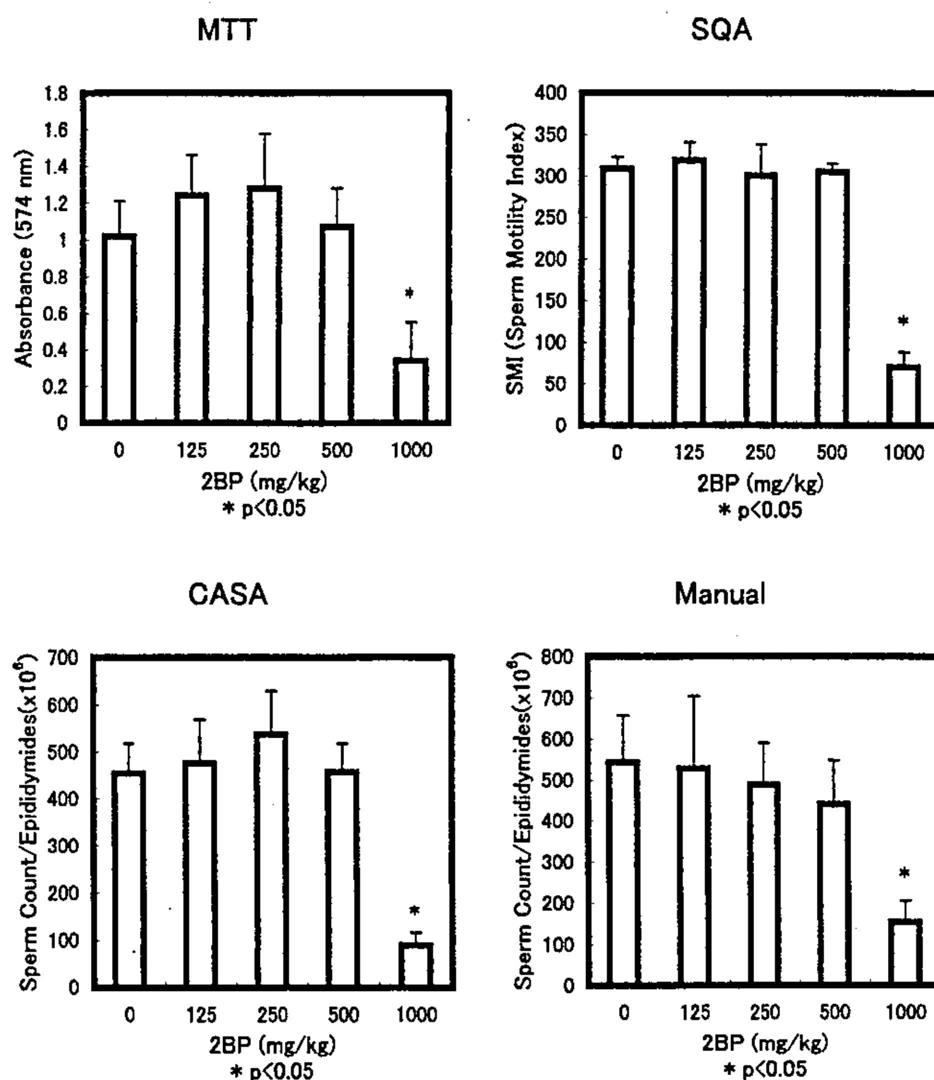

Fig. 3. Effect on rat sperm of 2BP (Comparison of results obtained by the MTT, SQA, CASA and Manual methods).

(R^2=0.9806). Similarly, MTT absorbance correlates well with the Sperm count by CASA (R^2=0.9725) and the manual method (R^2=0.9657). All the methods had common linearity mutually.

Detection of sperm toxicity of 2BP by several methods

As shown in Fig. 3, sperm count by the manual method, sperm count by CASA, SMI by the SQA method and absorbance by the MTT method were similarly low only in the 1,000 mg/kg group as compared with the control group. There was no significant reduction in the parameters in the groups given 2BP at the dose of less than 500 mg/kg.

Discussion

Only the testes weight could indicate sperm toxicity in the 500 mg/kg dose group, whereas while other methods failed to detect the change (Table 1 and Fig. 3). Although the weight of testes was the most sensitive parameter indicating the sperm toxicity, this requires autopsy and therefore has no clinical usefulness. A non-invasive method such as sperm count under an optical microscope is more practical and is widely accepted, but inherent shortcomings of this method are obvious: counts are susceptible to subjective variations and their inter-institutional or inter-observer comparisons and analyses are unreliable if ever possible, and it is not suitable for processing a large number of specimens in mass screening.

SQA is a simple and practical method, but it is no match in efficiency for the MTT method. In comparison, the MTT method excels SQA on account of rapidity and simplicity and therefore its ability to handle a large number of samples simultaneously. CASA gives not only the sperm count, but other information such as the motility rate and even morphological indexes[8-13]. Unfortunately this requires rather expensive equipment and has not achieved wide acceptance. MTT, one of the tetrazolium salts, is known to form formazan and is turned blue in the somatic cells by mitochondrial reductase[16]. The same reaction is observed in a suspension of sperm and the extent of coloring reflects the number and viability of the sperm[17]. The MTT method is utilized in many toxicity studies on somatic cells, but its use in sperm cell studies has been reported in only one paper. We introduced the use of the microplate and established a distinctly more efficient measurement system. If only qualitative analysis is required, direct observation may suffice, dispensing with absorption spectrometry. Possibly other tetrazolium salts may be found equally or more useful as the substrate and may replace MTT. We proposed the use of the MTT method for sperm analysis and established the protocol with microplates to facilitate processing a large number of samples rapidly as is required in mass screening. In the sperm count we obtained high correlation between the results by the MTT method and those by SQA, CASA, and the manual method as shown in Fig 2. We believe that the MTT method can replace these other methods where only the sperm count is required, but the MTT reaction is dependent on the activity of mitochondrial reductase in the sperm. This method cannot be expected to distinguish those sperm with abnormal morphology or diminished motility from normal, healthy sperm, as long as they have metabolic activity. On the other hand, the SMI value which is obtained by SQA has a positive correlation with both sperm count and sperm motility and is recognized as a strong predictor of fertility of the semen. The manual method can distinguish sperm deformity as well as give the sperm count. The advantage of the MTT method, on the other hand, is the efficiency in processing a large number of specimens and therefore may be a powerful tool for preliminary screening. The results of our animal experiments also established that the MTT method could detect sperm toxicity caused by introduced chemical agents to an extent comparable to other methods such as the manual method or CASA.

In conclusion we assert that the MTT method using the microplate reader provide a new tool in detecting sperm toxicity with sensitivity comparable to conventional or more expensive methods and is especially suitable for workplace mass screening. But manual dissolution of formazan is an extra step required in the MTT method. It is not readily amenable to automation. And this is a cause of errors in measurement. Recently tetrazolium salts which produce water-soluble formazan have been developed and their usefulness in toxicity tests on somatic cells has been reported. When these newer salts are used the process of dissolution of formazan is not necessary. Enhanced simplicity and improved accuracy of the method are expected. We plan to continue further studies on sperm toxicity using various tetrazolium salts to replace MTT.

In our experiments, distinct sperm toxicity was observed only in the group of rats given a large dose (1,000 mg/kg) of 2BP whereas a significant reduction in the weight of testes had already been found in the 500 mg/kg group (Table 1). This suggests that 2BP exerts its effect in the spermatogenesis stage. And reverse effects were observed in several cases in organ weight (EPCR: 125 mg/kg, EPCL: 250 mg/kg). This phenomenon is thought to result in a transitory effect.

Furthermore, we have started the study of reproductive toxicity induced by bromopropanes other than 2BP and have found that the MTT method is equally applicable to in these studies. We plan to present additional data on 2BP and other chemicals and further discussion on the mechanism of reproductive toxicity in our ensuing reports.

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Comparative Study on Picryl Chloride (PCL)-Induced Contact Dermatitis in Female IQI/Jic and BALB/c Mice

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Abstract: *Ear skin responses to picryl chloride (PCL)-induced contact dermatitis were compared in detail between IQI/Jic mice developed in Japan and BALB/c mice often used for the investigation of contact dermatitis. PCL was applied to the left ear of each mouse 4 (1st), 11 (2nd), 18 (3rd) and 25 days (4th) after sensitization of the abdominal skin with PCL. Time course examinations were carried out on the ear swelling responses, total IgE levels, skin histology and immunohistochemistry for infiltrated cells after the 1st and 4th application. In IQI mice, the peak time of the ear swelling responses tended to shift from 24 h to 9 h with marked elevation of total IgE levels and marked increase of mast cells showing degranulation after the 4th application when $CD8^+$ cells as well as $CD4^+$ cells also prominently increased. In BALB/c mice, except for the total IgE levels and the number of mast cells, the degrees of ear swelling responses, histological changes and increase of $CD4^+$ and $CD8^+$ cells were much less severe. Female IQI mice are considered to be a useful mouse strain for further investigations on the role of $CD4^+$ and $CD8^+$ T cells in the pathogenesis of contact dermatitis.*

Key words: *BALB/c mice, contact dermatitis, IQI/Jic mice, picryl chloride.*

Introduction

Contact dermatitis is a common and important occupational health problem. It develops in susceptible individuals, who have been sensitized with a certain haptenic chemical, after epicutaneous exposure to the same chemical. The number of patients suffering from contact dermatitis is increasing year by year, and many chemicals are thought to participate in skin sensitization. This suggests the necessity of an accurate hazard evaluation, but the exact etiology and pathogenesis of contact dermatitis have not fully been clarified yet. To find clues to solve these problems, comparative studies using various animal models are necessary.

IQI/Jic (IQI) mice are an ICR-derived inbred strain developed in Japan [10], and accumulated evidence suggests the existence of certain abnormalities in their immune system. Namely, they induce a high level of antinuclear auto-antibody following mercuric chloride treatment [21], have thymic B cells [19], and show an age-related development of Sjögren's syndrome-like sialadenitis [20]. In addition, the prevalence of allergic

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skin lesions of a spontaneous nature has been noticed in aged female IQI mice kept in an animal room of the National Institute of Industrial Health, Japan, suggesting that female IQI mice would be useful animals for the induction of contact dermatitis [8].

The present study was carried out to compare the ear skin responses to picryl chloride (PCL)-induced contact dermatitis in detail between female IQI/Jic mice and BALB/c mice, which are most often used for the induction of contact dermatitis [12, 13]. The present study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Materials and Methods

Mice: Fifty-six 8-week-old female IQI/Jic (IQI) mice (National Institute of Industrial Health, Kawasaki, Japan) and 56 age-matched female BALB/c CrSlc (BALB/c) mice (Japan SLC Co., Hamamatsu, Japan) were used for examination at 10 weeks of age after acclimatization for 2 weeks. The animals were individually kept in polycarbonate cages in an air-conditioned animal room (temperature: 23 ± 2°C; relative humidity: 55 ± 5%; light/dark cycle:12/12 h). They were fed standard laboratory pellets (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* throughout the experimental period.

Treatments: The animals were sensitized with an application of 150 µl of 5% picryl chloride (PCL) (Nakalai Tesque Inc., Kyoto, Japan) to the shaved skin of the abdomen. At 4 (1st), 11 (2nd), 18 (3rd) and 25 days (4th) after the sensitization, 20 µl of 0.8% PCL was topically applied to the left ear. PCL was dissolved in acetone and olive oil (4:1). Vehicle alone was applied to the right ear of each animal in the same way.

Ear swelling response: Ear thickness of each mouse was measured at 0, 1, 3, 6, 9, 12, 24 and 48 h after each application. The ear swelling response was judged by the difference between the ear thickness of the left and right ears of each animal at each time point.

Total serum IgE levels: Total serum IgE levels were measured at 0, 9 and 24 h after the 1st and 4th applications by the sandwich ELISA method using a mouse IgE measuring kit "YAMASA" EIA (Yamasa Shoyu Co., Ltd., Choshi, Japan).

Histopathology: At 0, 3, 6, 9, 12 and 24 h after the 1st and 4th applications, 4 mice of each strain were sacrificed by heart puncture under deep CO_2/O_2 anesthesia. The ears to which PCL or vehicle had been applied were taken from each mouse and fixed in 10% neutral buffered formalin. Paraffin sections (5 µm) were stained with hematoxylin and eosin (HE) or toluidine blue (TB). The number of mast cells was calculated on TB-stained sections (5 areas/section/animal) obtained from each animal at 0, 9 and 24 h after the 1st and 4th application under a light microscope (× 200), and the mean value was calculated for each animal.

Immunohistochemistry: Pieces of the left ear sample obtained from each animal at 0, 9 and 24 h after the 1st and 4th applications were embedded in O.T.C. compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan), rapidly frozen in dry ice-acetone, and then stored at −80°C until used. Cryosections (6 µm) were fixed in acetone for 10 min before immunohistochemical staining by the avidin-biotin-peroxidase complex (ABC) method using Vectastain ABC kit (Vector Laboratories, California, USA). As the primary antibodies, rat monoclonal antibodies against mouse CD4 (clone RM4–5), CD8 (clone 53–6.7), Mac-1 (clone M1/70), CD19 (clone 1D3), and MHC class II (I-A^d/I-E^d, clone 2G9) (Pharmingen, California, USA) were used. After ABC reaction, sections were visualized in 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis MO, USA) solution. Sections were counterstained with methyl green. The number of positive cells for each antibody was calculated on immunostained sections (5 areas/section/animal) under a light microscope (× 400), and the mean value was calculated for each animal.

Statistical analysis: The data of ear swelling responses, total serum IgE levels, and the numbers of mast cells, and $CD4^+$, $CD8^+$, Mac-1^+, $CD19^+$ and MHC class II^+ cells were expressed as mean ± standard deviation (SD) of 4 animals at each point of measurement. The data obtained at 0 h after the 1st application, i.e. just before application, were used as controls. Statistical analysis was done using Student's t-test.

Results

Ear swelling response: The severity of ear swelling responses was much stronger in IQI mice than in BALB/

c mice, and the ear thickness increased with the number of applications in both strains (Fig. 1). In IQI mice, although not significant, the peak time of ear swelling response tended to shift from 24 h to 9 h after the 4th application (Fig. 1a). On the other hand, BALB/c mice showed an acute-type response, and the peak time did not show such a shift even after the 4th application (Fig. 1b).

Total serum IgE levels: There were no significant increases in the total serum IgE levels after the 1st application in both strains. After the 4th application, the levels were greatly elevated in both strains without strain difference (Fig. 2).

Histopathological findings: There were no histopathological changes in the ear skin which was topically applied with vehicle alone.

In the dermis of the left ear of IQI mice, edema slightly occurred at 3 h after the 1st application and progressed towards 24 h (Fig. 3a). Infiltration of inflammatory cells mainly composed of neutrophils started at 6 h and progressed thereafter (Fig. 3a). Neutrophil infiltration was also observed in the epidermis. After the 4th application, edema with inflammatory cell infiltration was most conspicuous at 9 h (Fig. 3b), and the infiltrated cells included mast cells, mononuclear cells and eosinophils as well as neutrophils. In addition, epidermal hyperplasia, intra-epidermal inflammatory cell infiltration and formation of immature granulation tissues in the superficial dermis were also observed (Fig. 3b). Edema and inflammatory cell infiltration decreased towards 24 h.

In BALB/c mice, as compared with IQI mice, neutrophil infiltration was almost similar, but edema was less severe and epidermal hyperplasia after the 4th application was apparently weaker (Figs. 3c and 3d). The changes were most prominent at 9 h after both the 1st and the 4th application.

The number of mast cells showed no significant increase after the 1st application in both strains. After the 4th application, the number increased to levels about 2 to 3 times higher than those after the 1st application in both strains (Fig. 4), and, as compared with after the 1st application, degranulation of mast cells was frequently observed (Fig. 5).

Immunohistochemical findings: Table 1 shows the outline of immunohistochemical findings. CD19+ cells were rarely seen in both strains throughout the experi-

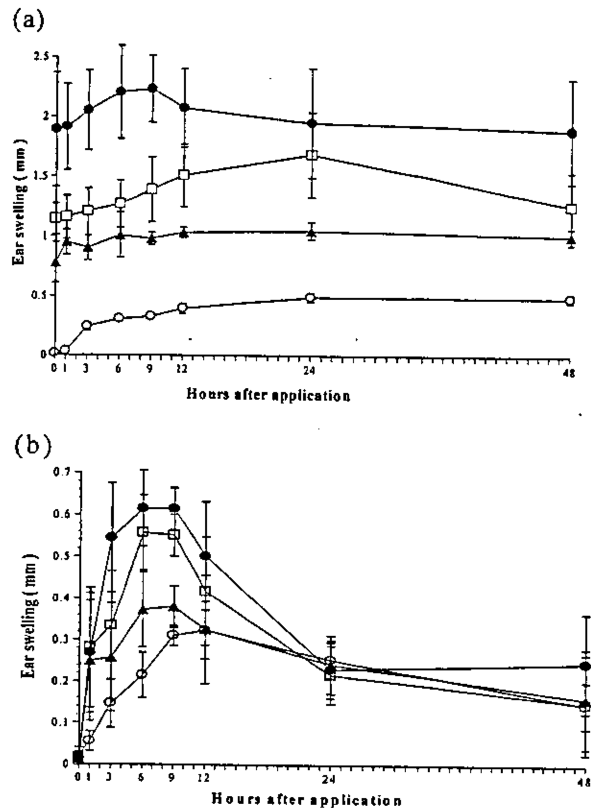

Fig. 1. Ear swelling responses in female IQI/Jic (a) and BALB/c (b) mice topically applied with PCL at 4 (○), 11 (▲), 18 (□) and 25 (●) days after the sensitization with PCL. Data are shown as mean ± SD (mm).

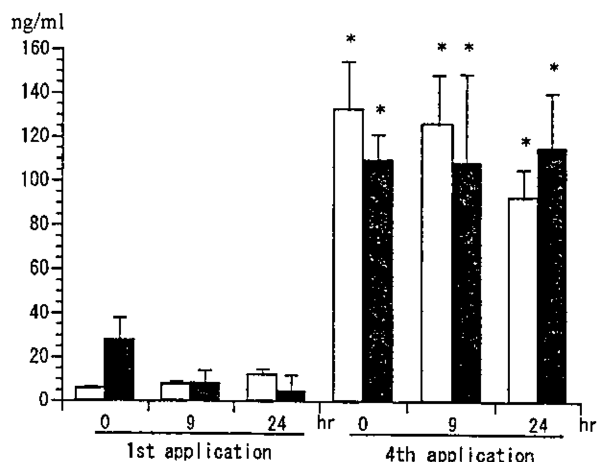

Fig. 2. Total serum IgE levels after the 1st and 4th applications of PCL following sensitization with PCL in female IQI/Jic (open column) and BALB/c (black column) mice. Data are shown as mean ± SD (ng/ml). * $P<0.05$: Significantly different from the value at 0 h after the 1st application.

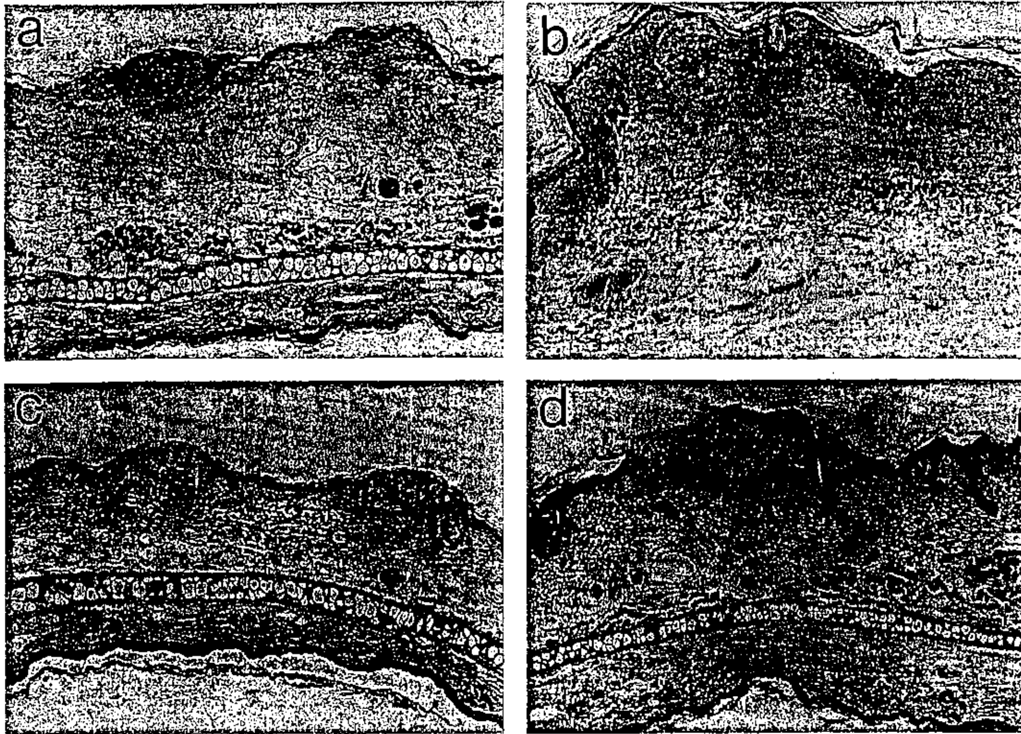

Fig. 3. Histopathological findings of the ear skin at 24 h after the 1st application (a, c) and at 9 h after the 4th application (b, d) of PCL following sensitization with PCL in female IQI/Jic (a and b) and BALB/c (c and d) mice. HE. (a) Prominent edema with neutrophil infiltration. × 120. (b) Besides intradermal edema and inflammatory cell infiltration, epidermal hyperplasia and intradermal connective tissue proliferation are seen. × 80. (c) Similar but less severe changes are seen compared with (a). × 120. (d) Similar but less severe changes are seen compared with (b). × 80.

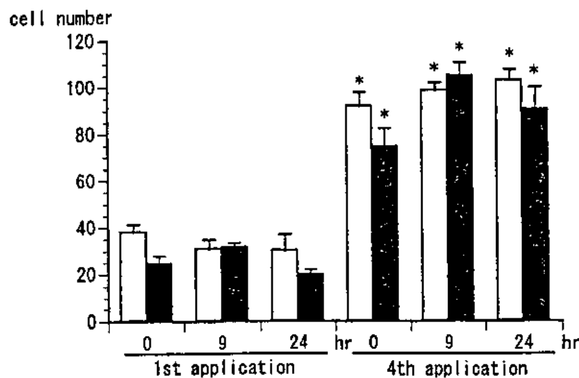

Fig. 4. The number of mast cells after the 1st and 4th applications of PCL following sensitization with PCL in female IQI/Jic (open column) and BALB/c (black column) mice. Data are shown as mean ± SD. * $P<0.05$: Significantly different from the value at 0 h after the 1st application.

mental period. At 0 h after the 1st application, the number of MHC class II^+ cells was significantly larger in IQI mice than in BALB/c mice, although the numbers of $CD4^+$, $CD8^+$ and Mac-1^+ cells were similar between the two strains.

After the 1st application, in IQI mice, the numbers of $CD4^+$ (Fig. 6a) and Mac-1^+ cells were significantly increased at 9 and 24 h, while the numbers of $CD8^+$ and MHC class II^+ cells did not show any significant changes (Table 1). BALB/c mice also showed similar changes except for a significant increase in the number of MHC class II^+ cells at 24 h (Table 1). In addition, the number of $CD4^+$ cells at 24 h was significantly larger in BALB/c mice than in IQI mice.

After the 4th application, in both strains, the numbers of $CD4^+$, $CD8^+$, Mac-1^+ and MHC class II^+ cells had markedly increased at 0 h (Table 1), and their numbers except for Mac-1^+ cells were maintained at almost the same levels thereafter. From the comparative view-

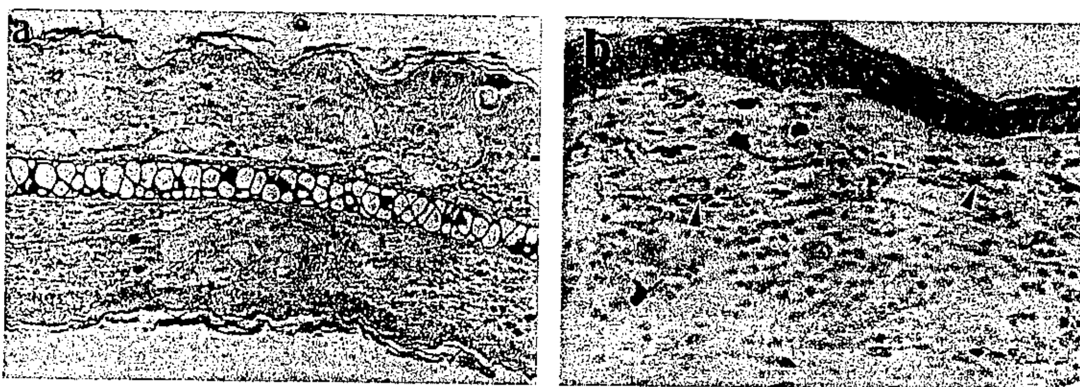

Fig. 5. Ear skin of female IQI/Jic mice at 24 h after the 1st application (a) and at 9 h after the 4th application (b). (a) Mast cells show no degranulation. TB, × 160. (b) There are many mast cells including those showing degranulation (arrowheads). TB, × 360.

Table 1. Immunohistochemistry of component cells in the ear of IQI/Jic and BALB/c female mice at 0, 9 and 24 h after the 1st and 4th applications of PCL following sensitization with PCL

Strain		1st application			4th application		
		0 h	9 h	24 h	0 h	9 h	24 h
IQI/Jic	CD4	1.3 ± 1.5	10.1 ± 3.2*	10.9 ± 1.6*-	40.5 ± 6.3*-	45.6 ± 4.2*-	48.4 ± 1.6*-
	CD8	0.7 ± 0.6	1.3 ± 0.8	1.7 ± 0.5	14.7 ± 1.8*-	17.7 ± 2.4*-	16.5 ± 1.7*-
	Mac-I	0.3 ± 0.4	22.0 ± 4.4*-	74.8 ± 5.3*	34.0 ± 5.5*	105.6 ± 9.9*‡	108.4 ± 14.4*‡
	MHC II	29.3 ± 3.5-	35.7 ± 3.3-	35.3 ± 4.7	59.7 ± 2.5*-	75.1 ± 8.7*‡-	66.7 ± 2.9*-
BALB/c	CD4	1.1 ± 1.4	8.3 ± 2.6*	24.4 ± 3.1*	25.5 ± 4.8*	29.3 ± 6.5*	35.2 ± 7.6*
	CD8	0.7 ± 0.5	1.6 ± 0.6	1.2 ± 0.7	7.3 ± 1.8*	10.7 ± 1.9*	10.2 ± 2.6*
	Mac-1	0.4 ± 0.5	38.1 ± 6.3*	78.6 ± 6.2*	31.0 ± 6.1*	111.3 ± 17.1*‡	104.0 ± 12.7*‡
	MHC II	12.3 ± 2.3	17.5 ± 5.3	27.7 ± 7.5*	45.7 ± 5.7*	49.1 ± 7.4*	50.8 ± 8.9*

Data are expressed as the mean ± SD of 4 mice per group. * P<0.05, Significantly different from the value at 0 h after 1st application. ‡ P<0.05, Significantly different from the value at 0 h after 4th application. - P<0.05, Significantly different from the value in BALB/c mice.

point, the numbers of CD4+, CD8+ and MHC class II+ cells were significantly larger in IQI mice than in BALB/c mice, while there was no significant difference in the number of Mac-1+ cells between the two strains (Table 1) (Figs. 6b-6f).

Discussion

Contact dermatitis results from epicutaneous sensitization and challenge with haptens [2, 12, 13, 16]. In the present study, the ear skin responses to PCL-induced contact dermatitis were examined in detail between female IQI mice and BALB/c mice, which have been often used for the induction of contact dermatitis.

The degree of the ear swelling response increased with the number of applications of PCL following sensitization with the same chemical in both strains, and it was much more severer in IQI mice than in BALB/c mice. In this study, the peak time of the ear swelling response tended to shift from 24 h to 9 h after the 4th application in IQI mice, whereas the ear swelling response in BALB/c mice was generally acute-type (peak time: 6–12 h). Kitagaki *et al.* [13] reported that immediate-type hypersensitivity response with increase of antigen-specific IgE levels followed by a late reaction was induced by repeated epicutaneous application of 2,4,6-trinitro-1-chlorobenzene or PCL following the sensitization with the same chemical in BALB/c mice [12, 13]. Nagai *et al.* [16] also reported similar findings in

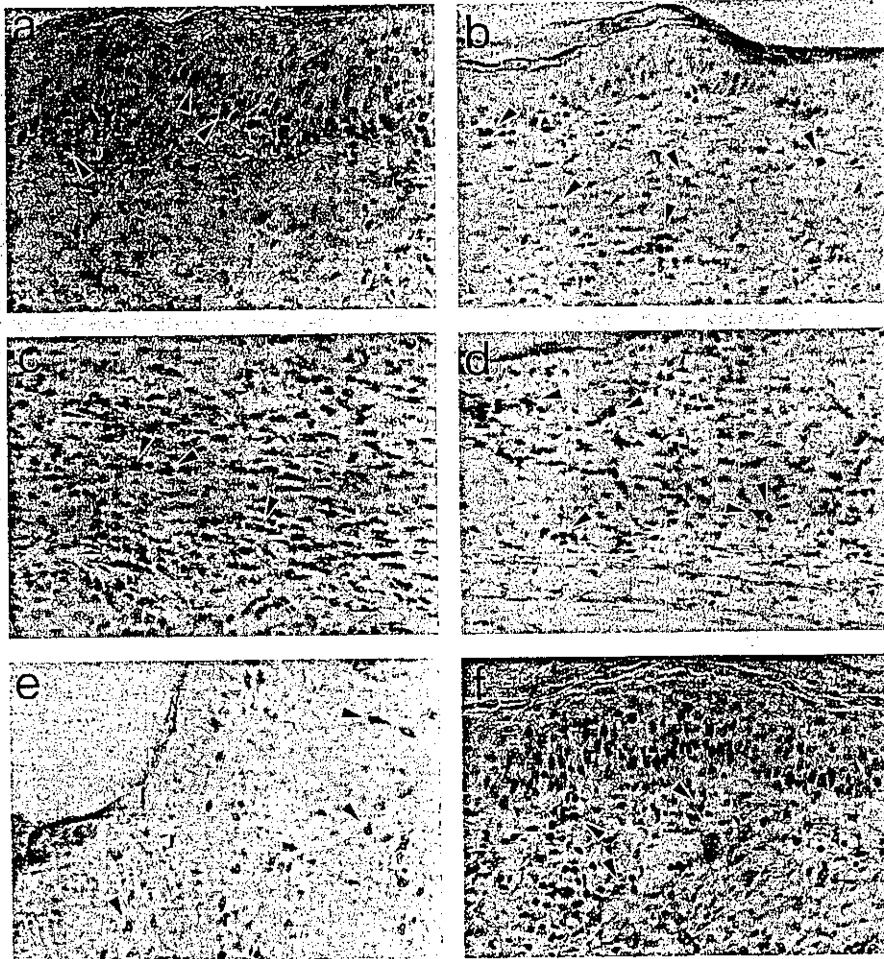

Fig. 6. Immunohistochemical staining of the ear at 24 h after the 1st application (a) and at 9 h after the 4th application of PCL (b-f) following the sensitization with PCL in female IQI/Jic (a-d, f) and BALB/c (e) mice. × 240. (a) Moderate numbers of CD4$^+$ cells (arrowheads). (b) Many CD4$^+$ cells (arrowheads). (c) Moderate numbers of CD8$^+$ cells (arrowheads). (d) Many Mac-1$^+$ cells (arrowheads). (e) Many CD4$^+$ cells (arrowheads). (f) Moderate numbers of MHC class II$^+$ cells (arrowheads).

mice treated with 2,4-dinitrofluorobenzene. They suggested that a shift from a Th1-dominant response to a Th2-dominant one might occur following repeated applications. Recent studies have also demonstrated that, in addition to allergic dermatitis, many immunological responses start as Th1-dominant type and then shift to Th2-dominant ones [2, 5, 18]. Further studies on cytokine profiles in PCL-induced contact dermatitis are now in progress in IQI and BALB/c mice.

The peak time of histopathological response characterized by edema and inflammatory cell infiltration corresponded well to that of the above-mentioned ear swelling response. After the 4th application, the number of mast cells increased prominently, and the total serum IgE level was also elevated markedly in both strains with no strain difference. IgE response differs with the sensitizing agents used. For example, trimellitic anhydride and diphenylmethane-4,4'-diisocyanate induced dermatitis with increase in total serum IgE while dermatitis induced by 2,4-dinitrochlorobenzene, dicyclohexylmethane-4,4'-diisocyanate and isophorone diisocyanate was not accompanied by the production of IgE [3, 4]. Degranulation of mast cells was conspicuous after the 4th application. Degranulation of mast cells was also confirmed by electron microscopic examination [9]. These findings suggest a relationship

between the elevation of IgE levels and the degranulation of mast cells. Mast cells are well known to produce a wide variety of mediators and cytokines [11] which may be important in the pathogenesis of contact dermatitis.

The immunohistochemical examinations showed that IQI mice had more MHC class II+ cells in the ear dermis than BALB/c mice by nature while the numbers of CD4+, CD8+ and Mac-1+ cells were similar between the two strains. There are several reports suggesting an important role of keratinocytes in the induction of contact dermatitis through expression of MHC class II [7, 17]. In the present study, the majority of MHC class II+ cells were however considered to be dermal cells in both strains throughout the experimental period.

After the 1st application, the numbers of CD4+ and Mac-1+ cells significantly increased in both strains at 9 and 24 h. After the 4th application, the numbers of CD4+, CD8+ and Mac-1+ cells as well as MHC class II+ cells were much more increased in both strains already at 0 h, and, except for that of Mac-1+ cells, they maintained almost the same levels thereafter. The absolute numbers of CD4+, CD8+ and MHC class II+ cells were significantly larger in IQI mice than in BALB/c mice, and this may be related to the difference in the severity of contact dermatitis between IQI and BALB/c mice. On the other hand, CD19+ cells, i.e. B lymphocytes, were however almost negligible throughout the experimental period.

Reports of T cell subsets infiltrating the skin lesion of contact dermatitis are conflicting. For example, some studies indicated that contact dermatitis is a CD4+ cell-mediated response [6, 15], and others concluded that CD8+ cells are the effectors and CD4+ cells function as negative regulators [1, 14, 22]. As mentioned above, although CD4+ cells were a predominant T cell subset after both the 1st and the 4th applications, a significant increase in the number of CD8+ cells was detected after the 4th application in both strains. This suggests that, although CD4+ cells play a main role, CD8+ cells may also participate in the induction of contact dermatitis at least after the 4th application with PCL.

In conclusion, PCL-induced contact dermatitis was markedly more prominent in female IQI mice than in female BALB/c mice, and the peak time of the ear swelling responses tended to shift from 24 h to 9 h in IQI mice while it was 6–12 h throughout the observation period in BALB/c mice. We considered female IQI mice will be a very useful animal for further investigation of the roles of CD4+ and CD8+ cells in the pathogenesis of contact dermatitis.

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Effects of 50Hz Electromagnetic Fields on Reproduction in Mice. A Three Generations Study.

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Abstract

Some epidemiological studies suggested that electromagnetic fields (EMF) might have adverse effects on reproduction in occupationally exposed workers. The aim of the present study is to elucidate the effects of 50Hz EMF on reproduction in experimental mouse systems. Three consecutive generations of IQI/Jic mice were kept continuously in 1mT, 0.1mT and control (less than 0.05μT) MF. In every generation, male and female mice weighing more than 33g and 28g at 10 weeks old, respectively, were selected and 5 or 6 pairs (1 male and 2 female) mated for 2 weeks, and related pregnancy outcome was observed. Sperm in cauda epididymus, testicular weights and plasma testosterone levels were examined in 12 weeks old male mice. In all generations, incidence of pregnant female mice, total number of weanlings, their ratio of male to female, and their weight gain were not different significantly between each group. Sperm motility, testicular weight, and plasma testosterone levels were similar in all groups. These results indicated that 50Hz EMF might not effect on male reproductive system or related pregnancy outcome in mice even after continuous exposure over 3 generations.

Introduction

Several epidemiological studies suggested potential effect of extremely low frequency electromagnetic fields (EMF) to human reproduction such as male infertility, reduced semen quality, early pregnancy loss, low birth weight and malformation. However, available epidemiological studies have limitations and there is no convincing evidence that occupational or daily life exposure to MF do any harm to human reproduction [1].

The results of animal studies on EMF are rather consistent and do not suggest adverse effects on reproduction nor development. On the other hand, there are a few reports indicating decrease of testicular weight and sperm account in mice after 4 weeks exposure to EMF [2], and increase of fetal loss in mice exposed during early pregnancy [3]. Therefore, this is an area that needs further investigation.

The aim of the present study is to elucidate the effects of 50 Hz MF on reproduction in mice through multiple generations.

Materials and Methods

Animals: IQI/Jic mice were originally introduced from the Central Institute for Experimental Animals (Kawasaki, Japan) and were maintained in the animal facilities of National Institute of Industrial Health.

EMF exposure: Three EMF exposure sets (Sho-Den, Tokyo Japan) were placed in an experimental house with a light cycle (12hrs light and 12 hrs dark). Each coil set consisted of vertically and horizontally oriented four square coils. Uniform magnetic fields were obtained in 80 cm high, 80 cm wide and 80 cm deep cubic area. Fifty Hz magnetic fields of 1 mT and 0.1 mT were generated for exposure. Exposure levels were checked continuously and were kept within 10% variation of target strength. Magnetic field of control area was kept as low as possible and its level was less than 0.05 μT during the experiment. Animal chambers were placed in the central area of each exposure set, and were supplied HEPA filter passed conditioned air (22±2 °C temperature and 50±15% relative humidity). Mice were kept in plastic cages (5 to 6 mice/cage) and allowed to get pellet (CE2, CLEA, Tokyo, Japan) and tap water *ad libitum*.

Experimental design: Mice were exposed to EMFs continuously, except for times of daily observation and cage exchange. Parental mice were transfered to exposure chambers at 10 weeks old. Three pairs (1 male and 2 female) mated for 2 weeks in each exposure group. Incidence of pregnancy and lactation were checked in female mice. Number of weanlings and their ratio of male to female were observed. In consecutive next 3 generations (F1 to F3), male and female mice weighting more than 33g and 28g at 10 weeks old, respectively, were selected and 5 or 6 pairs mated for 2 weeks and related pregnancy outcome as mentioned above were observed. The body

weight of weanlings were examined at 4 weeks old. At 12 weeks old, 15 male mice were anesthetised in chroloform and bled by heart puncture, and then testes were removed and thier weights were recorded. Sperms in cauda epididymus were also examined in 5 of them.

Plasma testosterone: Plasma was seperated and kept in freezer (-20°C) before titration of testosterone. Plasma testosterone levels were measured duplicately with the time-resolved fluoroimmunoassay using a commertial testosterone reagents kit (DELFIA, Wallac, Turku, Finland).

Sperm analysis: Cauda epididymus was minced in medium 199 containing 0.5% bovine serum albumin and was gently stirred to make sperm suspension. The sample was stained by a staining kit (Supra Vital IDENT stain kit) and then obsereved by sperm analyser (Hamilton Thorne Bioscience, MA, USA). More than 200 sperms were counted, and motile and progressive ratios were determined.

Results and Discussion

Incidence of pregnant female mice and lactating dams in parental to F3 were shown in Table 1. In all generations, more than 75% of female mice became pregnant and more than 80% of them successfully suckled their pups, while 4 of 10 dams of 0.1 mT group in F1 did not nurse their babies. The average numbers of suckling mice per dam were similar in all exposed groups in every generation. The numbers of weanlings in EMF exposed groups were equal to or more than those of control group in every generation except for 0.1 mT group in F1. The ratio of male to female was similar in all exposure groups, though male mice number was 1.5 times of female ones in 1 mT group in F1. Body weights of weanlings at 4 weeks old were similar in all groups in F1 to F3 generation. Any body abnormality or malformation could not be found by gross observation in weanlings. These results strongly indicated that EMF did not have any adverse effect on reproduction of both male and female mice, and embryonic and fetal development during pregnancy when they were continuously exposes to EMF over 3 generations.

Table 1. Related pregnancy outcome in over 3 generations

Generation (No. of mated female mice)	EMF	Dams		Weanlings		Body weight (g) of weanlings at 4weeks old	
		Pregnant	Lactating	Total number	Male : Female	Male	Female
Parent	Control	6	5	37	17 : 20	ND	ND
(6)	0.1 mT	6	4	35	19 : 16	ND	ND
	1 mT	5	5	39	18 : 21	ND	ND
F1	Control	12	10	81	47 : 34	22.0±3.4	20.4±2.0
(12)	0.1 mT	10	6	44	21 : 23	23.9±2.6	23.2±3.1
	1 mT	12	10	85	51 : 34	24.5±2.3	21.6±1.5
F2	Control	8	7	67	37 : 30	23.3±2.1	20.9±1.5
(10)	0.1 mT	10	10	91	49 : 42	22.8±3.0	20.3±2.8
	1 mT	10	8	85	40 : 45	23.2±2.9	20.0±2.9
F3	Control	9	8	59	22 : 37	25.6±1.7	22.2±1.5
(12)	0.1 mT	9	7	64	26 : 38	25.2±1.9	20.8±1.6
	1 mT	10	8	91	46 : 45	24.7±2.2	21.1±1.7

Control area was kept less than 0.05 μT. ND: not determined

Testicular weight and plasma testosterone of 12 weeks old male mice are shown in Table 2. Through F1 to F3 generations, total testicular weight of male mice at 12 weeks old were around 230 mg in all groups in every generation. There was not a testis weighting less than 100 mg in all groups, suggesting that EMF exposure from embryo to sexual maturation did not disturb testicular development. The plasma testosterone levels were variable (ranging from 0.2 to 35 ng/ml) in the present study. Though mean testosterone level of 1 mT group were lower than that of control group in F1generation, the level was equal to those of all groups in F2 generation. On the contrary, testosterone level of control group in F1 was similar to those of all groups in F3 generation. Therefore, we considered that testosterone levels were kept in a reasonable normal range in all groups in every generation, suggesting that there might not be adverse effect of EMF on testosterone production.

Table 2. Testicular weight and plasma testosterone of 12 weeks old male mice

Generation	Total testicular weight (mg)			Plasma testosterone (ng/ml)		
	Control	0.1mT	1 mT	Control	0.1mT	1 mT
F1	232 ± 16	227 ± 19	234 ± 19	13 ± 8	11 ± 8	6 ± 6
F2	230 ± 14	227 ± 20	228 ± 18	6 ± 12	7 ± 9	6 ± 10
F3	237 ± 13	231 ± 22	226 ± 13	12 ± 14	13 ± 15	10 ± 15

Mean ± SD

Motile and progressive sperms in cauda epididymus were 35 to 55% and 14 to 24%, respectively (Table 3)

except for a case having only 1% motile sperm in 0.1 mT group of F1. Comparing the results between groups, ratios of motile sperms were similar in all groups in all generations. These results suggested that EMF exposure might not effect on sperm development or sperm activity. On the other hand, however, there is a tendency that progressive sperms in EMF exposed groups were a little less than those of control group in all generations (Table 3). The significance of the decreased progressiveness in sperm was not certain and further studies are needed.

Table 3. Motile and progressive sperms in cauda epididymus of 12 weeks old male mice

Generation	Motile (%)			Progressive (%)		
	Control	0.1 mT	1 mT	Control	0.1 mT	1 mT
F1	49.8 ± 11.2	35.4 ± 19.6	40.6 ± 11.9	19.3 ± 5.3	14.0 ± 7.4	14.0 ± 3.7
F2	56.2 ± 6.4	55.3 ± 13.6	47.0 ± 12.8	24.3 ± 5.9	20.5 ± 5.9	18.2 ± 3.7
F3	35.8 ± 11.9	35.2 ± 9.4	38.2 ± 11.8	19.4 ± 10:3	16.2 ± 5.9	14.8 ± 4.9

Mean ± SD

In the present study, we observed reproduction of IQI/Jic mice continuously exposed to up to 1 mT EMF over 3 generations. In all generations, female mice mated under EMF exposure became pregnant, and successfully delivered pups and lactated well as non-exposed dams. Suckling mice grew well in both exposed and non-exposed group. Testicular weights, plasma testosterone levels and sperm activity were similar in exposed and non-exposed male mice in all generations. Based on these results, we concluded that 50Hz EMF does not affect on both male and female reproduction and embryonic and fetal development in mice. It is suggested furthermore that there could not be any cumulative and genetic adverse effects through multiple generations.

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ATP Amplification for Ultrasensitive Bioluminescence Assay: Detection of a Single Bacterial Cell

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ATP Amplification for Ultrasensitive Bioluminescence Assay: Detection of a Single Bacterial Cell

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We developed an ultrasensitive bioluminescence assay of ATP by employing (i) adenylate kinase (ADK) for converting AMP + ATP to two molecules of ADP, (ii) polyphosphate (polyP) kinase (PPK) for converting ADP back to ATP (ATP amplification), and (iii) a commercially available firefly luciferase. A highly purified PPK-ADK fusion protein efficiently amplified ATP, resulting in high levels of bioluminescence in the firefly luciferase reaction. The present method, which was approximately 10,000-fold more sensitive to ATP than the conventional bioluminescence assay, allowed us to detect bacterial contamination as low as one colony-forming unit (CFU) of *Escherichia coli* per assay.

Key words: ATP; polyphosphate; ATP amplification; bioluminescence assay; hygiene monitoring

ATP plays a central role in all aspects of metabolism, and therefore the development of methods to detect very low concentrations of ATP is very important in many areas of pure and applied biochemistry. The firefly luciferase-based (bioluminescence) assay for detection of ATP is a well-established technique[1] and has been used as a way to monitor the hygiene of food and non-food contact surfaces rapidly.[2] The bioluminescence assay may also be used for warning and detection of a biological warfare attack.[3] This assay technique, however, has a detection limit of approximately 10^4 colony-forming units (CFUs) of *Escherichia coli* per assay (equivalent to approximately 10^{-14} mol ATP), which is not sensitive enough for some industrial applications.

To increase the sensitivity of bioluminescence assay and its application to hygiene monitoring, several methods have been developed.[4–9] Sakakibara *et al.*, developed an effective method of treating biological samples with a combination of adenosine phosphate deaminase and apyrase to reduce extracellular ATP, which is a major impediment in improving the sensitivity of the bioluminescence assay for intracellular ATP.[4] The bioluminescence reaction degrades ATP to AMP. Therefore, enzymatic ATP cycling to convert AMP back to ATP with high-energy phosphoanhydride compounds increases the intensity of the luminescence by signal integration.[5,6] The instrument has been improved too. For example, the MicroStar™-RMDS (Nihon Millipore, Tokyo, Japan) consists of a highly sensitive camera that detects bioluminescence and performs computer-driven data analysis.[7] Cells are trapped on a special membrane, and ATP is extracted by dispersing extraction reagent onto the membrane. Cell numbers are quantified by counting luminescence spots displayed on the monitor.[8] Very recently Sakakibara *et al.*, have improved this filter-based bioluminescence assay to detect a single bacterial cell with the concomitant use of enzymes to reduce the extracellular ATP background, enzymatic ATP cycling, and the bioluminescence reaction.[9]

Based on the results of computer simulation, it has been proposed that ATP amplification using adenylate kinase (ADK) and pyruvate kinase has the potential to detect very low levels of ATP without using a photon detector as sensitive as described above.[10] Inorganic polyphosphate (polyP) is a linear polymer of many hundreds of phosphate residues linked by high-energy phosphoanhydride bonds. Its cellular function has recently been unveiled.[11] In this study we designed ATP amplification by employing (i) ADK as the first enzyme for converting AMP + ATP to two molecules of ADP and (ii) polyP kinase (PPK)[12] as the second enzyme for converting ADP back to ATP using polyP (Fig. 1). In this reaction, excess AMP and polyP were added to the reaction mixture to drive ADK and PPK equilibrium toward ADP and ATP formation respectively. The amplified ATP was then detected by bioluminescence in the firefly luciferase reaction. The present method, which was approximately 10,000-fold more sensitive to ATP than the bioluminescence assay without ATP amplification, allowed us to detect bacte-

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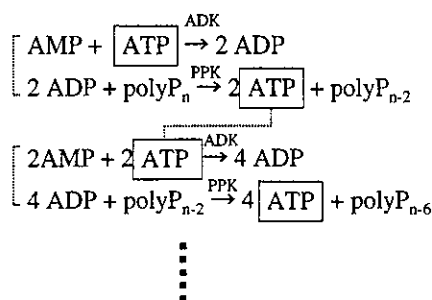

Fig. 1. ATP Amplification.

ADK, PPK, AMP, and excess polyP were prepared in the reaction mixture. ATP amplification started when ATP was added to the reaction mixture and ended when endogenous AMP was converted to ATP.

rial contamination as low as one CFU of *E. coli* per assay (equivalent to approximately 10^{-18} mol ATP).

Materials and Methods

Enzyme preparation. Genes encoding *E. coli* PPK (*ppk*)[12] and ADK (*adk*)[13] were amplified by PCR with primers (GGATCTAGATGAATAAAACGGAGTAAA-AGT and GGAGGATCCGCCGCCGCCGCCTTCAG-GTTGTTCGAGTGATTT) for *ppk* and (GGAGGATC-CATGCGTATCATTCTGCTTGGC and GGAAAGCT-TGCCGAGGATTTTTTCCAG) for *adk*, respectively. Each of the amplified DNA fragments was inserted into pGEMT vector (Promega, Wisconsin, U.S.A.), and the resultant plasmids were designated pGEMTppk and pGEMTadk respectively. A fusion gene of *ppk-adk* in that order with a C-terminal His-tag was constructed by ligating a 2.1-kb *Xba*I and *Bam*HI fragment of pGEMTppk, a 0.6-kb *Bam*HI and *Hin*dIII fragment of pGEMTadk, and *Xba*I and *Hin*dIII digested pET vector (Stratagene, California, U.S.A). *E. coli* BL21 carrying the resultant plasmid pETppkadk was incubated in 2xYT medium[14] for 2 h, and then 1 mM IPTG was added to the growing culture. After 4 h of incubation, *E. coli* BL21 cells were harvested by centrifugation and suspended in a 20 mM phosphate buffer (pH 7) containing 0.5 M NaCl. Cells were lyzed with a B-PER reagent (Pierce, Illinois, U.S.A) and then treated with DNase and RNase in the presence of 1 mM phenylmethylsulfonyl fluoride. The supernatant was obtained by centrifugation, filtered through a 0.2-μm membrane filter, and then applied to a Hitrap chelating column (Amersham Bioscience, Piscataway, U.S.A). The column was washed with 0.1 M pyrophosphate, 20 mM phosphate, and 0.5 M NaCl (pH 7.4). PPK-ADK fusion protein was eluted from the column with 20 mM phosphate, 0.5 M NaCl, 0.5 M imidazole, and 20% glycerol (pH 7.4). One unit of PPK synthesizes 1.0 μmol of ATP from ADP and polyP per min at 37 °C, while one of ADK produces 1.0 μmol of ATP from ADP per min at 37 °C. To remove ADP bound to PPK-ADK, 180 μg of PPK-ADK was incubated with 1 mM polyP for 30 min at 37 °C and then apyrase (200U) was added into the reaction mixture. After 1 h incubation, PPK-ADK was purified again by using the Hitrap chelating column. One unit of apyrase liberates 1.0 μmol of phosphate from ATP per min at 30 °C.

ATP amplification and bioluminescence assay. ATP amplification was started by adding a 2-μl ATP sample to 48 μl of a reaction mixture containing 0.16 μg of ADK-PPK, 10 μM of AMP, 400 μM of polyP, 8 mM $MgCl_2$, and 60 mM Tris–HCl (pH 7.4). The reaction mixture was incubated at 37 °C. After an appropriate period of incubation, a 5-μl reaction mixture was sampled and mixed with 40 μl of the ATP bioluminescence assay reagent (Roche, Basel, Switzerland). When the reaction reached equilibrium under these conditions, about 50% of the AMP was converted to ATP. The ATP sample from bacterial culture and water was prepared according to a protocol of the ATP bioluminescence assay (Roche). The *E. coli* culture (early stationary) was appropriately diluted with distilled water and heated at 100 °C for 2 min to release ATP from the cells. Bioluminescence was measured by using a multiplate luminometer (Wallac, Massachusetts, U.S.A.).

Chemicals. AMP and ATP were purchased from Wako chemical (Osaka, Japan) and Sigma (Missouri, U.S.A.), respectively. AMP was further purified by using a SAX column (Tohso, Tokyo, Japan) with 0.2 M KCl and 1% EDTA (pH10) as a solvent. PolyP (average chain-length: 65) was purchased from Sigma. The bioluminescence assay kit (CLSII) containing luciferin and luciferase was purchased from Roche. Apyrase was purchased from Sigma.

Results and Discussion

ATP amplification by PPK-ADK

A fusion protein of PPK and ADK with a C-terminal His-tag, designated PPK-ADK, was expressed and purified from *E. coli* recombinants. This fusion protein showed both ADK (43 U/mg) and PPK (38 U/mg) activities, generating ATP from AMP and polyP. Unexpectedly, however, ATP amplification was detected even when no exogeneous ATP was added to the reaction mixture (Fig. 2). We found that ADP, which could be converted to ATP by PPK in the presence of polyP, bound to PPK-ADK even after the first purification. To eliminate ADP contamination, we treated PPK-ADK with polyP, and then with apyrase which is capable of degrading ATP to AMP. PPK-ADK was further purified with a chelating column (Hitrap chelating). The addition of 0.1 M pyrophosphate to a washing buffer was also effective to release ADP from PPK-ADK. The apyrase-treated PPK-ADK showed both ADK and PPK activities. After this treatment, ATP amplification was not observed for at least the initial 60 min if exogenous ATP was not added to the reaction mixture (Fig. 2).

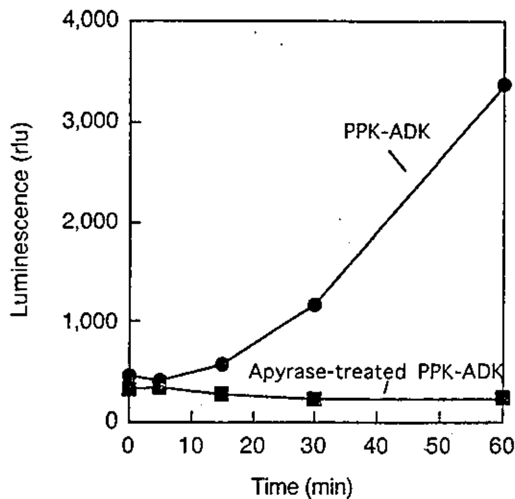

Fig. 2. Removal of ADP from PPK-ADK by Apyrase Treatment.
Before and after apyrase treatment, 0.16 μg of ADK-PPK was added to a reaction mixture containing 10 μM of AMP, 400 μM of polyP, 8 mM $MgCl_2$, and 60 mM Tris–HCl (pH 7.4). Five μl of the reaction mixture was sampled and mixed with 40 μl of ATP bioluminescence assay reagent (Roche). Bioluminescence was measured by using a multiplate luminometer.

Ultrasensitive bioluminescence assay

The highly purified PPK-ADK successfully amplified ATP depending on its initial concentration (Fig. 3A). The ATP amplification for 60 min prior to the bioluminescence assay enabled us to detect ATP as low as 10^{-18} mol, while the conventional bioluminescence assay requires ATP as high as 10^{-14} mol to detect bioluminescence (Table 1). Thus, the sensitivity of the bioluminescence assay was increased approximately 10,000-fold by ATP amplification.

Table 1. Effect of ATP Amplification on Bioluminescence Assay

ATP (10^{-15} mol)	Luminescence (rlu)	
	Without ATP amplification	With ATP amplification
330	810 ± 22	28,200 ± 1,600
33	110 ± 14	18,800 ± 240
3.3	50 ± 6	8,770 ± 440
0.33	52 ± 9	4,460 ± 36
0.033	53 ± 12	2,730 ± 230
0.0033	62 ± 12	1,550 ± 100
none	51 ± 2	229 ± 26

ATP amplification was performed for 60 min prior to the bioluminescence assay as described in Materials and Methods. The luminescence values are the means ± standard deviations of three separate measurements.

Detection of E. coli

The *E. coli* culture (early stationary) was appropriately diluted with distilled water and heated to 100 °C for 2 min to release ATP from the cells. After heating, the suspensions were subjected to the ultrasensitive bioluminescence assay. High levels of bioluminescence were detected, depending on the bacterial numbers subjected to the assay (Fig. 3B). The levels of luminescence were drastically increased by ATP amplification (Table 2). Without ATP amplification, several 10,000 CFUs of *E. coli* cells were necessary for the detection of significant levels of bioluminescence. On the other hand, the present technique enabled us to detect as little as one CFU of *E. coli* per assay (Table 2). The intracellular levels of ATP in viable *E. coli* cells were reported as approximately 7 μmol/g dry cell.[15)]

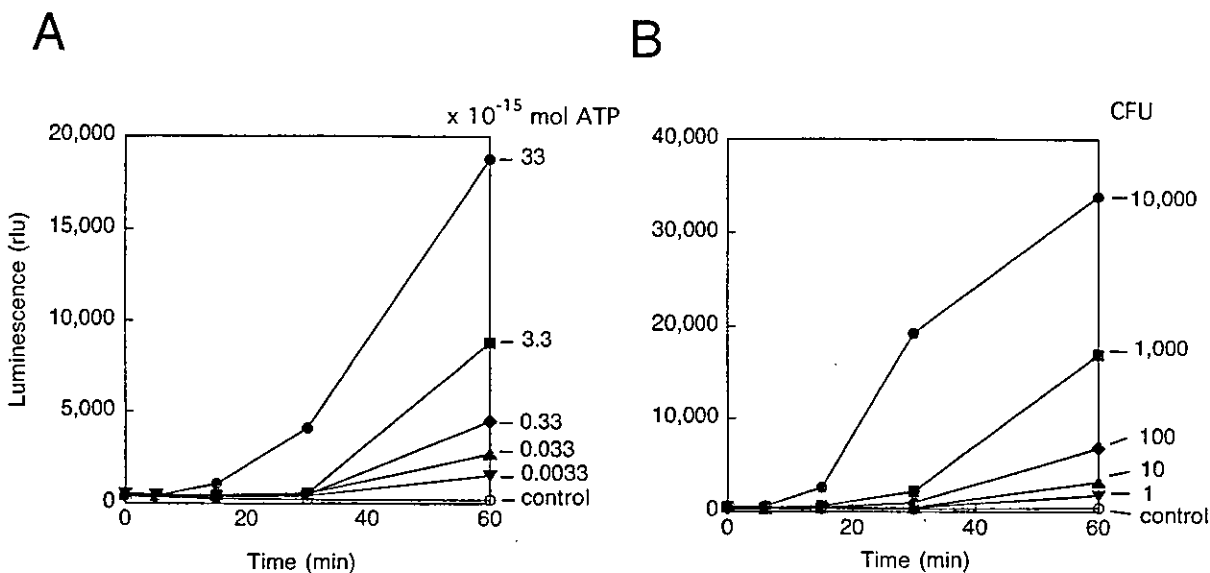

Fig. 3. Bioluminescence Time Courses during ATP Amplification.
(A) The amount of ATP that was initially present in the 5-μl reaction mixture is indicated. (B) *E. coli* cells (early stationary, 2.0 × 10^9 CFU/ml) were appropriately diluted. The cell suspensions (500 μl) were mixed with a 500-μl lysis-buffer (Bioluminescence assay, Roche) and then incubated at 100 °C for 2 min. Heated samples (2 μl) were subjected to ATP amplification before the bioluminescence assay. The number of *E. coli* (CFU) that were present in the 5-μl reaction mixture of ATP amplification is indicated.

Table 2. Detection of *E. coli* Cells by Bioluminescence Assay with and without ATP Amplification

E. coli cells (CFU per assay)	Luminescence (rlu)	
	Without ATP amplification	With ATP amplification
100,000	1,130 ± 260	39,700 ± 1,600
10,000	290 ± 34	33,900 ± 2,200
1,000	52 ± 4	16,900 ± 1900
100	37 ± 4	6,820 ± 200
10	39 ± 6	3,280 ± 600
1	37 ± 7	1710 ± 44
none	43 ± 12	364 ± 73

The *E. coli* culture (early stationary) was appropriately diluted with distilled water and heated to 100 °C for 2 min to release ATP from the cells. ATP amplification was performed for 60 min prior to the bioluminescence assay. The luminescence values are the means ± standard deviations of three separate measurements.

Since the dry weight of one *E. coli* cell is approximately 2.8×10^{-13} g,[16] it can be estimated that *E. coli* contains approximately 2×10^{-18} ATP per cell. This level of ATP is almost equal to that of the detection limit of our ultrasensitive bioluminescence assay.

Application to hygiene monitoring

To further examine the sensitivity of our assay for bacterial contamination, an *E. coli* cell suspension was spread on a polystyrene petri dish, air-dried, and then swabbed with a cotton swab. Since commercially available cotton swabs contain significant levels of ATP, the swabs were autoclaved at 121 °C for 75 min to decompose ATP to AMP. The sample swabbed from a surface area of 4 cm^2 was dipped into a lysis buffer (Roche) in a plastic tube and then heated to 100 °C for 2 min to release ATP from the cells. Swab monitoring coupled with the ultrasensitive bioluminescence assay enabled us to detect approximately 12 CFUs of *E. coli*/cm^2 (data not shown).

We also examined the sensitivity of our assay for detecting bacterial contamination in drinking water. Water samples were heated to 100 °C for 2 min in a plastic tube and then subjected to our assay. It enabled us to detect bacterial contamination in drinkable tap water at levels at which the conventional bioluminescence assay failed to detect contamination (Table 3). Bacterial contamination of tap water has been reported occasionally.[17] As shown in Table 3, our assay allowed us to detect bacterial contamination of one CFU per ml within 60 min. The conventional method using a nutrient agar medium typically requires several days to detect bacterial contamination (Table 3).

In the dairy industry, a rapid and reliable test for bacterial contamination in raw milk has been sought, because such contamination often causes extensive damage. We therefore examined the sensitivity of our assay for detecting *Staphylococcus aureus* in milk. The growing culture of *S. aureus* was appropriately diluted and added to milk. To remove non-bacterial ATP derived from mammary gland and somatic cells, the milk was first passed through a 0.45-μm membrane filter. The membrane filter was then washed with a solution containing 0.2% Triton X-100 to disrupt somatic cells but not *S. aureus*.[18] Then the membrane filter was dipped into a lysis buffer (Roche) and heated to 100 °C for 2 min, and then subjected to bioluminescence measurement. Our assay increased sensitivity in the detection of *S. aureus* in milk approximately 10,000-fold compared with that of the conventional bioluminescence assay, and enabled us to detect 75 CFUs of *S. aureus* per 0.5-ml of milk (data now shown).

Concomitant use of ATP cycling and the bioluminescence assay increases luminescence by signal integration without forfeiting the linear correlation between luminescence and ATP concentration.[5,6] But at the same time, the background noise (dark current) also increases. On the other hand, ATP amplification before the bioluminescence assay increased the luminescence without signal integration, thus increasing the sensitivity of the bioluminescence assay for ATP dramatically. But the significant linear correlation between luminescence and ATP concentration was not observed. Hence it may be difficult to enumerate cells precisely by the ATP amplification method. The rate of amplification in ATP amplification depends mostly on the amount of initial AMP that is converted to ATP after amplification. Therefore it is possible to amplify very low levels of ATP extracted from a single bacterial cell to high levels

Table 3. Detection of Bacterial Contamination in Water Samples by Bioluminescence Assay with and without ATP Amplification

Water sample	Luminescence (rlu)		Bacterial numbers (CFU/ml)[d]
	Without ATP amplification	With ATP amplification	
Tap water I	15	1,400	33
Tap water II	13	413	1
Bottled water[a]	30	239	<1
Sterilized water[b]	23	254	<1
Pond water[c]	9	3,100	59

Heated water samples (2 μl) were subjected to ATP amplification for 60 min. Tap water I was supplied from a public waterworks (Hiroshima, Japan). Tap water II was supplied from the waterworks at Hiroshima University. [a]Bottled water was purchased commercially. [b]Sterilized water was prepared by autoclaving distilled water at 121 °C for 75 min. [c]Pond water was sampled at Hiroshima University. [d]One ml of a water sample was spread on a nutrient agar plate (1.6 g of tryptone, 1 g of yeast extract, 0.5 g of NaCl, and 15 g of agar per liter). CFU was determined by counting a colony formed on the nutrient agar plate after 3 d of incubation at 28 °C.

that we can detect without using a sensitive photometer. In conclusion, we have demonstrated for the first time that ATP amplification increased the sensitivity of the bioluminescence assay by approximately 10,000-fold. This ultrasensitive bioluminescence assay is applicable to the detection of bacterial contamination at very low levels in a wide range of hygiene monitoring tasks.

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