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研究分担報告書

婦人科腫瘍、胎児組織の移植維持系の確立

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研究要旨：婦人科癌における抗癌剤の有効性に関わる蛋白質を網羅的に解析し、Annexin A4 を同定した。また、抗癌剤の有効性を、抗癌剤投与後の癌細胞におけるグルコースの取り込み能の変化をみることで、癌の形態上の変化の前に予測できることを明らかにした。

A. 研究目的

婦人科癌に対しては抗癌剤治療が行われることが多いが、その有効性は限られている。生活習慣の変化などによって増加している婦人科癌の制御は急務であり、当研究においては、婦人科癌の移植系を確立し、抗癌剤の有効性の予測や感受性に関わる遺伝子を同定することなどを目的とする。

B. 研究方法

1. 婦人科癌で抗癌剤が奏効した症例としなかった症例における癌組織での蛋白質発現を網羅的に解析し、抗癌剤感受性の鍵となる蛋白質の同定を試みた。

2. 癌細胞を抗癌剤で処理した場合に起こる変化を細胞株および臨床検体を用いて解析した。

（倫理面への配慮）

研究に際し、細胞株以外に臨床検体の採取・利用も不可欠であるが、インフォームドコンセントを得たうえで、手術で摘出された組織を用いるため、患者に危害が及ぶことはなかった。

C. 研究結果

1. 卵巣癌および子宮内膜癌における抗癌剤の有効性に関わる重要な蛋白質として Annexin A4 を同定した。そのメカニズムとして、Annexin A4 が抗癌剤の排出を促進することで抗癌剤耐性を来たすことを解明した(Kim A, Enomoto T, et al. Int J Cancer. 2009 125(10):2316-22)。

2. 卵巣癌細胞を抗癌剤処理した場合、細胞死を来たす前にグルコースの取り込みが減少することを見い出した（投稿中）。

D. 考察

卵巣癌・子宮内膜癌の治療において抗癌剤は重要であり、その感受性に関わる蛋白質として Annexin A4 を同定した。今後、同マウスを用いて Annexin A4 を target とした分子標的治療の開発に向けた研究を行う予定である。

また、卵巣癌における抗癌剤の有効性を、抗癌剤投与後の癌細胞におけるグルコースの取り込み能の変化をみることで、癌の形態上の変化の前に予測できることが判明した。今後、同マウスを用いて、抗癌剤感受性予測システムを確立したい。

E. 結論

婦人科癌において、Annexin A4 は抗癌剤の排出を促進することで抗癌剤耐性を来たすことがはじめて示された。また、抗癌剤の有効性を、抗癌剤投与後の癌細胞におけるグルコースの取り込み能の変化をみることで、癌の形態上の変化の前に予測できる可能性も示唆された。

F. 健康危険情報

G. 研究発表

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2. 学会発表

H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 特になし

III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

雑誌

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Distribution of Receptor Glycolipids for *Lactobacilli* in Murine Digestive Tract and Production of Antibodies Cross-reactive with them by Immunization of Rabbits with *Lactobacilli**

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In the digestive tract of mice (HR-1 strain), glycolipids belonging to the ganglio-series were revealed to be expressed in region-specific manners, *i.e.* FGA1 and FGM1 in the stomach, GA1 in the small intestine, and FGA1 and sulphatides in the cecum. The amount of GA1 as a receptor glycolipid for *Lactobacilli* was especially higher in the small intestine than in the other regions, it comprising 1.6–2.8 μg/mg dry weight. On immunization of rabbits with *Lactobacillus johnsonii* and *Lactobacillus intestinalis*, both of which are murine intestinal bacteria, antibodies toward bacterial glycolipids, *i.e.* Galα1–2Glcα1–3DG, and tri- and tetrahexaosyl DGs, were effectively generated and, in addition, they were found to cross-react with GA1 and GalCer, but not with structurally related glycolipids such as Lc_4Cer, nLc_4Cer and $IV^3Gal\alpha$-nLc_4Cer, indicating that GA1 is a preferable antigen for anti-lactobacillus antisera and suggesting the presence of epitopes common to both *Lactobacilli* and the host. In fact, molecules reacting with anti-GA1 antibodies were detected among bacterial proteins on Western blotting, indicating a possible occurrence of the carbohydrate structure mimicking GA1 in bacterial proteins.

Key words: asialo GM1, bacterial receptor, digestive tract, glycoglycerolipids, TLC-immunostaining.

Abbreviations: CL, cardiolipin; CMH, ceramide monohexoside; CS, cholesterol sulphate; DG, diacyl glycerol; Hep, L-glycero-D-mannoheptose; FGA1, fucosyl asialo GM1; FGM1, fucosyl GM1; GA1, asialo GM1; PG, phosphatidyl glycerol; Sul, sulphatide.

Glycolipids are ubiquitous membrane components of mammalian tissues and cells, and their carbohydrate moieties are included in antigens concerning blood group, species specificity and cellular differentiation and transformation, and receptors for bacteria, bacterial toxins and viruses (*1*). In the murine digestive tract, glycolipids belonging to the globo- and ganglio-series are separately distributed in the mesenchymal and epithelial tissues, respectively, and GA1, as the backbone structure of ganglio-series glycolipids, is expressed in association with cellular differentiation from the crypt to the villus in the intestinal microvilli (*2*), and provides receptors for several bacteria, such as *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus johnsonii* (*L. acidophilus*), *Bifidobacterium bifidum*, *Pseudomonas aeruginosa*, *Actinomyces maeslundii* and *Neisseria gonorrhoeae* (*1*). However, the carbohydrate moiety of GA1 has been revealed to be modified through transcriptional regulation of the sugar transferase gene on bacterial infection (*3–6*). Under conventional breeding conditions, expression of FGA1 from the fucosyltransferase gene in the small intestine occurs during the postnatal period from early suckling to weaning (*7–9*), and is enhanced by food containing dietary fibre at the time of weaning (*10*). Fucosylation does not occur under germ-free conditions, but infection by indigenous filamentous bacteria and wild-type *Bacteroides thetaiotaomicron* in germ-free mice triggers expression of the FUT2 gene, a counterpart of the human secretor (Se) gene, for synthesis of FGA1 only in the small intestine, *i.e.* not in other regions of the digestive tract (*5*, *6*). In fact, differences in the regulation of gene expression among different regions have been revealed by targeted deletion of the FUT1 and FUT2 genes (*6*, *11*). Although fucosylated glycolipids completely disappear from the antrum, cecum and colon of FUT2-null mice, those in the small intestine of FUT2-null mice are maintained at similar levels to those in the wild-type (*6*). In contrast, the amounts of fucosylated glycolipids in the stomach, cecum and colon are not affected by targeted deletion of the FUT1 gene, but those in the small intestine of FUT1-null mice are rather increased compared to in wild-type and

*The nomenclature for glycolipids and gangliosides is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [IUPAC-IUB Commission on Biochemical Nomenclature. (1977) The nomenclature of lipids. *Eur. J. Biochem.* **179**, 11–21] and Svennerholm [Svennerholm, L. (1963) Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**, 613–623], respectively.

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FUT2-null mice (*6*). These findings indicate that fucosylation in the antrum, cecum and colon is preferentially due to the FUT2 gene, but that in the small intestine to either the FUT1 or FUT2 gene. Accordingly, fucosylation of glycolipids in the stomach, cecum and colon due to the FUT2 gene occurs in a development-dependent manner, but that in the small intestine is regulated by the surrounding circumstances in mice, *i.e.* suppression of FUT2-gene expression under germ-free conditions, and activation of FUT2-gene expression under conventional breeding conditions and targeted deletion of the FUT1 gene. Thus, expression of GA1 in the epithelial cells of the digestive tract is actively regulated through modification of the carbohydrate structures, and is thought to influence the colonization of bacteria having the ability to bind with GA1 as a receptor, particularly of symbiotic bacteria, *i.e. Lactobacillus* and *Bifidobacterium* (*12–16*), which play a role in protection from infection by harmful bacteria passing into the digestive tract together with food.

However, the profile of glycolipid expression under region-specific regulation in the digestive tract is not clearly understood yet, and therefore we determined the glycolipid compositions in different regions of the digestive tract of conventional breeding mice, with special reference to the receptor distribution for intestinal *Lactobacilli*, the receptor glycolipids for *L. johnsonii* having been characterized as GlcCer, Gb_3Cer, GA1, nLc_4Cer and Lc_4Cer (*13*). In addition, we prepared antisera by immunization of rabbits with *L. johnsonii* and *L. intestinalis*, both of which are murine intestinal bacteria, and found that the antisera cross-reacted with GalCer and GA1, indicating the occurrence of an intestinal glycolipid-like structure in *Lactobacilli*, which might be related with a mechanism for evading immunological surveillance by the host for symbiosis with *Lactobacilli* in the digestive tract of mice.

MATERIALS AND METHODS

Lactobacilli—Lactobacillus johnsonii (*L. acidophilus*) (JCM No. 1022), *Lactobacillus casei* (JCM No. 1134) and *Lactobacillus intestinalis* (JCM No. 7548) were purchased from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center (Wako, Saitama, Japan), and cultured in *Lactobacilli* MRS broth (Difco, Beckton-Dickinson, Sparks, MD, USA).

Glycolipids and phospholipids—The glycolipids used in this experiment were purified from various sources in our laboratory: Galα1–2Glcα1–3DG from *L. casei* (*17*), GlcCer, LacCer, Gb_3Cer, Gb_4Cer, GM3 and IV^3NeuAc-nLc_4Cer from human erythrocytes, IV^3GalNAcα-Gb_4Cer (Forssman antigen) from equine kidney, Gg_3Cer from guinea pig erythrocytes, GalCer containing non-hydroxy fatty acids (GalCer NFA) and 2-hydroxy fatty acids (GalCer HFA), sulphatides and GM1 from bovine brain, FGM1 from bovine thyroid, Lc_4Cer from human meconium and IV^3Galα-nLc_4Cer from rabbit erythrocytes (*18*). GA1, FGA1 and nLc_4Cer were prepared from GM1, FGM1 and IV^3NeuAc-nLc_4Cer, respectively, by treatment with *Arthrobacter ureafaciens* sialidase (*19*). *N*-Stearoyl glycolipids, as standards, for TLC-densitometry were prepared by deacylation with sphingolipid ceramide *N*-deacylase (*Pseudomonas sp.* TK4), followed by reacylation with stearoyl chloride. Dioleoyl derivatives of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl glycerol (PG), phosphatidic acid (PA) and sphingomyelin (SM) from human erythrocytes were kindly provided by Alfresa Pharma Co. (Osaka), and cardiolipin (CL) and phosphatidyl inositol (PI) were purchased from Sigma (St Louis, MO, USA). The concentrations of standard phospholipids in chloroform/methanol (1:1, v/v) were determined by the phosphomolybdate procedure after decomposition of the lipids with 70% $HClO_4$ and H_2O_2 (*20*).

Antisera—Rabbit polyclonal antibodies toward *L. johnsonii* and *L. intestinalis* were generated by immunizing rabbits (Japanese White; Japanese Biological Materials, Tokyo) intradermally with a water-in-oil emulsion prepared by mixing 15 mg of each bacterium in 1 ml of phosphate-buffered saline (PBS) with 1 ml of Freund's complete adjuvant (Sigma), and the antibody titres were subsequently monitored by enzyme-linked immunosorbent assaying (ELISA) with the respective bacterium (2 μg/well) as the antigen. In a similar way, polyclonal antibodies toward Forssman antigen and GA1 were generated by immunizing rabbits with 1 mg of glycolipids together with Freund's complete adjuvant, and also monoclonal antibodies toward FGM1 and FGA1 were prepared by immunizing mice with 20 μg of glycolipids together with *Salmonella minnesota* as the adjuvant, followed by hybridization of lymphocytes with murine myeloma P3X63Ag8. Anti-Forssman, anti-GA1, anti-FGM1 and anti-FGA1 antibodies generated characteristically reacted with the respective glycolipid antigens and no cross-reaction to structurally related glycolipids were observed (*5*, *21*).

Quantitation of lipids in murine tissues and bacteria—Mice (HR-1, 10 weeks of age) were kept under conventional breeding conditions at a room temperature of 24 ± 1°C and a humidity level of 55 ± 10% with food and water *ad lib.* Animal treatment followed the animal care guidelines of Kinki University.

Tissues, *i.e.* stomach, duodenum, jejunum, ileum, cecum and colon, were rinsed with PBS and then lyophilized. Total lipids were extracted from the lyophilized tissues with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1:0, v/v/v), and then fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; GE Healthcare Bioscience, Piscatway, NJ, USA). Then, the neutral glycolipids were separated from the unabsorbed neutral lipid fraction by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the absorbed acidic lipid fraction containing gangliosides and cholesterol sulphate (CS) was saponified with 0.5 M NaOH in methanol to remove the ester-containing lipids, followed by dialysis (*22*, *23*).

The total lipids, and acidic and neutral lipids thus obtained were examined by TLC. The following solvent mixtures were used as the developing solvents for TLC, chloroform/methanol/water (65:35:8, v/v/v) for phospholipids and neutral glycolipids, chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v/v) for cholesterol sulphate and sulphatides, and chloroform/methanol/0.5%

$CaCl_2$ (55:45:10, v/v/v) for gangliosides, and the detection reagents were as follows, cupric acetate–phosphoric acid reagent for neutral lipids, phospholipids and CS, Dittmer's reagent for phospholipids, orcinol–sulphuric acid reagent for glycolipids and resorcinol–hydrochloric acid reagent for gangliosides. The density of spots on TLC plates was determined by image analysis (NIH image). Standard lipids, *i.e. N*-stearoyl derivatives of GalCer, LacCer, Gb_3Cer, GM3, Gb_4Cer, Forssman glycolipid, GA1, GM1, FGM1 and GD1a, and dioleoyl derivatives of PE, PS, PC, PG and PA, and CS (0.1–1.5 μg), were developed on the same TLC plates for the preparation of standard curves (*24*). Analysis of lipids from *Lactobacilli* was carried out by the same procedure, but quantitation of mono- to tetrahexaosyl DGs in *Lactobacilli* was performed using the standard curves for mono- to tetrahexaosyl ceramides as described above.

TLC-immunostaining—Lipids were developed on plastic-coated TLC plates (Macherey-Nagel, Düren, Germany), which were then blocked with blocking buffer (PBS containing 1% polyvinylpyrrolidone and 1% ovalbumin), and the spots were visualized by immunostaining with the above anti-glycolipid and anti-lactobacilli antibodies (1:500) diluted with dilution buffer (PBS containing 3% polyvinylpyrrolidone), followed by immunostaining with peroxidase-conjugated anti-rabbit IgG and IgM (1:1,000; Jackson Immunoresearch Lab., PA, USA), and anti-murine IgG and IgM antibodies (1:1,000; Sigma), and peroxidase substrates, 4-chloro-1-naphthol and H_2O_2, according to the procedure reported previously (*25*). Control staining with normal rabbit serum was performed simultaneously under the same conditions.

SDS-PAGE and Western blotting—*Lactobacilli* were suspended in PBS by ultrasonication, and the protein concentration of the resulting solution was measured by Bradford's procedure with bovine serum albumin as the standard protein (*26*). Then the solution, corresponding to 8 μg of protein, was denatured with 15 μl of sample buffer [1% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.125% bromophenol blue in 0.06 M Tris–HCl (pH 6.8)] at 95°C for 4 min, and then electrophoresed on an acrylamide gel (12%), which was stained with Coomassie brilliant blue (CBB) for proteins (*27*). Also, proteins on a SDS–PAGE gel were electrically transferred to a nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Lab., Hercules, CA, USA), and the membrane was immunostained with anti-lactobacilli and anti-GA1 antibodies according to the procedure for TLC-immunostaining described above.

RESULTS AND DISCUSSION

Glycolipid receptors for Lactobacilli in the murine digestive tract—As shown in Figs 1 and 2, the glycolipids, particularly ganglio-series ones, were revealed to be distinct in different regions of the murine digestive tract. Although Forssman glycolipid was present in all regions of the tract, amounting to 0.12–1.11 μg/mg dry weight, GA1 as a receptor for intestinal *Lactobacilli* was predominant in the duodenum, jejunum and ileum, in comparison to in the stomach, cecum and colon, where it was not detectable or was only present in a trace amount, as judged on TLC-immunostaining with anti-GA1 antibodies (Fig. 2). The amounts of GA1 in the duodenum, jejunum and ileum were 1.6, 2.8 and 2.2 μg/mg dry weight, respectively, corresponding to ~36–50% of the total neutral glycolipids. In contrast, FGA1 was abundant in the stomach and cecum, amounting to 0.9 and 1.6 μg/mg dry weight, respectively, corresponding to ~20% of the total neutral glycolipids. On comparison of the ratio of FGA1 to GA1 plus FGA1 as the rate of fucosylation of GA1, GA1 in the stomach, cecum and colon was revealed to be almost completely converted to FGA1, but the fucosylation rates in the duodenum, jejunum and ileum were only 8, 0.3 and 10%, respectively (Table 1). On the other

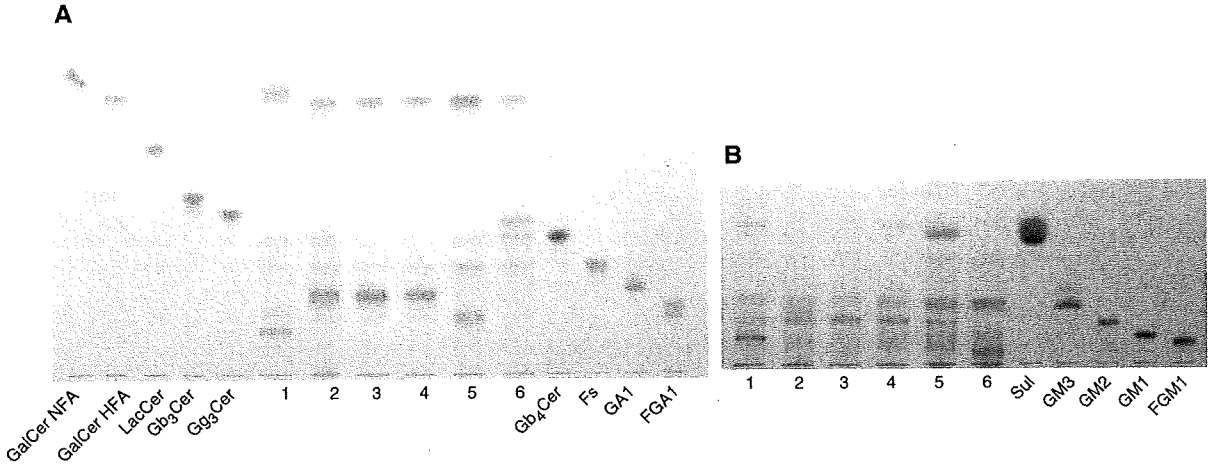

Fig. 1. **TLC of neutral (A) and acidic (B) glycolipids from different regions of the murine digestive tract.** Neutral glycolipids and gangliosides, corresponding to 0.5 mg dry weight, were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v) for A and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) for B, and were detected with orcinol–sulphuric acid reagent. 1, Stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon.

hand, FGM1 was abundant in the stomach, in which the concentration of FGM1 was similar with that of FGA1, and fucosylation of GM1 was almost complete like that of GA1, suggesting that the fucosyltransferase equally acts on GA1 and GM1 in the stomach (Fig. 1) (*5*).

Ceramide monohexosides (CMH) were the most abundant neutral glycolipids in all regions, amounting to 1.7–4.1 μg/mg dry weight. As shown in Fig. 1, gastric CMH migrated to a similar position on a TLC plate to GalCer containing 2-hydroxy fatty acyl sphingosine, but to a higher position than intestinal CMH, which is composed of 2-hydroxy fatty acyl phytosphingosine, as reported in the literature (*2*).

As to the acidic lipids, the distribution of sulphatides was restricted to the stomach and cecum, and the amount in the cecum was significantly higher than those in the other regions. Also, CS and GM3 were uniformly distributed in the tract, their highest amounts being observed in the ileum and colon, respectively.

Then the molar ratios of individual lipids to CMH were calculated using their mean molecular weights [behenic acid (22:0)-containing ones], that is, 783 for CMH, 1,310 for Gb_4Cer, 1,513 for Forssman glycolipid, 1,310 for GA1, 1,456 for FGA1, 466 for CS and 1,236 for GM3, the CMH:Gb_4Cer:Forssman glycolipid:GA1:FGA1:CS:GM3 ratio in the jejunum being found to be 1.00:0.05:0.04:0.73:0.002:0.17:0.05, which resembled the reported ratio (*2*), showing that the jejunum as well as the duodenum and ileum contain GA1 in relatively high molar proportions.

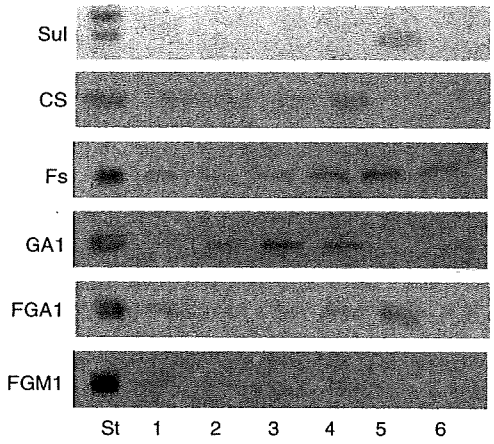

Fig. 2. **TLC- and TLC-immunostaining of lipids from different regions of the murine digestive tract.** For CS and sulphatides (Sul), acidic lipids, corresponding to 0.5 mg dry weight, were developed on TLC plates with chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, v/v/v), and were detected with cupric acetate–phosphoric and orcinol–sulphuric acid reagents, respectively. For TLC-immunostaining of glycolipids, total lipid extracts, corresponding to 0.1 mg dry weight, were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v), followed by detection with anti-Forssman (Fs), anti-GA1, anti-FGA1 and anti-FGM1 antibodies. 1, Stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon.

Thus, the following glycolipids were abundant in the murine digestive tract in region-specific manners, FGA1 and FGM1 in the stomach, GA1 in the small intestine, FGA1 and sulphatides in the cecum. Accordingly, the small intestine seemed to be the site for colonization by *Lactobacilli*, because GA1 was characterized as the receptor with strongest affinity toward *L. johnsonni*, *L. casei* and *L. reuteri* (*13*).

Bacterial lipids—Extensive studies on the structures of bacterial lipids including those of *Lactobacillus* species have appeared in the literature (*28*, *29–33*). In accord with previous reports, CL and PG were the major phospholipids in *L. johnsonii* and *L. intestinalis*, amounting to 0.12–0.65 μg of dry weight (Table 2) (*33*). Also, the major glycolipid, whose mobility on a TLC plate was similar with that of Galα1–2Glcα1–3DG from *L. casei*, was present in both bacteria, amounting to 1.03 μg/mg in *L. johnsonii* and 0.41 μg/mg in *L. intestinalis*, as determined by TLC-densitometry with *N*-stearoyl LacCer as the standard for quantitation. The other glycolipids in *L. johnsonii* were supposed to be Glcα1–3DG, Glcβ1–6Galα1–2Glcα1–3DG and Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG on the bases of their mobilities on a TLC plate,

Table 2. **Amounts of lipids in *Lactobacilli*.**

	L. johnsonii (μg/mg dry weight)	*L. intestinalis* (μg/mg dry weight)
CL	0.13	0.65
PG	0.12	0.21
Monohexaosyl DG (Glcα1–3DG)	0.15	tr
Dihexaosyl DG (Galα1–2Glcα1–3DG)	1.03	0.41
Trihexaosyl DG (Glcβ1–6Galα1–2Glcα1–3DG)	0.39	0.03
Tetrahexaosyl DG (Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG)	0.61	–

Quantitative determination of lipids was performed by TLC-densitometry with the following standards, CL from bovine heart, dioleoyl PG and N-stearoyl derivatives of GalCer, LacCer, Gb_3Cer and Gb_4Cer. tr, trace amount. Values are the means for three different experiments.

Table 1. **Amounts of lipids in the murine digestive tract (microgram/milligram dry weight).**

	Cho	CL	PE	PG	PC/PS	SM	CMH	Gb_4Cer	Fs	GA1	FGA1	FGM1	Sul	CS	GM3
Stomach	6.5	0.5	5.9	1.5	7.3	1.6	3.5	0.37	0.25	–	0.87	0.79	0.20	0.19	0.40
Duodenum	7.4	0.7	6.1	1.6	11.1	1.3	1.7	0.21	0.12	1.6	0.17	–	–	0.16	0.25
Jejunum	5.5	0.8	4.7	1.2	8.1	1.1	2.3	0.21	0.16	2.8	0.01	–	–	0.23	0.20
Ileum	6.1	0.2	4.0	1.0	6.6	0.9	2.4	0.69	0.33	2.2	0.28	–	tr	0.42	0.39
Cecum	6.3	0.3	5.3	1.2	7.5	1.2	4.1	0.53	1.11	tr	1.56	–	0.88	0.11	0.90
Colon	7.1	0.3	5.3	0.8	7.1	1.2	2.4	0.82	0.57	–	0.08	–	–	0.11	1.20

Cho, cholesterol; Fs, Forssman glycolipid; Sul, sulphatides; tr, trace amount. Values are the means for three different experiments.

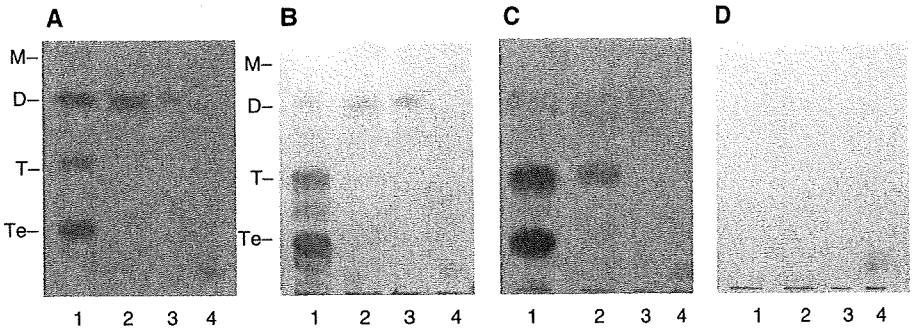

Fig. 3. **TLC-immunostaining of lipids from *Lactobacilli*.** Lipid extracts of *L. johnsonii* (*1*) and *L. intestinalis* (*2*), corresponding to 1 mg dry weight for A, and 0.1 mg dry weight for B–D, were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v), and the spots were visualized with cupric acetate–phosphoric acid (A), anti-*L. johnsonii* (B), anti-*L. intestinalis* (C), and anti-GA1 (D) antisera. 3, Galα1–2Glcα1–3DG from *L. casei*; 4, GA1. M, D, T and Te indicate the positions of mono-, di-, tri- and tetrahexaosyl DGs, respectively.

as described in the literature (*28*, *29–32*). Tri- and tetraglycolipids were present in *L. johnsonii* at significantly high concentrations, but were only present in trace amounts in *L. intestinalis*.

Bacterial antigens reacting with anti-L. johnsonii and anti-L. intestinalis antisera—Immunization of rabbits with *L. johnsonii* and *L. intestinalis* yielded antisera with ELISA titres of more than 1:12,800. Both the anti-*L. johnsonii* and anti-*L. intestinalis* antisera reacted with Galα1–2Glcα1–3DG in *L. casei*, as well as that in *L. johnsonii* and *L. intestinalis*, but not with monohexaosyl DG (Glcα1–3DG). Although tri- and tetrahexaosyl DGs were not detectable in *L. intestinalis*, even on spotting of lipids corresponding to 5 mg dried bacteria, those in *L. johnsonii* were intensively stained with anti-*L. intestinalis* antisera to a similar levels to in the case of anti-*L. johnsonni* antisera, indicating their strong antigenicities (Fig. 3). In fact, the relative densities of spots/μg of Galα1–2Glcα1–3DG were significantly lower than those of tri and tetrahexaosyl DGs, *i.e.* Glcβ1–6Galα1–2Glcα1–3DG and Glcβ1–6Glcβ1–6Galα1–2Glcα1–3DG, respectively (*28*,*32*). However, since Galα1–2Glcα1–3DG was widely distributed in *Lactobacillus* species in relatively higher amounts than those of tri- and tetrahexaosyl DGs, it was revealed to contribute to a *Lactobacillus* antigen, as already reported by others (*30*, *34*).

Similarly, the strong antigenicity of diglucosyl DGs, *i.e.* kojibiosyl (Glcα1–2Glc) DG in *Acholeplasma laidlawi* and gentibiosyl (Glcβ1–6Glc) DG in *Mycoplasma neurolyticum*, has been well studied (*34*), and antibodies towards digalactosyl DG have been reported not to cross-react with GalCer or gangliosides (*35*, *36*), but to be involved in the production of natural antibodies in patients suffering from multiple sclerosis (*37*). Accordingly, one can suggest that *Lactobacillus* antigens including glycolipids are also involved in the production of natural antibodies.

Lipid antigens reacting with anti-lactobacillus antisera in murine tissues—Anti-*L. johnsonii* and anti-*L. intestinalis* antisera contained antibodies that reacted with GA1, but glycolipids that reacted with anti-GA1 antibodies were not present in the lipids from either bacterium (Fig. 3). Therefore, antigens reactive with antisera were explored by TLC-immunostaining with lipids from the murine digestive tract and several standard glycolipids. Among the glycolipids examined, GalCer and GA1 were reactive with antisera, but structurally related glycolipids such as GlcCer, LacCer, Gb_3Cer, Gg_3Cer, Gb_4Cer, Forssman glycolipid, Lc_4Cer, nLc_4Cer and $IV^3Gal\alpha$-nLc_4Cer were not reactive, indicating that terminal galactose moieties are not always included in the epitope and that gangliotetraose is a preferable antigen for anti-lactobacillus antisera (Fig. 4). On comparison of the densities of spots per microgram of glycolipids stained with antisera, the intensities of Galα1–2Glcα1–3DG and GA1 were found to be similar, but that of GalCer was lower than those of Galα1–2Glcα1–3DG and GA1 (Fig. 4). Thus, symbiotic *Lactobacilli* were shown to generate antibodies toward receptor glycolipid GA1.

To determine why anti-GA1 antibodies were yielded on immunization with *Lactobacilli*, the antigens reacting with anti-GA1 antibodies in *Lactobacilli* were explored by Western blotting. As shown in Fig. 5, although the protein-profiles on SDS–PAGE differed between the two bacteria, anti-lactobacillus antisera reacted with the same antigenic proteins with molecular weights of 43, 50, 55 and 75 kDa. In addition, a protein with a molecular weight of 26 kDa in both bacteria exhibited a positive reaction with anti-GA1 antibodies, indicating the presence of gangliotetraose-like glycans in their protein fractions. If the molecules mimicking GA1 in *Lactobacilli* to generate anti-GA1 antibodies exhibit receptor activity for a ligand, binding of bacteria to GA1 in the small intestine and bacterial aggregation through the ligand might allow effective colonization on the surface of the small intestine. In this connection, modification of GA1 through fucosylation might regulate the number of colonies of *Lactobacilli*.

The production of antibodies to GA1 and gangliosides in human autoimmune diseases such as Guilain–Barré syndrome has been reported to be due to infection by *Campylobacter jejuni*, which causes gastroenteritis (*38*). In fact, an oligosaccharide mimicking GA1, that is, Gal-GalNAc-Gal-(Glc)HepII-(Glc)HepI, was detected in the lipooligosaccharides (LOS) of a gram-negative bacterium, *C. Jejuni* (*39*), and an immune response to the bacterial LOS was thought to result in autoimmune diseases through a reaction with gangliosides in neural tissues,

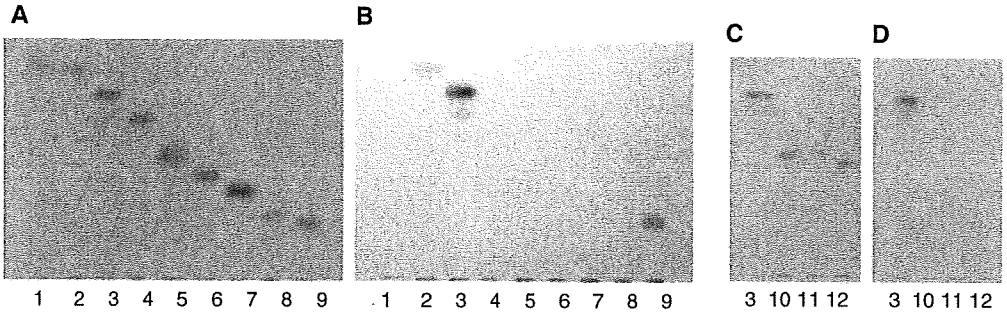

Fig. 4. **TLC and TLC-immunostaining of standard glycolipids.** Glycolipids (0.5–1.5 μg) were developed on TLC plates with chloroform/methanol/water (65:35:8, v/v/v) for A and B, and with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v/v) for C and D, and the spots were visualized with orcinol–sulphuric acid for A and C, and with anti-*L. johnsonii* antisera for B and D. 1, GlcCer; 2, GalCer NFA; 3, Galα1–2Glcα1–3DG from *L. casei*; 4, LacCer; 5, Gb_3Cer; 6, Gg_3Cer; 7, Gb_4Cer; 8, Forssman antigen; 9, GA1; 10, Lc_4Cer; 11, nLc_4Cer; 12, IV^3Galα-nLc_4Cer.

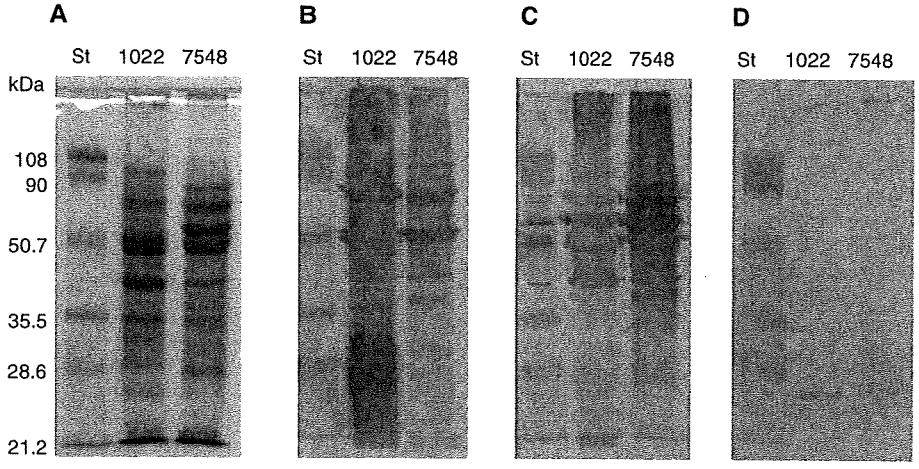

Fig. 5. **SDS-PAGE and Western blotting of proteins from *Lactobacilli*.** *L. johnsonii* (1,022) and *L. intestinalis* (7,548) suspended in PBS by sonication were denatured by heating with the sample buffer, and the resultant solutions were analysed by SDS–PAGE with CBB-staining (A), and by Western blotting with anti-*L. johnsonii* (B), anti-*L. intestinalis* (C), and anti-GA1 (D) antibodies. No band was obtained by staining with normal rabbit serum (1:500). St, protein molecular markers.

playing a crucial role in the pathogeneses of the diseases. In this connection, *Lactobacilli*, gram-positive bacteria, also carry glycans mimicking GA1 in the digestive tract of mice, and the resemblance in the epitope structure between bacteria and the host might be essentially related with a mechanism for evading immune responses to establish symbiosis with *Lactobacilli* in the digestive tract.

CONFLICT OF INTEREST

None declared.

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No Evidence of Increased Mutation Rates at Microsatellite Loci in Offspring of A-Bomb Survivors

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Kodaira, M., Ryo, H., Kamada, N., Furukawa, K., Takahashi, N., Nakajima, H., Nomura, T. and Nakumura, M. No Evidence of Increased Mutation Rates at Microsatellite Loci in Offspring of A-Bomb Survivors. *Radiat. Res.* 173, 205–213 (2010).

To evaluate the genetic effects of A-bomb radiation, we examined mutations at 40 microsatellite loci in exposed families (father-mother-offspring, mostly uni-parental exposures), which consisted of 66 offspring having a mean paternal dose of 1.87 Gy and a mean maternal dose of 1.27 Gy. The control families consisted of 63 offspring whose parents either were exposed to low doses of radiation (< 0.01 Gy) or were not in the cities of Hiroshima or Nagasaki at the time of the bombs. We found seven mutations in the exposed alleles (7/2,789; mutation rate 0.25 × 10^{-2}/locus/generation) and 26 in the unexposed alleles (26/7,465; 0.35 × 10^{-2}/locus/generation), which does not indicate an effect from parental exposure to radiation. Although we could not assign the parental origins of four mutations, the conclusion may hold since even if we assume that these four mutations had occurred in the exposed alleles, the estimated mean mutation rate would be 0.39 × 10^{-2} in the exposed group [(7 + 4)/2,789)], which is slightly higher than 0.35 × 10^{-2} in the control group, but the difference is not statistically significant.

INTRODUCTION

Studies on the genetic effects of radiation have been pursued for many years at the Atomic Bomb Casualty Commission (ABCC) and its successor, the Radiation Effects Research Foundation (RERF). While somatic mutations have been documented to occur in a dose-related manner, as seen by chromosome aberrations in blood lymphocytes (*1*), no indication of genetic (trans-generational) effects of radiation has been seen in several end points such as malformation, stillbirth or chromosome aberration (*2–5*). Currently, in addition to epidemiological (*6, 7*) and clinical studies (*8*), laboratory studies to determine whether A-bomb radiation caused genetic effects at the DNA level, that is, whether a significant increase in mutation rate is observed among the children of A-bomb survivors, are also being conducted using several molecular biology techniques (*9–15*). One of those molecular studies relates to the instability of repeated DNA sequences. Polymorphic tandem repeat sequences, such as minisatellites and microsatellites, are known to exhibit high spontaneous mutation rates (i.e., alterations in the number of repeats) in germ cells, and it has been suggested that these may serve as versatile tools for genetic studies because it might be possible to draw conclusions by studying a relatively small number of the offspring.

Mini- and microsatellites are conventionally classified based on the number of nucleotides that comprise the unit of repeat sequence. Minisatellites are composed of repeat-sequence units of 10 to 100 bp, and the number of repeats may exceed 500. They are located at around 1,000 sites in the human genome, some of which are known as hypervariable minisatellites in humans due to their high spontaneous mutation rates in germ cells (0.5 × 10^{-2} to 10 × 10^{-2}/locus/generation) (*16*). The mechanisms of mutagenesis acting at these hypervariable loci are complex, but they appear to involve gene conversion in meiosis (*17, 18*). Several studies measured the mutation induction rates at hypervariable minisatellite loci in humans, with results that sometimes appeared to be discordant. For example, increased mutation rates were reported in residents of contaminated areas after the Chernobyl accident (mean estimated dose of around 0.03 Sv), in areas exposed to atmospheric nuclear tests at Semipalatinsk (mean estimated dose may be over 1 Sv), and in areas along the Techa River exposed to the release of radionuclides (mean dose of about 0.1 Sv) (*19–22*). In contrast, no significant increase in mutation rates was observed in

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studies of A-bomb survivors who suffered from a one-time acute exposure at a mean dose of 1.9 Sv or in the Chernobyl cleanup workers, who were also exposed to relatively acute radiation at doses of up to over 1 Sv (*9–12, 23, 24*). A study using DNA from sperm to evaluate mutation rates in three patients before and after radiotherapy for seminoma (estimated doses of 0.38 to 0.82 Gy) reported no significant increase in the mutation rates at two hypervariable minisatellite loci examined (*25*).

On the other hand, microsatellites comprise shorter repeat units of 1 to 6 bp, with repeat numbers of 100 copies or less. Their entire length can range up to several hundred bases, and they are dispersed and found at over 100,000 sites in the human genome. Spontaneous mutation rates at microsatellite loci in germ cells range from 10^{-2} to 10^{-4}/locus/generation which are much higher than those at unique protein-coding loci (10^{-5} to 10^{-6}/locus/generation) but are considerably lower than those seen at hypervariable minisatellite loci (*26–28*). In contrast to mutations in minisatellites, microsatellite mutations are thought to derive from "slippage" during DNA replication that results in gains or losses of repeat units (*29*). Therefore, mini- and microsatellites may differ in mutation behavior after exposure to radiation. Several studies examined the genetic effects of radiation and found significantly increased mutation rates at microsatellite loci; one study examined barn swallows and wheat grown in highly contaminated sites after the Chernobyl accident (*30, 31*), and another examined Japanese medaka fish that were irradiated chronically at low dose rates (*32*). However, it is not clear whether microsatellite mutations are induced in mammals after radiation exposure. In humans, only three small-scale studies have been reported; in studies of the survivors of A-bombs and the Chernobyl cleanup workers, there was no clear evidence of increased mutation rates (*11, 33*). The third study reported an elevated mutation rate in offspring born to parents who were exposed to ^{137}Cs γ rays at Goiania, Brazil, but these results do not appear to be conclusive, because only 12 microsatellite loci were examined for 10 exposed families (*34*).

As a part of the effort to take advantage of the relatively low spontaneous mutation rates at microsatellite loci compared with the mutation rate at hypervariable minisatellite loci to increase the statistical power of mutation studies, Furitsu *et al.* (*35*) developed assays to examine as many as 72 microsatellite loci and studied 64 families of cleanup workers and 66 controls after the Chernobyl accident. Those results again indicated no genetic effects of radiation, although the possibility remained that the radiation effects were too low to detect because the mean radiation dose was only 0.039 Sv.

In view of the results of the past studies in humans, it is important to examine not only as many loci as possible but also as many people as possible who were exposed to large doses. In the present study, the offspring of A-bomb survivors (doses to the exposed parents are mainly 1 Gy or larger) were screened for mutations at 40 microsatellite loci that are known to exhibit relatively high mutation rates.

MATERIALS AND METHODS

Subject Families and Ethical Considerations

Blood samples were collected from A-bomb survivors who participated in the Adult Health Study (AHS) and from their family members (spouses and children). They provided written informed consent for examining DNA following the form approved by the Ethics Committee for Genome Research in RERF established in accordance with the "Guidelines from Three Ministries" in Japan (*36*). Subsequently, mononuclear cells were isolated and a fraction of the cells were infected with Epstein-Barr virus (EBV) to establish lymphoblastoid cell lines, while the remaining cells were stored in liquid nitrogen without culture. White blood cells were also isolated and stored at −80°C.

DNA from family members (father-mother-children) was examined in 49 families in the exposed group (highest-dose cases were selected) and 51 families in the control group. The protocol for this study was reviewed and approved by the Institutional Review Board of both RERF and Osaka University.

Radiation Dose

The dose information for the parents (gonadal dose) is summarized in Table 1. The doses were estimated using the Dose System 2002 (DS02) with a relative biological effectiveness (RBE) of 10 for the neutron component (*37*). Of 129 offspring examined, 66 were in the exposed group and 63 were in the control group, and 115 of these subjects were the same ones examined in our previous minisatellite studies (*9–12*). The exposed families consisted mostly of uni-parental exposures but also included two families (a single offspring in one family and three siblings in the other) that had high-dose-exposed fathers (>2 Gy) and low-dose-exposed mothers (<0.1 Gy). The total number of offspring derived from irradiated germ cells was 70 (mean dose 1.56 Gy); of these, 34 were derived from exposed fathers (mean dose 1.87 Gy, range 0.78–3.09 Gy) to 23 daughters and 11 sons and 36 from exposed mothers (mean dose of 1.27 Gy, range 0.02–2.51 Gy) to 16 daughters and 20 sons. The total number of offspring derived from unexposed germ cells was 188 (95 from fathers to 40 daughters and 55 sons and 93 from mothers to 47 daughters and 46 sons).

The mean parental age at the time of exposure was 15.3 ± 5.1 years (mean ± SD) in the exposed group and 14.2 ± 5.7 years in the control group. The mean interval between radiation exposure and the birth of the offspring was 14.5 years (range 1.5–28.9 years) in the exposed group and 15.4 years (range 3.1–32.0 years) in the control group. The mean age of the fathers at the time of having the offspring was 30.1 ± 4.3 years (range 20.5–39.9 years) in the exposed group and 31.3 ± 5.2 years (range 21.3–51.6 years) in the control group. As for the mothers, the mean age at the time of delivery was 28.8 ± 5.3 years (range 20.2–41.8 years) in the exposed group and 27.4 ± 4.2 years (range 19.4–39.4 years) in the control group. There was no significant difference in the age-related parameters between the exposed and the control group.

Microsatellite Analysis

1. DNA samples

For screening candidate mutations at microsatellite loci, DNA from later passages of lymphoblastoid cells was first used. Mutations

TABLE 1
Parental Doses in the 129 Study Subjects

Testis doses of fathers in Gy (mean)	Ovary doses of mothers in Gy (mean)					
	< 0.01 (0[a])	0.01–0.10 (0.05)	0. 50–0.99 (0.87)	1.00–1.99 (1.42)	> 2.00 (2.38)	Total
<0.01 (0[a])	63	0	5	24	3	95
0.50–0.99 (0.85)	2	0	0	0	0	2
1.00–1.99 (1.43)	18	0	0	0	0	18
>2.00 (2.63)	10	4	0	0	0	14
Total	93	4	5	24	3	129

[a] Zero dose includes those parents who were not in the cities of Hiroshima or Nagasaki at the time of the bombs (classified as not in city, NIC).

at microsatellites occur somatically in the process of EBV transformation and/or during culture, and cells having such mutations sometimes proliferate clonally during culture. The errors in mistyping of microsatellites may be due to clonal expansion of cells having those somatic mutations (*38*). To avoid this possibility, in this study, we did not use DNA samples from cell lines with high clonal expansion.

When putative mutations were detected, DNA from another independent lymphoblastoid cell line from the same donor was used for confirmation. We ultimately confirmed *de novo* mutations by using DNA from uncultured cells.

2. Microsatellite loci

We selected 40 microsatellite loci that were previously documented to show relatively high mutation rates ($\geq 0.5 \times 10^{-2}$/locus/generation) or high heterozygosity rates in a population (>70%). The genomic location and size of the repeat unit of each locus are shown in Table 2.

3. Detection of microsatellite mutations

Microsatellite sequences were amplified with PCR using fluorescence-labeled primers flanking the repeat sequence, and the amplicon sizes in offspring were compared with those of both parents. Most of the primer sequences and PCR conditions used in this study are described in Furitsu *et al.* (*35*). We examined ten loci for which PCR conditions were not described or that were modified from the descriptions of Furitsu *et al.* The primer sequences and annealing temperatures for PCR are summarized in Supplementary Table 1. Five microsatellites in that table (D2S1338, D3S1358, D5S818, D13S317, D16S539) were amplified by multiplex PCR in the first screening.

Fluorescence-labeled PCR products from offspring and their parents were subjected to capillary electrophoresis with size markers and were analyzed with software (GeneScan or GeneMapper Analysis) provided by Applied Biosystems.

When the allele size for an offspring was found to differ from the sizes of the parental alleles, the new allele was considered as a putative mutation. Since it was reported that more than 85% of microsatellite mutations result from addition or deletion of a single repeat unit (*39*), we assumed that the mutant allele was derived from the parental allele of the closest size (*26–28*).

4. Exceptional cases

Occasional failures of PCR amplification were encountered and were apparently caused by sequence variations in regions that corresponded to the primer sequences. In such cases, one parent might show only one band, and thus appear to be homozygous, although he or she could actually be heterozygous bearing one "non-amplifying" (apparently null) allele (*28, 40*). Consequently, the offspring would appear to have inherited no allele from one parent and two copies of one of the two alleles from the other, a condition analogous to uni-parental disomy. Three such cases were discovered: one at DS19S47 and two at D10S1214 (in siblings). However, an alternative possibility also exists but is less likely to happen. That is, a mutation occurred at the mother's allele to give rise to the same size of the allele that was transmitted from the father. To discriminate the two possibilities, we examined the existence of hidden non-amplifying alleles by PCR amplification using new primer sets that were specific to sequences outside of the original primer regions. We found that none of the cases were caused by new mutations but rather were caused by the non-amplifying allele.

Mosaicism was also encountered; namely, individuals can show three bands as the result of a somatic mutation that occurred at one allele soon after fertilization. Two such cases were encountered, one at D21S1245 and the other at ACTBP2. Those were not considered to be germ-cell mutations. In the present study, both mosaic offspring were encountered only in the control group, thus we do not discuss the effect of a parental exposure in the post-zygotic mutation rate in offspring.

5. Mutation rates

The mutation rate is defined as the number of mutations per allele per generation.

6. Statistical analysis

Statistical analyses were conducted using Fisher's exact test. To evaluate the possibility that A-bomb radiation exposure increases the germline mutation rate, P values were obtained from one-sided likelihood-ratio tests, with values <0.05 indicating statistical significance.

RESULTS

Characteristics of the Mutations

In 70 exposed haploid sets (2,789 alleles) and 188 unexposed haploid sets (7,465 alleles) (Supplementary Table 2), 58 putative mutations were found in the first screening. Subsequently, second cell lines from 17 families were used to confirm the putative mutations but 9 out of 23 were not detected, indicating that these mutations arose during *in vitro* cell culture, while 14 were detected, counting results of the first screening. Next, we examined 49 putative mutations (i.e., 58 – 9) by using stored, uncultured whole blood samples; we found that 12 had occurred during *in vitro* culture of the cells and 37 were true *de novo* mutations (Table 3). The number of mutations detected at each locus is presented in Table 2. Possible parental origins were successfully assigned in the majority of the cases except for four

TABLE 2
Microsatellite Loci Screened in this Study with Mutations Detected at each Locus

Microsatellites	Type of repeat unit	Chromosome no.	Gene name[a]	Number of mutations derived from		
				Exposed allele	Unexposed allele	Undetermined allele
D9S58	2	9			2	
D9S63	2	9	FNBP1 (intron)			
D13S120	2	13	KIAA0774 (intron)*			
D13S128	2	13	FARP1 (intron)		2	1
D19S47	2	19				
D1S389	4	1				
D1S1612[b]	4	1	RPL7AP18*			
D2S1338[b]	4	2		1	1	
D3S1358[b]	4	3	LARS2 (intron)			
D3S1359[b]	4	3	MON1A (intron)			1
D3S1744	4	3			1	
D4S2431	4	4				
FGA	4	4	FGA (intron)			
D5S2501	4	5	FLJ43080 (intron)*		1	
D5S818[b]	4	5				
CSF1R (TAGA)	4	5	CSF1R (intron)		1	
CSF1R (CCTT/CTTT)	4	5	CSF1R (intron)		1	
ACTBP2	4	6	ACTBP2	2		
D7S1517	4	7	HYAL4		1	
D7S1482[b]	4	7		2	4	
D8S1179	4	8			1	
D9S748	4	9				
D10S1214	4	10		1	4	1
D10S1237	4	10	AFAP1L2 (intron)		1	
D12S66	4	12		1		
D12S67	4	12	MGAT4C (intron)*			
D12S391	4	12			1	
D12S1090	4	12			1	
VWA	4	12	VWA			
D13S317[b]	4	13				
D15S657	4	15	LOC100132798 (intron)*			
D16S539[b]	4	16				
D18S51	4	18	BCL2 (intron)			
D18S1270	4	18				
D19S244	4	19	CYP4F3 (intron)			
D19S245	4	19			1	1
D19S247	4	19	GNA15 (intron)			
D21S11	4	21			1	
D21S1245	4	21	TTC3 (intron)			
DXS981	4	X			2	
Total number of mutations				7	26	4

[a] Microsatellite loci that are mapped in coding (transcribed) regions are denoted by the appropriate gene name, but these with an asterisk are not registered in the Online Mendelian Inheritance in Man (OMIM). Those mapped in non-coding regions are shown as blanks.
[b] These eight microsatellite loci were added to the 32 loci described by Furitsu *et al.* (*34*).

TABLE 3
Mutations in the Alleles from Exposed and Unexposed Parents

	Exposed group		Control group	Statistical test (one-sided)
	Exposed alleles	Unexposed alleles	Unexposed alleles	
Number of alleles examined	2,789	2,462	5,003	
Mutations of parental origin determined	7	9	13	
Mutations of parental origin undetermined	4		4	
Total number of mutations (mean mutation rate: %)	11[a]/2,789 (0.39%)	26[a]/7,465 (0.35%)		$P = 0.43$[a]
	7[b]/2,789 (0.25%)	30[b]/7,465 (0.40%)		$P = 0.91$[b]

[a] All four mutations with unassigned parental origin in the exposed group were assumed to be radiogenic in origin.
[b] Four mutations with unassigned parental origins in the exposed group were assumed to be spontaneous.

TABLE 4
Gain or Loss of Repeat Units at Mutated Microsatellite Loci

	Exposed alleles	Unexposed alleles
Expansion (gain of repeat units)	6	12
Contraction (loss of repeat units)	1	11
Undetermined	0	3
Total number of mutations	7	26

mutations in the exposed group and four in the control group.

Mutations at di- or tetranucleotide repeats. Among the 37 mutations, five occurred at dinucleotide repeat loci (5 mutations/5 loci) and 32 at tetranucleotide repeat loci (32 mutations/35 loci) (Table 2). Thus there appears to be no preferential induction of mutations at di- and tetranucleotide repeat loci.

Mutations showing gain or loss of the repeat units. The direction (expansion or contraction) of the 33 mutations was examined (four mutations whose parental origin could not be assessed in the exposed families are not included here) (Table 4). Among the mutations that occurred at unexposed alleles, gains and losses seemed to occur equally (12 gains and 11 losses), but mutations at exposed alleles appeared to consist of more gains than losses (6 and 1, respectively). However, the latter difference was not statistically significant ($P = 0.14$, two-sided test).

Sex differences in mutation rates. It was possible to determine the parental origins of 22 mutations in the unexposed alleles, and the spontaneous mean mutation rates were 0.45×10^{-2}/locus/generation (17/3,745, Table 5) for the paternal alleles and 0.13×10^{-2}/locus/generation (5/3,720, Table 6) for the maternal alleles. The paternal mean mutation rate was about three times higher than that in the maternal alleles ($P < 0.05$), which is consistent with the data of other researchers showing that microsatellite mutation rates in humans are three to six times higher in males than in females (*27, 28, 39*). Among the exposed alleles, the mean mutation rates were 0.30×10^{-2}/locus/generation (4/1,349, Table 5) for the paternal alleles and 0.21×10^{-2}/locus/generation (3/1,440, Table 6) for the maternal alleles, but this difference was not statistically significant.

Effects of parental age. The mean parental ages at the birth of their offspring were compared between couples whose offspring exhibited or did not exhibit microsatellite mutations; they were 30.1 and 31.3 years old for the fathers and 27.9 and 27.8 years for the mothers, respectively. This difference was not statistically significant for either parent.

Effects of Parental Exposure to Radiation

1. Mutation rates in the exposed and unexposed alleles

In offspring from the exposed and the control families, 20 and 17 mutations were found, respectively (Table 3). Among those, we could not determine the parental origins of four mutations in the exposed group and four mutations in the control group. Those non-assigned mutations created a problem in estimating radiation-induced mutation rates, because only one parent was exposed in most of the exposed families (this is not a problem in the control families because neither parent was exposed). Thus two extreme cases were assumed: All non-assigned mutations in the exposed group occurred either in the exposed alleles or in the unexposed alleles. In the former case, the mean mutation rate in the exposed alleles was 0.39×10^{-2}/locus/ generation [(4 + 7)/2,789], which was slightly higher than 0.35×10^{-2}/locus/generation (26/7,465) in the control group (Table 3), but this difference is not statistically significant ($P = 0.43$). In the latter case, the mean mutation rate was 0.25×10^{-2}/locus/generation (7/2,789) in the exposed group, which is lower than the value of 0.40×10^{-2}/locus/generation [(4 + 26)/7,465] (Table 3) in the control group.

TABLE 5
Mutations in Paternal Alleles

	Exposed group		Control group	
	Exposed paternal alleles	Unexposed paternal alleles	Unexposed paternal alleles	Statistical test (one-sided)
Number of alleles examined	1,349	1,262	2,483	
Number of mutations of paternal origin	4	6	11	
Number of mutations of undetermined parental origin	4 (1 in a family of father's exposure and 3 in families of mother's exposure)		4	
Total number of mutations (mean mutation rate: %)	5[a]/1,349 (0.37%) 4[b]/1,349 (0.30%)		17[c]/3,745 (0.45%)	$P = 0.73$[d]

[a] It was assumed that one unassigned mutation in a family with father's exposure was derived from the exposed paternal allele.
[b] It was assumed that one unassigned mutation in a family with father's exposure was derived from the unexposed maternal allele.
[c] It was assumed that all of the seven unassigned mutations (three in the exposed and four in the control group) were derived from maternal alleles to estimate the smallest spontaneous mutation rate in paternal alleles of the control group.
[d] Statistical test of the differences between *a* and *c*.

TABLE 6
Mutations in Maternal Alleles

	Exposed group		Control group	
	Exposed maternal alleles	Unexposed maternal alleles	Unexposed maternal alleles	Statistical test (one-sided)
Number of alleles examined	1,440	1,200	2,520	
Number of mutations of maternal origin	3	3	2	
Number of mutations of parental origin undetermined	4 (3 in families of mother's exposure and 1 in a family of father's exposure)		4	
Total number of mutations (mean mutation rate: %)	6[a]/1,440 (0.42%)	5[c]/3,720 (0.13%)		$P = 0.06$[e]
	3.8[b]/1,440 (0.26%)	6.3[d]/3,720 (0.17%)		$P = 0.30$[f]

[a] It was assumed that three unassigned mutations in families with mother's exposure were derived from the exposed maternal alleles.
[b] It was assumed that only 1/4 of three unassigned mutations in families with mother's exposure were derived from exposed maternal alleles.
[c] It was assumed that all five unassigned mutations (one in a family with father's exposure and four in the control group) were derived from paternal alleles and were excluded from calculating the spontaneous mutation rate in females.
[d] It was assumed that only 1/4 of five unassigned mutations were derived from maternal alleles.
[e] Statistical test of differences between cases *a* and *c*.
[f] Statistical test of differences between cases *b* and *d*.

2. Mutation rates in paternal and maternal alleles

Since the mutation rate in paternal alleles was about three times higher than that in the maternal alleles in the control group, radiation effects on paternal and maternal germ cells were assessed separately. Tables 5 and 6 summarize the possible mutations detected in paternal and maternal alleles, respectively.

Mutations in paternal alleles. In the exposed group, 14 mutations were detected. Four occurred in the exposed alleles, six occurred in the unexposed alleles, and four could not be assigned a parental origin (one in a family with father's exposure and three in families with mother's exposure, Table 5). In the control group, 15 mutations were detected but for four of them, the parental origin could not be determined. Therefore, if we restrict the confirmed cases regarding the parental origin, the mean mutation rate is 0.30×10^{-2}/locus/generation in the exposed group (4/1,349, Table 5), which is not higher than 0.45×10^{-2}/locus/generation in the control group (17/3,745, Table 5). Even if we consider that the one unassigned mutation in a family with father's exposure occurred in the paternal allele, and the remaining unassigned mutations occurred in the maternal alleles (i.e., three mutations in the exposed group but in families with mother's exposure and four mutations in the control group), which gives the lowest estimation of the spontaneous mutation rate in the paternal alleles, the difference is not statistically significant: 0.37×10^{-2}/locus/generation [(4 + 1) = 5/1,349] in the exposed paternal alleles compared to 0.45×10^{-2}/locus/generation (17/3,745) in the unexposed paternal alleles ($P = 0.73$, Table 5).

Mutations in maternal alleles. In the exposed group, 10 mutations were found. Three occurred in the exposed alleles, three occurred in the unexposed alleles, and four could not be assigned a parental origin (three in families with mother's exposure and one in a family with father's exposure, Table 6). In the control group, six mutations were detected, but the parental origin could not be determined in four of them.

Mutation rates were estimated under two different sets of hypothetical conditions. Under the first set of conditions, the minimum estimate of the spontaneous mutation rate was made by assuming that all five unassigned mutations (one in a family with father's exposure and four in the control group) occurred in paternal alleles, which gave an estimate of 0.13×10^{-2}/locus/generation (5/3,720, Table 6). The corresponding maximum rate of mutation induction after radiation exposure was estimated by assuming that all three unassigned mutations in families with maternal exposure occurred in the exposed maternal alleles, which gave an estimate of 0.42×10^{-2}/locus/generation [(3 + 3) = 6/1,440, Table 6]. That rate was nearly three times higher than the spontaneous rate of 0.13×10^{-2} but the difference was only marginally significant due to the small number of the mutations ($P = 0.06$, Table 6). Under the second set of conditions, unassigned mutations were allocated to paternal and maternal origins in proportion to the spontaneous rate, namely, 3 to 1. In that situation, the mutation rate in the exposed alleles was 0.26×10^{-2}: [3 + (3/4)]/1,440 in the exposed alleles and 0.17×10^{-2}; [5 + (1/4) + (4/4)]/3,720 in the unexposed alleles (Table 6). The difference was not statistically significant ($P = 0.30$, Table 6).

DISCUSSION

Different Radiation Response in Mice and Humans

The present results indicated no mutagenic effect of parental exposure to ionizing radiation at microsatellite loci in the offspring of A-bomb survivors. The results are at variance with the results of mutagenesis studies obtained at hypervariable tandem repeat sequences in male mice (*41*).

Mouse hypervariable loci consist of arrays of 4 to 6 base repeats as do human microsatellites but contain a much larger number of repeats which often exceed 1,000 (>several kb in allele length) and are thus referred to as expanded simple tandem repeats (m-ESTRs) (*41–45*). Although mutations at both m-ESTRs and human microsatellites (h-microsatellites) are thought to occur via replication slippage, spontaneous mutation rates differ considerably between the two species, e.g., as high as 10×10^{-2}/locus/generation in the ESTRs compared to less than 1×10^{-2}/locus/generation in h-microsatellites (*26–28, 42–45*). Since the mutation induction rate at m-ESTR loci may reach as high as a few percent per Gy, which is too high to be explained by radiation-induced damage to the target loci themselves, it is generally believed that the ESTR mutations are induced through genomic instability (*41, 46–48*). After irradiation of mouse spermatogonia cells, the mutation rate increased in a dose-dependent manner at the m-ESTR loci, and the estimated doubling dose (the dose required to double the spontaneous level of mutations) ranged from 0.4 to 1.1 Gy in most studies (*41, 47, 48*). Thus we expected that if the radiosensitivity for mutagenesis at m-ESTR and h-microsatellite loci were not largely different, genetic effects of parental exposure to radiation would have been detected in the present study. However, in contrast to high radiosensitivity at m-ESTR, no radiation-associated mutation induction at h-microsatellites was observed. It is noted that with our sample size, 2,789 alleles (mean dose 1.56 Gy) in the exposed group and 7,465 alleles in the control group, we could have detected the radiation effect with a 70% probability if the mean mutation rate had doubled in the exposed group compared with that in the control group (0.35%; 26/7465, Table 3).

In the mouse specific locus test, which is believed to detect mutations via direct damage of the genes by irradiation, the X- or γ-ray-induced mutation rate in the offspring mated at 6 to 7 weeks after irradiation did not differ from that in the offspring mated 8 to 40 weeks after irradiation (*49*). In the m-ESTR mutation studies, however, the radiation effect has not been studied beyond 15 weeks after the irradiation. In this regard, it should be noted that most of the A-bomb survivors had their children more than 15 years after their radiation exposure. Thus it is possible that either the instability is less pronounced in humans than in mice and/or unstable conditions that might have been induced in human germ cells immediately after radiation exposure did not persist beyond 15 years after the exposure.

Last, ionizing radiation can induce mutations in somatic and germ cells of various species tested so far, and hence it is unlikely that humans are an exception. Radiation doses in animal studies are considerably higher than those received by the survivors of the bombs. For example, specific locus tests in mice mainly used acute doses of 3 to 5 Gy whereas only a small fraction of A-bomb survivors have estimated doses of 2 Gy or larger. The mean parental dose of the survivors (exposed group) in the F_1 epidemiological cohort is about 0.4 Gy (*50*). Nonetheless, a clear dose response has been observed in m-ESTR studies for a dose range of 0.5 to 1 Gy (*41, 47, 48*), which corresponds to the dose range of the A-bomb survivors.

Feasibility of Detecting Radiation Effects in Females

Although no discernible effect from radiation exposure was observed, the observed mutation rates after maternal exposures appeared to be elevated compared with spontaneous rates ($0.26–0.42 \times 10^{-2}$/locus/generation and $0.13–0.17 \times 10^{-2}$/locus/generation, respectively, Table 6) although the difference was not significant. If we take the mutation rates at face value, then we may estimate the sample size to detect the possible difference with a probability of 0.8, i.e., 48,000 unexposed alleles and 16,000 exposed alleles, about 10 times larger than the present study. Unfortunately, obtaining such a large number of samples is not realistic at this time.

Biological Implications of New Mutations in Human Genome

Recent reports on genome sequencing revealed that even apparently healthy individuals carry thousands of nonsynonymous single nucleotide polymorphisms that cause amino acid substitutions and nearly 1000 insertions or deletions that involve protein-coding sequences in their genome (*51–55*). Some of those variations may be old and are either neutral or even advantageous, whereas others may be rather new and may cause a genetic burden to their hosts. In any event, when we present the data on the genetic effects of radiation, the risk would be better understood if we were able to know the number of pre-existing mutations in our genome and the ones that were added as a consequence of radiation exposure. Three different approaches, epidemiological, clinical and molecular studies, are indispensable for an unbiased understanding of the genetic effects of A-bomb radiation.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. Primer sequences and annealing temperature for ten loci that were not described previously. **Supplementary Table 2**. Number of alleles examined. http://dx.doi.org/10.1667/RR1991.1.S1

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