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Full Paper

Analysis of the gut microbiome to validate a mouse model of pellagra

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Pellagra is caused by an abnormal intake and/or use of niacin, but its phenotypes are diverse. The phenotypes of pellagra can also be atypical, such as nausea. We previously reported a mouse model of pellagra-related nausea. However, the mechanism of this model is unclear. In this study, we found that the gut microbiota, which is thought to be a source of niacin, played an important role in the development of pellagra-related nausea in germ-free mice. We also investigated the gut microbiome. We compared urinary niacin metabolite levels and the dermal response between mice fed a normal diet and those fed a low-niacin diet to investigate the putative trigger of pellagra. Epoxyeicosatrienoic and hydroxyeicosatetraenoic acid levels were higher in mice fed a low-niacin diet compared with those fed a normal diet. Furthermore, histological studies indicated a dermatological response to the low-niacin diet. Interestingly, higher levels of oxidised fatty acids in response to the germ-free state were also observed. These findings indicate successful establishment of our newly established mouse model of pellagra via the gut microbiota. We believe that this model could enable the discovery of the putative cause of pellagra and phenotypes of pellagra that have not been recognised yet.

Key words: animal model, microbiome, niacin, pellagra

INTRODUCTION

Pellagra is a clinical syndrome characterised by dermatitis, diarrhoea, dementia, and nausea [1–4]. It is caused by malnutrition and has two types. One type may result from insufficient intake of dietary niacin or tryptophan (primary pellagra), and the other results from insufficient use of niacin or tryptophan (secondary pellagra) [5]. Although the cause of pellagra is clear, its diagnosis usually relies on severe clinical symptoms. Previously, we found that feeding a low-niacin diet was required to construct mouse models of mild and secondary pellagra using isoniazid (INH) [6]. A niacin-free diet causes body weight (BW) loss and severe pathological conditions in rats and humans [7, 8]. In our studies, we did not use these diets, as we wished to avoid inducing severe pellagra, which sometimes causes death.

All living organisms, including bacteria, require nicotinamide adenine dinucleotide (NAD) to live and regulate intracellular processes [9]. Bacteria obtain NAD via *de novo* synthesis and the salvage pathway. However, some bacteria cannot synthesise NAD *de novo* and thus must use the salvage pathway to import niacin or nicotinamide riboside through substrate importers [10]. Some water-soluble vitamins, including niacin, are synthesised by certain types of gut bacteria, which are thought to be important sources of B vitamins for their host [11, 12]. Therefore, dietary niacin and the gut microbiome appear to mutually control each other [13].

Based on this, we used model mice to investigate whether the gut microbiota plays an important role in the development of pellagra-related nausea. Furthermore, we also focused on the symptoms of pre-pellagra, which is defined as the condition before the onset of prominent symptoms and has obvious potential to cause disease, to gain mechanistic insights with respect to pellagra. In the future, our newly validated and established mouse model will hopefully be useful for identifying putative pellagra-inducing drugs that have not yet been recognised as triggers or causes of this disease.

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MATERIALS AND METHODS

Experimental animals

The Institutional Animal Care and Use Committee at Shionogi & Co., Ltd. approved the animal experiments. The experiments were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (S21041C-0002). Female Balb/c mice (6 weeks of age) were obtained from Japan SLC (Hamamatsu, Japan) and housed five per cage under controlled environmental conditions (24°C ± 2°C; 50% ± 20% relative humidity; 12-hr light/dark cycle, lights on at 8:00 a.m.). The mice were fed a normal or low-niacin diet (Supplementary Tables 1, 2), and their BWs were recorded as previously described [6]. The mice were fed the normal or low-niacin diet for 15 or 17 days (D0–D15 or D0–D17). Germ-free Balb/c and their control counterparts were obtained from CLEA Japan, Inc. (Tokyo, Japan) to investigate the role of the gut microbiota in the profile of oxidised fatty acids (oxFAs) in tissue.

All animal experiments were conducted in accordance with the ARRIVE guidelines (Animal Research: Reporting *In Vivo* Experiments) [14].

Procedures of the animal experiments

As previously reported [15], antibiotic-treated animals were provided ampicillin (1 g/L; Sigma Aldrich, St. Louis, MO, USA), vancomycin (0.5 g/L; Sagent Pharmaceuticals, Schaumburg, IL, USA), neomycin (0.5 g/L; Thermo Fisher Scientific, Waltham, MA, USA), gentamycin (100 mg/L; Sigma Aldrich), and erythromycin (10 mg/L; Sigma Aldrich) in drinking water for 15 days beginning at 6 weeks of age to investigate the role of the gut microbiota in the development of pellagra-related nausea (Fig. 1a). Mice received the normal or low-niacin diet for 15 days to investigate the role of a low-niacin diet on the development of mild pellagra or on the gut microbiota (Fig. 1b). After mice were fed the low-niacin diet for 15 days, they were kept under the normal diet for 2 days to investigate whether the effect of the low-niacin diet was transient (Supplementary Fig. 1a).

To investigate the effect of the low-niacin diet on the profile of the gut microbiota or tissue levels of oxFAs, faeces and ears were harvested immediately from 16 days onwards after the experiments began. These samples were stored at −80°C or in liquid nitrogen for later analysis of the gut microbiota and lipid production. To investigate whether the effect of the low-niacin diet was a transient or plastic response, faeces and ears were harvested from 18 days onwards. These samples were stored at −80°C or in liquid nitrogen for later analysis of the gut microbiota and the amount of lipids.

Urinary samples were collected from all mice each day and stored at −80°C for later analysis of tryptophan–nicotinamide pathway metabolites. Briefly, each mouse was administered saline once daily for 5 days starting at 10 days after the start of feeding of the normal or low-niacin diet. At 3 hr after saline administration, each mouse was moved to a 500-mL glass beaker, and urine was harvested from the mice within 5 min.

Histopathological analyses

Paraffin sections were prepared from skin (ear) samples obtained from Balb/c mice that were fed a normal or low-niacin diet as previously described [16]. The sections were stained with haematoxylin and eosin and/or acidic toluidine blue for histopathological analysis by light microscopy.

Analysis of the gut microbiome

Faeces were collected in 2-mL tubes (ST-0250F-0, Yasui Kikai, Osaka, Japan) with Metalcorn (MC-0218R(S), Yasui Kikai), freeze-dried using a freeze dryer (VD-250R; Taitec, Koshigaya, Japan), and crushed using a Multi-beads Shocker (MB2000, Yasui Kikai) at 1,500 rpm for 2 min at room temperature. DNA was extracted and purified from the crushed faeces in accordance with the procedures of ISOSPIN Fecal DNA (Nippon Gene, Tokyo, Japan) and MPure Bacterial DNA Extraction (MP Bio Japan, Tokyo, Japan) kits. A DNA library was then constructed using two-step tailed polymerase chain reaction (PCR) methods. The sequences of the first primers for the first PCR were ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNC CTA CGG GNG GCW GCA G (5′-3′) and GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN GAC TAC HVG GGT ATC TAA TCC (5′-3′). The sequences of the first primers for the second PCR were ATT GAT ACG GCG ACC ACC GAG ATC TAC AC -INDEX2- ACA CTC TTT CCC TAC ACG ACG C (5′-3′) and CAA GCA GAA GAC GGC ATA CGA GAT -INDEX1- GTG ACT GGA GTT CAG ACG TGT G (5′-3′). The quality of the constructed libraries was checked using a Fragment Analyzer (BioTek, Winooski, VT, USA) and dsDNA 915 Reagent Kit (Agilent, Santa Clara, CA, USA) and analysed using a MiSeq

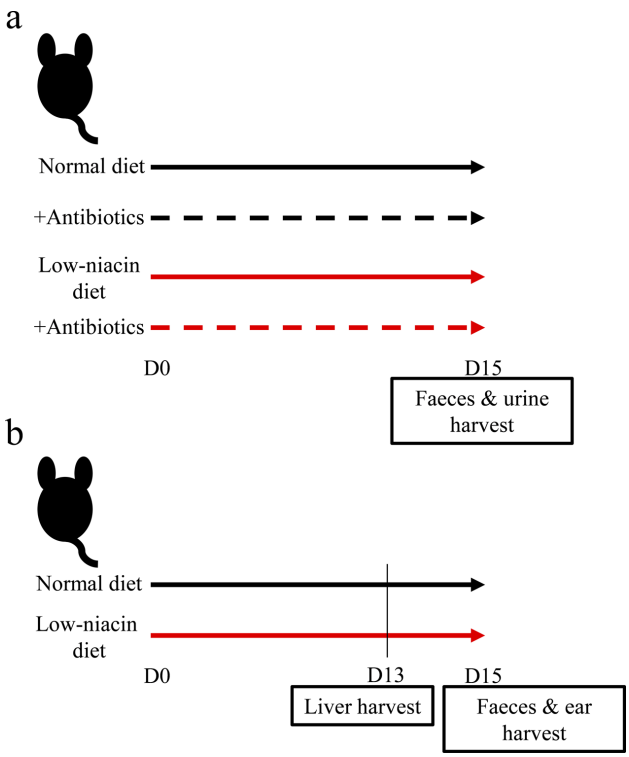

Fig. 1. Study procedures. The black arrow indicates mice on the normal diet. The red arrow indicates mice on the low-niacin diet. The experiments started on day 0 (D0). (a) The solid line indicates mice not treated with antibiotics, and the dotted line indicates those treated with antibiotics. Faeces and urine were harvested on D15. (b) Livers were harvested on D13. Faeces and ears were harvested on D15. Four or five mice in each group were housed in plastic cages under appropriate conditions.

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system and MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA; Supplementary Table 3). Each read sequence was extracted when the beginning of the sequence exactly matched the primer used. The primer sequence was then deleted from the extracted sequence, and read sequences with the appropriate quality were selected and joined with others having at least 10-bp overlap.

Analysis of skin epoxyeicosatrienoic acid, hydroxyeicosatetraenoic acid, and prostaglandin levels

Harvested ears were immediately moved to liquid nitrogen, dried using a freeze dryer (VD-550R, Taitec, Kumagaya, Japan), and stored at −80°C until lipid extraction to investigate the tissue levels of prostaglandin E2, epoxyeicosatrienoic acid (EET), and dihydroxyeicosatrienoic acid in accordance with previous studies [16, 17]. In some experiments, harvested ear samples were directly stored at −80°C.

Analysis of gene expression in the liver

Each liver was harvested at 13 days after the beginning of the experiments to investigate the cause of pre-pellagra. Total RNA from the liver was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with a standard procedure in accordance with the manufacturer's instructions. After the extraction, the quantity of RNA was determined using a Quantus Fluorometer (Promega, Madison, WI, USA) and QuantiFluor RNA system (Promega). The quality of RNA was determined using a 5200 Fragment Analyzer System (Agilent Technologies, Santa Clara, CA, USA) and Agilent HS RNA Kit (Agilent Technologies). After checking the RNA, the libraries were constructed using these RNAs in accordance with the manufacturer's instructions (MGIEasy RNA Directional Library Prep Set, MGI Tech Co., Ltd., Shanghai, China). The concentrations of the libraries were measured with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and dsDNA HS Assay Kit (Thermo Fisher Scientific). The quality was checked with a Fragment Analyzer (Agilent Technologies) and dsDNA 915 Reagent Kit (Agilent Technologies). Circular DNA was then prepared using the constructed libraries and an MGIEasy Circularization Kit in accordance with the manufacturer's instructions (MGI Tech Co., Ltd.). After construction, DNA Nanoballs (DNBs) were prepared with a DNBSEQ-G400 RS High-throughput Sequencing Kit (MGI Tech) in accordance with the manufacturer's instructions. Sequencing analysis of these DNBs was carried out using the DNBSEQ-GS400 under 2 × 100-bp conditions. Low-quality sequences were omitted using the Cutadapt (ver. 1.9.1) and Sickle (ver. 1.33) tools. Finally, we performed mapping using high-quality sequences and Hisat2 (ver. 2.2.0) and obtained data in SAM format. Each gene, which was <50 reads in both groups of mice, was omitted. The numbers of reads were compared between mice fed the normal diet and those fed the low-niacin diet.

Statistical analysis

The appropriate sample size for each group was determined as previously reported [6] and found to be n=3–5 for each group. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The groups were compared using the paired t-test, Wilcoxon rank-sum test, or Tukey's multiple comparisons method.

RESULTS

Effect of the gut microbiota on pellagra-related nausea

We previously reported that INH treatment caused pellagra-related nausea in mice fed a low-niacin diet but not in those fed a normal diet [6]. In the present study, mice were treated with antibiotics to investigate the role of the gut microbiota in pellagra-related nausea. Metabolic flow from tryptophan to 1-methyl-2-pyridone-5-carboxamide (2-Py), N'-methyl-4-pyridone-3-carboxamide (4-Py), and nicotinamide N-oxide (NNO) was observed (Fig. 2a). Some of the observed metabolites were thought to be important in our mouse model of pellagra. An increase in BW was not observed in mice fed the normal or low-niacin diet and treated with antibiotics compared with those not treated with antibiotics (Fig. 2b). Faeces were harvested from each group at 16 days onward, and pica, which is thought to represent human nausea, was only observed in mice fed the low-niacin diet and treated with antibiotics (Fig. 2c). Urinary levels of 1-methylnicotinamide (MNA), NNO, 2-Py, and 4-Py were significantly lower in mice fed the low-niacin diet, with or without antibiotic treatment, compared with those fed the normal diet (Fig. 2d). Furthermore, higher MNA, NNO and 2-Py responses to antibiotics were observed in mice fed the normal or low-niacin diet compared with those not treated with antibiotics (Fig. 2d). On the other hand, lower kynurenic acid (KA) and xanthurenic acid (XA) responses to antibiotics were observed in mice fed the normal or low-niacin diet compared with those not treated with antibiotics (Fig. 2e).

Effect of a low-niacin diet on the gut microbiota

BW did not differ between mice fed the normal diet and those fed the low-niacin diet (Fig. 3a). Although the orders *Clostridiales*, *Bacteroidales*, *Desulfovibrionales,* and *Lactobacillales* dominated the bacterial compositions of both groups, their gut microbiome profiles differed (Fig. 3b). These differences were statistically analysed according to the procedure described in Supplementary Table 4. The delta score was significantly higher in mice fed the low-niacin diet compared with those fed the normal diet (Fig. 3c). At the family level, the microbiomes of mice fed the normal diet were significantly enriched with *Bacteroidaceae* (% frequency; normal diet, 10.00 ± 1.60; low-niacin diet, 6.40 ± 1.61; p=0.019) and *Streptococcaceae* (normal diet, 10.34 ± 2.12; low-niacin diet, 4.17 ± 0.57; p=0.001). The microbiomes of mice fed the low-niacin diet were significantly enriched with S24-7 (normal diet, 6.36 ± 1.01; low-niacin diet, 10.54 ± 1.96; p=0.009) and *Ruminococcaceae* (normal diet, 3.55 ± 0.51; low-niacin diet, 4.31 ± 0.32; p=0.044). Sixteen bacterial taxa were significantly more abundant in mice fed the normal diet compared with those fed the low-niacin diet, including four families, four genera, and one strain (Fig. 3d and Supplementary Table 5).

Pellagra-like (pre-pellagra) responses to a low-niacin diet

Patients with pellagra tend to recover after niacin supplementation, and urinary levels of niacin metabolites, specifically MNA and 2-Py [18], are good biomarkers for pellagra. Therefore, we investigated the urinary levels of MNA, NNO, 2-Py, and 4-Py from both mouse groups as previously described [6]. Figure 2a shows the metabolic flow from tryptophan to its metabolites. To collect the required amount of urine, mice were administered 400 µL of saline before collection.

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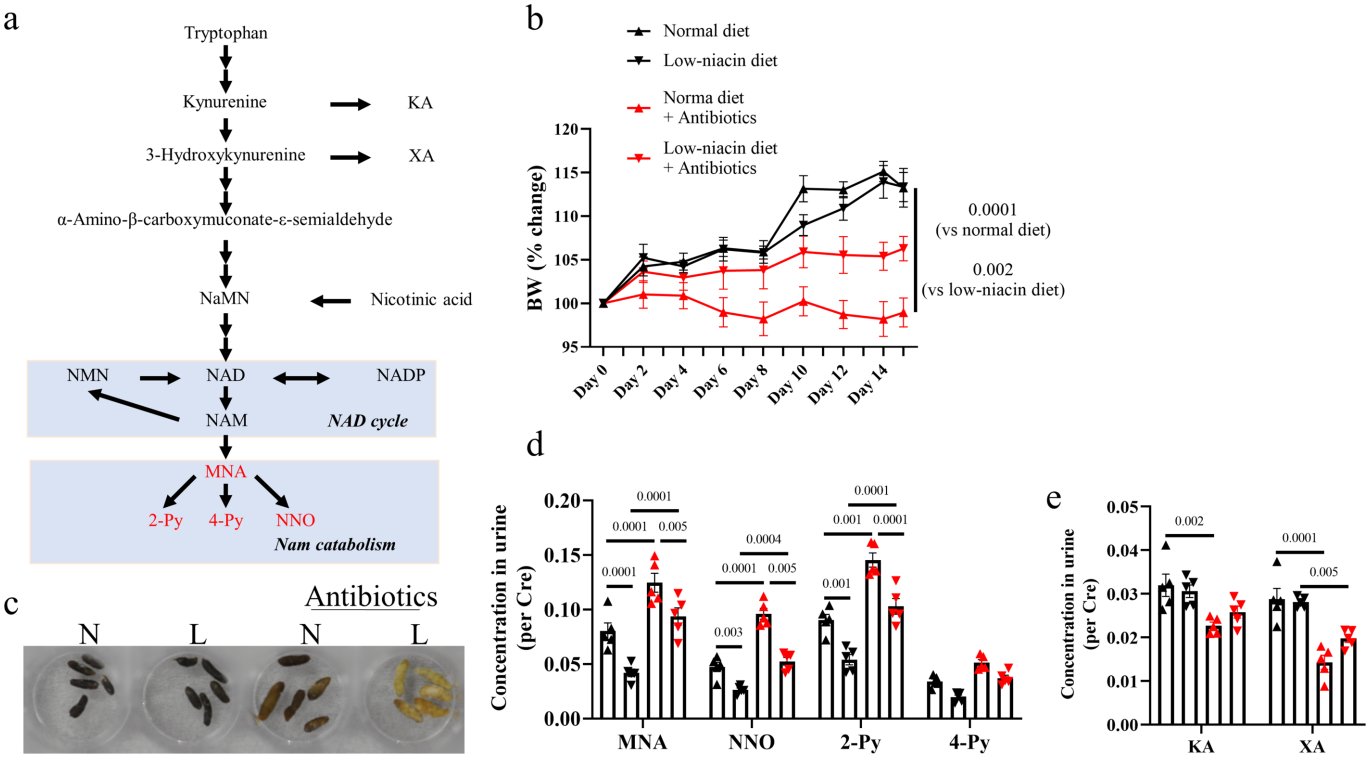

Fig. 2. Effect of germ-free status on pellagra-related symptoms. (a) Metabolic flow from tryptophan to its metabolites, with the investigated metabolites indicated in red. Abbreviations for the niacin metabolites are as follows: kynurenic acid (KA), xanthurenic acid (XA), nicotinic acid mononucleotide (NaMN), nicotinamide adenine dinucleotide (NAD), nicotinamide (NAM), N1-methylnicotinamide (NMN), nicotinamide adenine dinucleotide phosphate (NADP), N-methylnicotinamide (MNA), N'-methyl-2-pyridone-3-carboxamide (2-Py), N'-methyl-4-pyridone-3-carboxamide (4-Py), nicotinamide-N-oxide (NNO). (b) Growth curves (% BW change) in the normal diet and low-niacin diet groups treated with or not treated with antibiotics (n=5). Each comparison was carried out between mice treated with and not treated with antibiotics and fed the same diet. ▲, Normal diet; ▼, low-niacin diet; ▲, normal diet + antibiotics; ▼, low-niacin diet + antibiotics. (c) Photographs of faeces show whether pica was observed. The image with white-coloured faeces reflects pica, which is thought to indicate nausea in humans. (d) Urinary levels of the niacin metabolites MNA, NNO, 2-Py, and 4-Py were determined in both groups on D15 (n=5 in each group). (e) Urinary levels of the niacin metabolites KA and XA were determined in both groups on D15 (n=5 in each group). The experiments were repeated twice. Each triangle represents an individual. Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods section.

Urine samples were collected after 3 hr rather than collecting 24 hr urine samples because we were concerned about the stability of these metabolites. We also wished to focus on rapid metabolite responses to certain types of drugs [6]. The MNA, NNO, 2-Py, and 4-Py responses to the low-niacin diet were significantly lower than those to the normal diet (Fig. 4a).

We then performed a histopathological analysis using ears from mice fed the normal or low-niacin diet to determine pellagra-like response because some dermal symptoms have been observed in patients with pellagra [1–4]. Although no prominent differences were observed in either group, the number of mast cells was significantly higher in mice fed the low-niacin diet compared with those fed the normal diet (Fig. 4b–d). We defined this phenotype as pre-pellagra.

Tissue oxidised fatty acid levels

Skin levels of arachidonic acid (AA) metabolites, such as hydroxyeicosatetraenoic acids (HETEs), play important roles in the development of dermatitis [16, 17, 19]. Furthermore, niacin deficiency increases the levels of prostaglandin E synthase, which produces prostanoids, in the skin of mice and humans [20].

Ear skin samples were harvested from the mice using an 8-mm biopsy punch (Kai Industries Co. Ltd., Gifu, Japan) and immediately frozen until use. The lipids were investigated as previously described [16, 17]. Unexpectedly, the prostaglandin D2, prostaglandin E2, and PGF2a levels in the skin did not differ between mice fed the low-niacin diet and those fed the normal diet (Fig. 5a). The 8-, 9-, 11- and 15-HETE responses in the ears were significantly higher in mice fed the low-niacin diet than in those fed the normal diet, but no clinical features, such as oedema or erythema, were observed in either group (Fig. 5b). Finally, we analysed tissue levels of EETs to determine whether a low-niacin diet causes quantitative changes in EETs. Ear skin from mice fed the low-niacin diet showed significantly more EETs than those in mice fed the normal diet (Fig. 5c).

Effects of the gut microbiota on skin fatty acid metabolite levels

Ears harvested from germ-free and control mice were used to investigate the role of the gut microbiota on skin levels of oxFAs. Significantly higher 9-HETE, 12-HETE, 15-HETE, 16-HETE, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET responses were observed in germ-free mice compared with those in control mice (Fig. 6).

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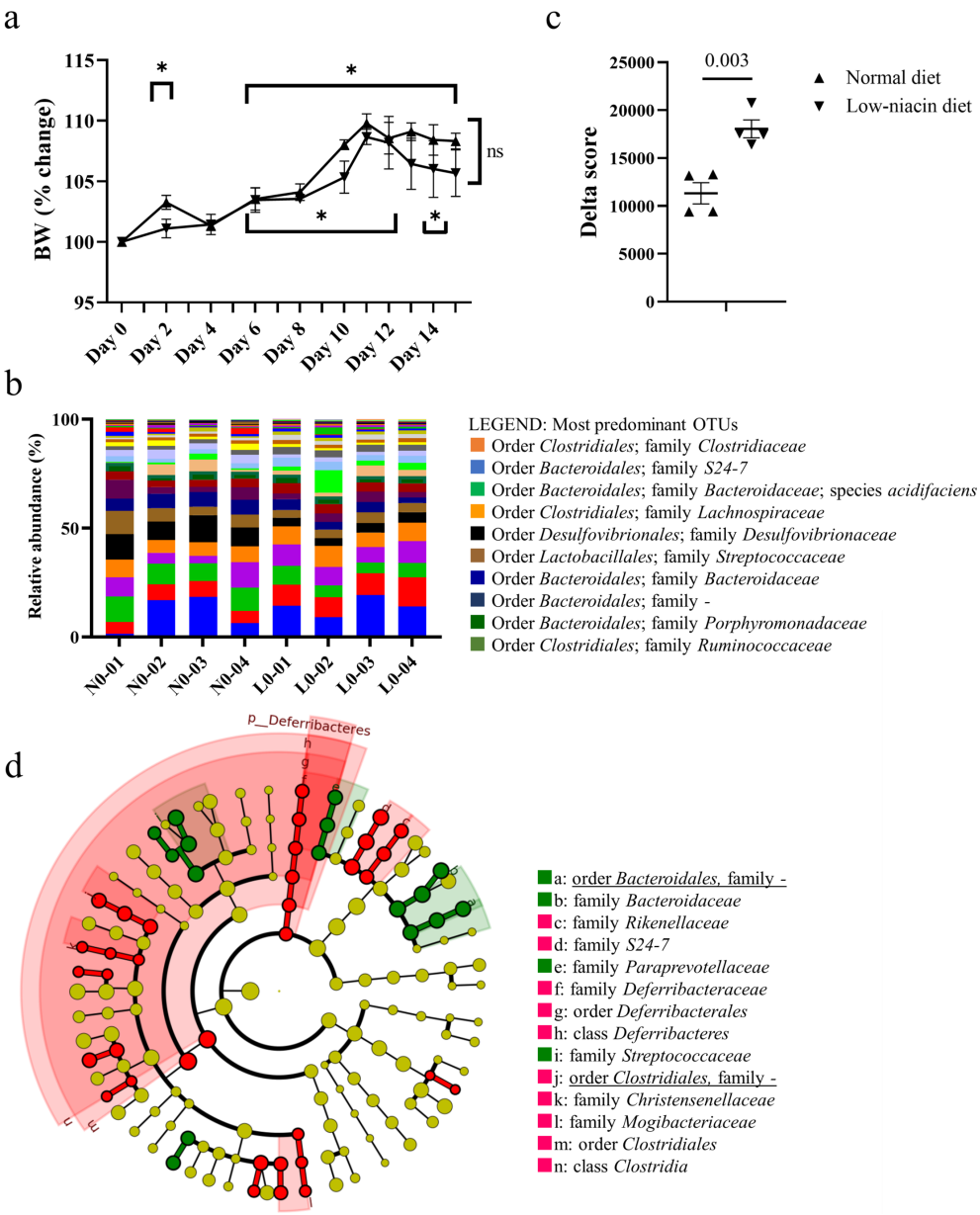

Fig. 3. Effect of a low-niacin diet on the gut microbiota. (a) Growth curves (% BW change) in the normal diet and low-niacin diet groups (n=4 in each group). Each comparison was carried out between both groups. ▲, Normal diet; ▼, low-niacin diet; ns: not significant; *: significant ($p<0.05$) vs. day 0. (b) Operational taxonomic units (OTUs) of the gut microbes were compared between mice fed the normal diet (n=4) and those fed the low-niacin diet (n=4 in each group). The most predominant OTUs are indicated. (c) We calculated the delta score to estimate the differences in the bacterial profile between both groups as follows: delta score of N0-1 = (|B1n1−B1 m | + | B2n1−B2 m | + |B3n1−B3 m | + |B4n1−B4 m | + |B5n1−B5m| + |B6n1−B6m| + |B7n1−B7m|) (Supplementary Table 4) [39]. Each triangle represents an individual. (d) Cladogram of gut microbial taxa in mice fed the normal or low-niacin diet. Green circles indicate increased taxa in the normal diet group; red circles indicate increased taxa in the low-niacin diet group. The experiments were repeated twice. Each triangle represents an individual. Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods.

Gene expression profile

Harvested liver samples from mice fed the normal or low-niacin diet were used for analysis of gene expression to better understand the mechanism of how low-niacin diet affects the development of mild pellagra. Table 1 shows the top 50 genes sorted by the significance of p values. The gene expression of Cyp enzymes and Elovl6 differed significantly between mice fed the normal diet and those fed the low-niacin diet. These genes are thought to be involved in fatty acid metabolism.

DISCUSSION

In this study, we found that the gut microbiota plays an important role in the development of pellagra and that pellagra induced by a low-niacin diet may also play a role in the gut microbiota. The gut microbiota may be directly involved in some dermal responses in our mouse model. A conceptional scheme of how niacin in involved in pellagra is shown in Fig. 7. Importantly, control of the gut microbiota might be a therapeutic

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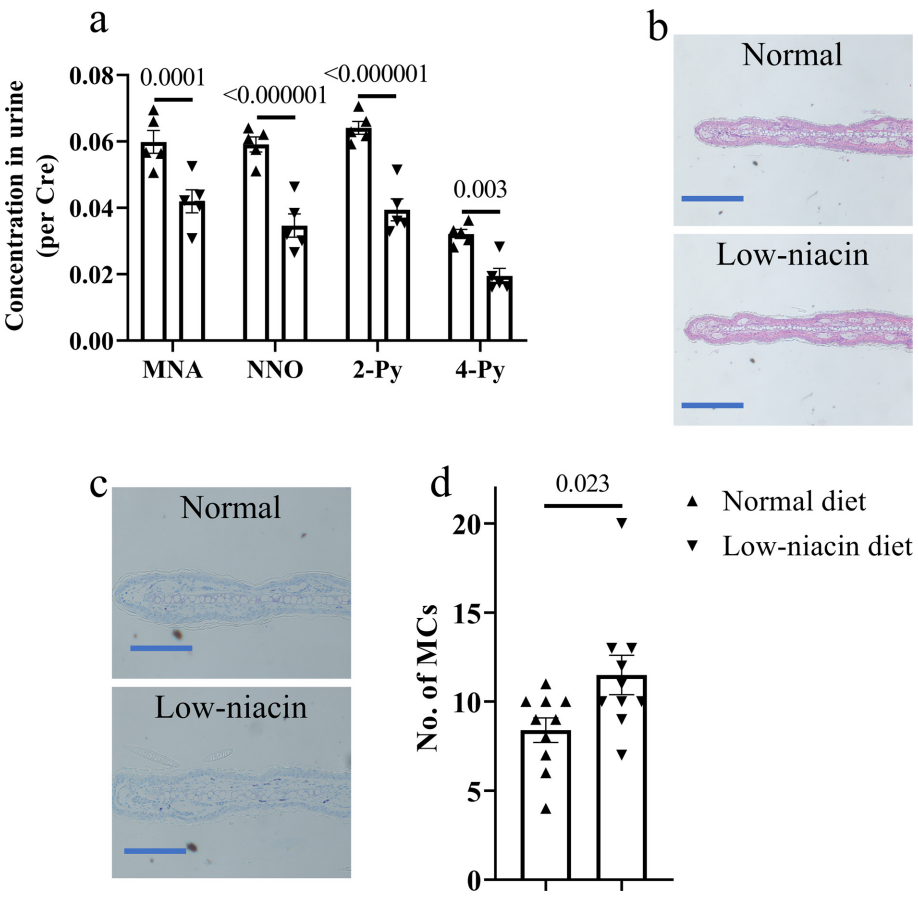

Fig. 4. Effect of the low-niacin diet on urinary levels of niacin metabolites and certain types of dermal response. (a) Urinary levels of the niacin metabolites MNA, NNO, 2-Py, and 4-Py were determined in both groups on D15 (n=5 in each group). The experiments were repeated twice. ▲, Normal diet; ▼, low-niacin diet. (b–d) Representative images of ear skin from mice fed the normal or low-niacin diet at D15. Tissues were subjected to haematoxylin eosin (HE) staining (b) to evaluate the pathology and to toluidine blue (TB) staining (c) to count the number of mast cells (MCs) (d). Scale bars, 200 μm for HE and 100 μm for TB. Values represent the number of MCs in one selected focus area (randomly selected). Each triangle represents an individual (n=10). Data represent the mean ± standard error. The experiments were repeated twice.

target for pellagra because the changes in the gut microbiota profile caused by a low-niacin diet are transient (Supplementary Fig. 1). Interestingly, the gut microbiota responses to the diets were quicker than those of oxFAs. Furthermore, the urine levels of metabolites responded to the diets the quickest (Supplementary Fig. 1).

Previous studies showed that bacterially produced vitamin B6 [13, 16], which is important for producing niacin, was insufficient to sustain the metabolism of the gut microbiota [11, 12]. These studies reported that vitamin B6 deficiency reduced the relative abundance of *Bacteroidaceae* and increased that of *Lachnospiraceae*. These results are mostly consistent with our results, indicating that a deficiency of vitamin B, including niacin, might significantly affect the host's gut microbiota. Furthermore, pellagra-related pica was observed in germ-free mice fed the low-niacin diet, although pica was not observed in non-germ-free mice fed the fed low-niacin diet or in germ-free mice fed the normal diet. These findings suggest that abnormal intake of niacin via food or bacteria might be the trigger that causes pellagra. Curiously, the urine levels of niacin metabolites in mice treated with antibiotics were elevated as compared with those not treated with antibiotics. In addition, no weight gain with growth was observed in the treated mice. The above suggests that NAD synthesis from tryptophan, which is essential for maintaining the body, is prioritized, and this may be a seemingly contradictory result. Upper metabolites such as KA and XA in the tryptophan-NAM pathway are derived from non-hepatic tissue [21]. Urine levels of KA and XA in mice treated with antibiotics were significantly lower compared with those not treated with antibiotics which might indicate that almost all the tryptophan was used up to produce NAD in the liver.

In contrast to our prediction, the BW loss in mice fed the normal diet and treated with antibiotics was more severe compared with that in those fed the low-niacin diet (Fig. 2). After the experiments, we compared the stomach contents between the groups. We found more paper containing water in the stomachs of mice with pica, which could explain why the mice did not lose more weight than expected.

Nicotinamide and nicotinic acid, which are biosynthesised from tryptophan in the liver [22], are generically referred to as niacin. Some developing countries, such as Malawi, are thought to have a higher incidence of secondary pellagra than most developed countries [23], partly because Malawians rely on maize as a staple food and maize is a cause of niacin deficiency.

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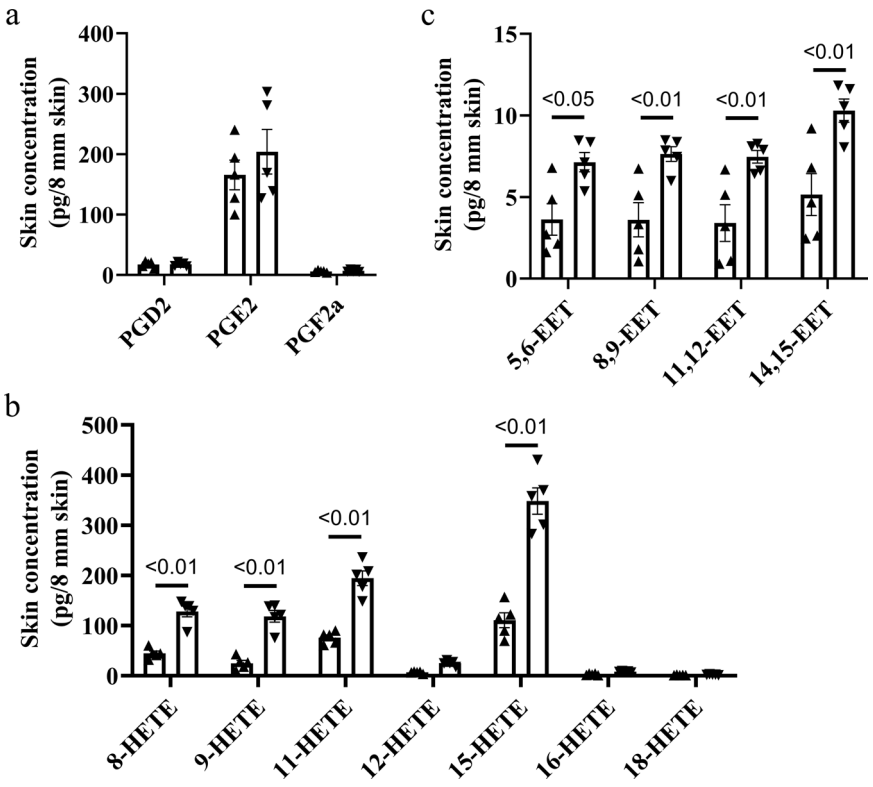

Fig. 5. Effect of the low-niacin diet on skin oxFA levels. Lipids were extracted from ear skin from 15 days onwards in each group (n=5). Prostaglandins (PGs) (a), HETEs (b), and EETs (c) were measured by liquid chromatography–mass spectrometry to investigate the response to the low-niacin diet. ▲, Normal diet; ▼, low-niacin diet. Each triangle represents an individual. Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods. The experiments were repeated twice.

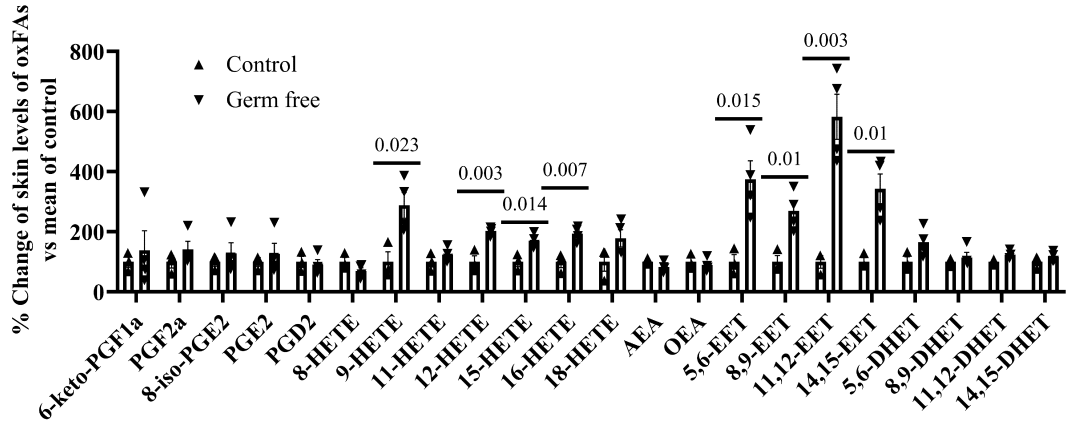

Fig. 6. Skin oxFA levels in germ-free and control mice. The values indicate the percentage change in skin oxFA levels relative to the mean for the normal diet in each group (Balbc mice). ▲, Control; ▼, germ-free mouse. Each triangle represents an individual. Data represent the mean ± standard error. Statistical analysis was conducted as stated in the Materials and Methods. The experiments were repeated twice.

However, we believe that there are many unrecognised patients with pellagra in developed countries. As an example of this, the nausea caused by INH appears to be like that caused by pellagra [6] and was originally not thought to be related to pellagra. Furthermore, some phenotypes of pirfenidone-related adverse effects, such as photosensitivity and nausea, appear to resemble those of pellagra. Currently, we are investigating whether these phenotypes are pellagra-like using our model mice [24, 25].

The World Health Organisation and Food and Agricultural Organization recommend consuming 11–12 mg of niacin daily [26]. Interestingly, some gut bacteria synthesise vitamin B3 from tryptophan [10, 27]. Therefore, vitamin B3 derived from the host, food, and commensal bacteria is important for maintaining homeostasis in the host. In this study, urinary levels of niacin-related metabolites and skin oxFA levels in mice fed the low-niacin diet recovered after their microbiota profiles were altered.

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Table 1. Comparison of gene expression in the liver between mice fed the normal diet and those fed the low-niacin diet

Gene	Gene ID	Normal	Low niacin	Log2 (fold change)	p-value
Dmbt1	NM_001347632	1,335	182	−2.87	0.00000
Mup1	NM_001163011	944	148	−2.67	0.00000
Pnpla3	NM_054088	64	517	3.01	0.00000
Serpina4-ps1	NR_002861	960	235	−2.03	0.00000
Cyp4a32	**NM_001100181**	**1,178**	**327**	**−1.85**	**0.00000**
Fasn	NM_007988	1,282	5,655	2.14	0.00000
Mup9	NM_001281979	4,435	1,499	−1.56	0.00000
Gm2788	NR_155436.1	713	206	−1.79	0.00000
Acly	NM_134037	1,037	4,391	2.08	0.00000
Cyp4a14	**NM_007822**	**3,317**	**1,155**	**−1.52**	**0.00000**
Insig1	NM_153526	2,202	8,757	1.99	0.00000
Slc25a30	NM_026232	755	237	−1.67	0.00000
Mup17	NM_001200006	19,758	7,406	−1.42	0.00000
Mup13	NM_001347134	1,122	391	−1.52	0.00000
Mup7	NM_001347129	1,003	357	−1.49	0.00000
Elovl6	**NM_130450**	**1,272**	**4,698**	**1.88**	**0.00000**
Vnn1	NM_011704	500	162	−1.63	0.00000
LOC115486422	XR_882082	373	114	−1.71	0.00000
Mup2	NM_001286096	1,776	704	−1.33	0.00000
Hsd3b5	NM_008295	221	61	−1.86	0.00000
Mup11	NM_001164526	2,628	1,103	−1.25	0.00000
Mup15	NM_001200004	1,398	579	−1.27	0.00001
Acot1	NM_012006	331	112	−1.56	0.00001
Socs2	NM_001168655	317	109	−1.54	0.00001
Nlrp12	NM_001033431	515	204	−1.34	0.00002
Rab30	NM_029494	429	166	−1.37	0.00002
Mup8	NM_001347131	455	181	−1.33	0.00003
8030431J09Rik	XR_866592	422	1,361	1.69	0.00003
Cyp51	**NM_020010**	**401**	**1,285**	**1.68**	**0.00004**
G0s2	NM_008059	1,674	781	−1.10	0.00004

Bold type is considered to be involved in fatty acid metabolism.

We consider a low-niacin diet to initially affect the gut microbiota and then change tissue oxFA levels and urinary levels of niacin metabolites (Fig. 7). Eventually, the gut microbiota might be a therapeutic target for pellagra-like symptoms.

Some types of dermatitis, such as photosensitivity, are major symptoms of pellagra [1–3]. Additionally, skin levels of AA metabolites, such as HETEs, play important roles in the development of dermatitis [16, 17, 19]. Furthermore, niacin deficiency increases the levels of prostaglandin E synthase, which produces prostanoids, in the skin of mice and humans [20]. The discrepancy between these previous findings and the findings of the present study may be the result of the different methods used to induce niacin deficiency. Previous authors used a niacin antagonist [20], whereas we used a low-niacin diet to induce mild pellagra or pre-pellagra. Previous studies showed that 15-HETE had a therapeutic effect on psoriasis [28] and inhibited T-cell proliferation and leukotriene B4 synthesis [29–31]. These results appear to be conflicting, as there were significant amounts of bioactive lipids but no dermatological symptoms, except for an increase in skin mast cells. A low-niacin diet causes mild dermatitis or pre-dermatitis, and consequently, the HETE response is believed to be a biological response for homeostasis. Further analysis of these responses is required. Finally, niacin modulates transient receptor potential vanilloid 4 [32], which plays an important role in the development of dermatitis, fibrosis, and pain

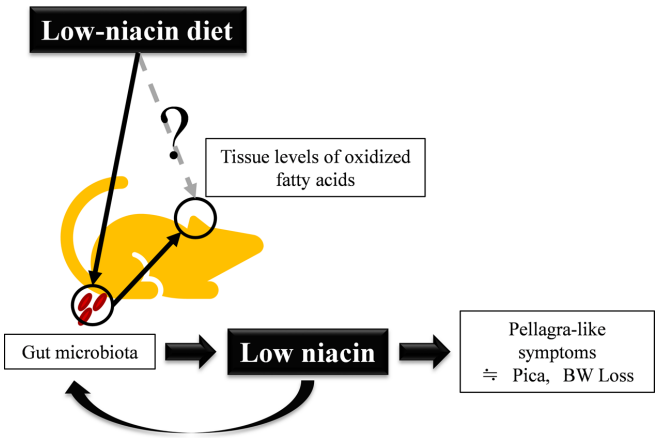

Fig. 7. Schema of how niacin is involved in pellagra. A low-niacin diet causes bacterial changes in the gut, which might play a role in skin oxFAs levels, and other changes to the gut microbiota. Continuous and sequential responses of the gut microbiota might have an important role in the course of pellagra.

[33–36]. Chronic deficiency of an endogenous modulator due to a low-niacin diet might cause quantitative changes in endogenous ligands, which are thought to be EETs [37], to maintain host homeostasis via transient receptor potential vanilloid 4. Dermal responses to excess niacin through AA metabolites have been previously reported [38]. In this study, the expression levels of genes that appeared to be involved in fatty acid metabolism were altered by the low-niacin diet (Table 1). Changes in the gut microbiota caused by a low-niacin diet may affect AA metabolites (Fig. 3) [39]. Although the dermal responses to reduced niacin remain unclear, we believe that our results are important for better understanding the relationship between niacin deficiency and the dermal response to it via the gut microbiota (Fig. 7).

Treatment of germ-free mice with antibiotics is too severe a model to understand the actual mechanisms in patients with pellagra. Our investigations were based on *in vivo* mouse experiments and not investigations in humans. The purchased germ-free mice, which had been bred in isolators that fully blocked exposure to microorganisms, could not be raised aseptically in our animal room because we did not have sufficient space to house them. Even with all the limitations listed above for our study, we believe that we successfully achieved a mouse model of pellagra induced by feeding a low-niacin diet, as shown by the results for the gut microbiota. Investigating the therapeutic or beneficial roles of the microbiota in treating patients with pellagra is challenging, but our findings could be useful. We intend to investigate the putative cause of pellagra using our mouse model in the future.

DATA AVAILABILITY

The raw metagenomic reads used in this study were uploaded to the DDBJ Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index.html).

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