth was not inhibited strongly in the medium containing 2-10 mg mL^{-1} of asbestos and was 88-98% of the control after 24 and 36 h cultivation. However, growth was inhibited strongly in the medium containing 20 mg mL^{-1} of asbestos, resulting 73 and 66% of the control growth after 24 and 36 h, respectively (Fig. 3). An adsorption to the asbestos of bacterial cells was only slightly noticeable during cultivation.

Effect of radical scabenger on growth inhibition: Figure 4 shows the growth of *A. radiobacter* in LB medium containing 20 mg mL^{-1} of asbestos and radical scabenger such as catalase and superoxide dismutase (SOD). Growth of strain after 24 h cultivation is shown as OD550. Growth of *A. radiobacter* is suppressed by 60-70%, similar to the results shown in Fig. 3, in the medium containing 20 mg mL^{-1} of asbestos. However, although not completely, growth inhibition was released when 210 U mL^{-1} of catalase was added to the culture medium. Moreover, growth inhibition was also released, when a SOD was added to the culture medium. Growth inhibition was recovered to 80 and 90% of the control by the addition of 250 and 500 U mL^{-1} of SOD, respectively. In *A. radiobacter*, those growth inhibition was not observed at all when only 420 U mL^{-1} of catalase or 500 U mL^{-1} of SOD alone was added to the culture medium.

Active oxygen species generated on the surface of asbestos may be one cause of growth inhibition by asbestos. These results support a former theory[10] that states that asbestos catalyzes formation of the active oxygen species.

DISCUSSION

One aim of this investigation was to minimize fiber penetration as a means of stimulating mutagenesis, since the greater thickness of the bacterial cell wall compared with the animal cell membrane, is viewed as a means of substantially discouraging mechanical penetration. One possible mutagenic mechanism of asbestos is that of fiber penetration through the cell membrane, resulting in physical damage to DNA[11]. Fiber size and shape are apparently critical to mutagenesis. Long, thick fibers cause fibrosis of the lung, whereas finer fibers are more tumorigenic. Some mechanism other than mechanical penetration by asbestos fibers appears to be operative, since mutations were amplified in bacteria after exposure not only to asbestos, but also to kaolinite and bentonite. The possibility that heavy metals in clay minerals could be responsible for its ability to cause mutation, though this is unlikely in bacteria[12], has not been ruled out by the present study. Furthermore, hydrocarbons or other undetermined impurities present in natural asbestos could account for the mutagenic properties seen in bacteria.

Recent studies on asbestos in cell-free systems have demonstrated by electron spin resonance that chrysotile, crocidolite and amosite asbestos generate active oxygen species in the presence of H_2O_2 or physiological saline[10]. Chrysotile asbestos complex has significant levels of ferric iron onto their surface. Under these circumstances, Fe^{2+} on the surface of the asbestos appears to drive a modified Haber-Weiss (Fenton) reaction that results in production of the toxic hydroxyl radical from H_2O_2 and O_2^- in the following manner: H_2O_2+Asb-Fe^{2+}→$^-$OH +OH^- +Asb-Fe^{3+}[10]. These reactions result in lipid peroxidation, which is prevented by the incubation of asbestos by the iron chelator, desferroxamine[13]. A role for active oxygen species-mediating asbestos toxicity is supported by the observation that antioxidant enzymes, such as catalase and SOD, attenuate asbestos-induced growth inhibition (Fig. 4). Iron is probably active by its ability to catalyze the production of reactive free radical oxygen species, such as the superoxide anion and hydroxyl radical. These species can react with DNA, causing DNA strand breaks[14] and base modifications[15] as well as the induction of cellular oxidative stress. These interactions might induce chromosomal misaggregation or abnormalities.

The present study showed that mutation in bacteria can be caused by asbestos and clay minerals such as kaolinite and bentonite, the latter two being universally extant as principal components of soil. Study also showed that asbestos mediates the transformation of *E. coli* cells by exogenous plasmid DNA (pUC18)[16]. This study summrized that fibrous and clay minerals may play roles in microbial evolution.

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