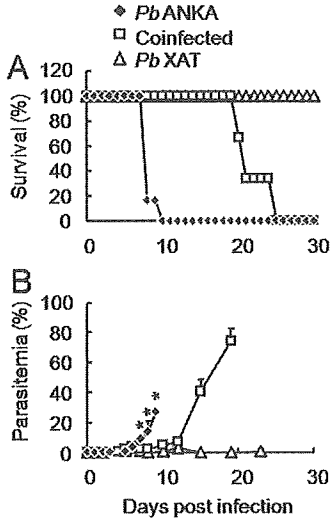

Fig. 1. Co-infection with non-lethal *Plasmodium berghei* (*Pb*) XAT strain suppresses the acute severe parasitemia caused by *Pb* ANKA strain infection in mice and prolongs their survival. Mice were infected with 1×10^4 parasitized red blood cells (pRBC) of *Pb* ANKA or *Pb* XAT. In the co-infected group, mice were infected with *Pb* XAT 1 day before infection with *Pb* ANKA. (A) Survival rates. (B) Course of parasitemia. An asterisk indicates a statistically significant difference compared with co-infected mice or *Pb* XAT-infected mice ($P < 0.05$). Results are expressed as means ± SD of three mice. Experiments were performed three times with similar results.

2.8. Statistical analysis

When comparing two groups, a Mann–Whitney *U*-test was used and when comparing more than two groups, a Kruskal Wallis test was used. Survival curves were compared using a log-rank test. All statistical analyses were performed using the Statcel program (OMS Ltd., Saitama, Japan). $P < 0.05$ was set as a statistically significant difference.

3. Results

3.1. Co-infection with Pb XAT prevents the development of ECM caused by Pb ANKA infection

To investigate whether the presence of non-lethal malaria parasites affects the outcome of *Pb* ANKA infection, B6 mice were co-infected with *Pb* ANKA and *Pb* XAT. Mice singly infected with *Pb* ANKA showed high levels of parasitemia and died within 8 days although mice singly infected with *Pb* XAT cured spontaneously at around 3 weeks p.i. (Fig. 1A and B). On the other hand, mice co-infected with *Pb* ANKA and *Pb* XAT survived longer than mice infected with *Pb* ANKA (Fig. 1A) and their parasitemia was suppressed until day 11 (Fig. 1B). In the late phase of infection, high levels of parasitemia developed in co-infected mice.

As shown in Fig. 2, hemorrhages containing pRBCs were observed in *Pb* ANKA-singly infected mice (number of hemorrhages, $8.83 \pm 6.43/4\ mm^2$ in cerebellum, Fig. 2C) and pRBCs and leukocytes accumulated within cerebral microvessels (Fig. 2D). In contrast, neither hemorrhages nor accumulation of pRBCs and leukocytes within cerebral microvessels were observed in mice co-infected with *Pb* ANKA and *Pb* XAT (Fig. 2E and F) similar to mice singly infected with *Pb* XAT (data not shown). In mice co-infected with *Pb* ANKA and *Pb* XAT, ECM was not apparent before mice died. These results suggest that co-infection with *Pb* XAT prevents the development of ECM caused by *Pb* ANKA infection.

3.2. Severe anemia is caused by co-infection with Pb ANKA and Pb XAT

Next, we determined levels of hematocrit during co-infection with *Pb* ANKA and *Pb* XAT. Co-infected mice showed low levels of hematocrit compared with those of *Pb* ANKA-singly infected mice on day 6 p.i. (Fig. 3A). The levels of hematocrit in co-infected mice were comparable to those in *Pb* XAT singly infected mice. In the late phase of infection, co-infected mice showed much lower levels of hematocrit than *Pb* XAT singly infected mice (Fig. 3A, on day 18 p.i.). These results suggested that mice co-infected with

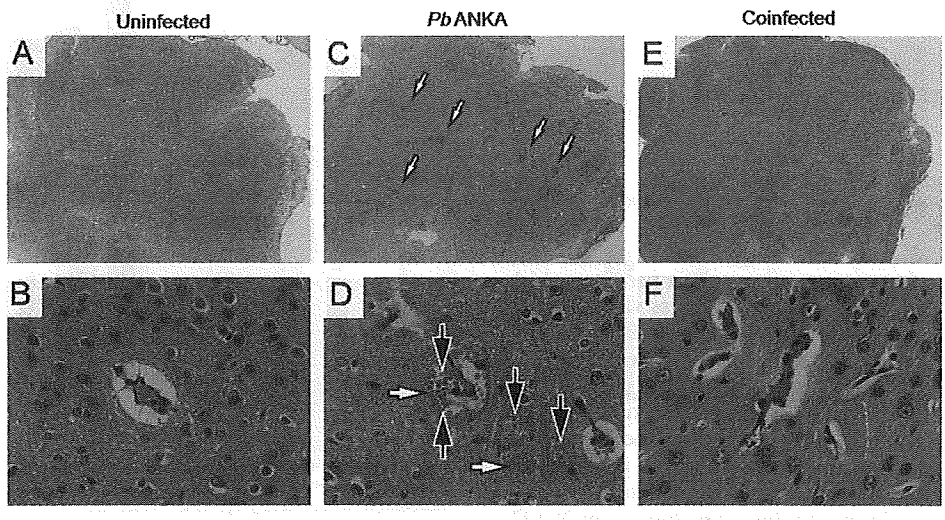

Fig. 2. The presence of *Plasmodium berghei* (*Pb*) XAT strain suppresses the development of experimental cerebral malaria (ECM) caused by *Pb* ANKA strain infection. Mice were infected with malaria parasites as described in the legend to Fig. 1. Brains were obtained from infected and co-infected mice on day 8 p.i. with *Pb* ANKA, and histological analyses performed. Typical results of uninfected mice (A and B), mice singly infected with *Pb* ANKA (C and D), and mice co-infected with *Pb* ANKA and *Pb* XAT (E and F) are shown. (A, C and E) Analyses of hemorrhages in cerebellum. H & E, magnification 20×. Open arrows indicate hemorrhages. (B, D and F) Analyses of hemorrhages and microvessels containing parasitized red blood cells (pRBC) and leukocytes. H & E, magnification 400×. Closed arrows indicate pRBCs. Experiments were performed three times with similar results and the representative data are shown.

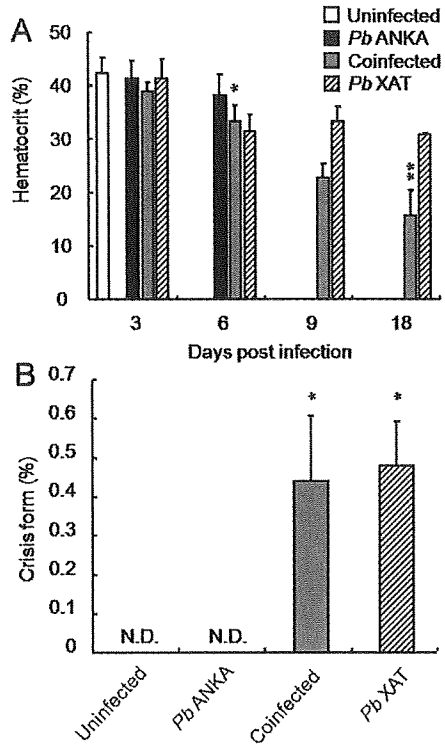

Fig. 3. During co-infection, lower levels of hematocrit and comparable percentages of crisis-form parasites are observed compared with *Plasmodium berghei* (*Pb*) XAT strain infection. Mice were infected with malaria parasites as described in the legend to Fig. 1. (A) Levels of hematocrit. Blood (50 μl) was collected from infected and co-infected mice on days 3, 6, 9 and 18 p.i. with *Pb* ANKA and hematocrit values were determined. Asterisks indicate a statistically significant difference ($^{*}P < 0.05$ compared with *Pb* ANKA strain-infected mice; $^{**}P < 0.05$ compared with *Pb* XAT-infected mice). (B) The percentage of crisis-form parasites. The results were obtained from blood smear samples from infected and co-infected mice on day 6 p.i. with *Pb* ANKA. The percentage of crisis-form parasites was calculated as follows: [(number of pRBC containing crisis-form parasites)/(total number of RBCs counted) × 100]. N.D., not detected; pRBC, parasitized red blood cells. Asterisks indicate a statistically significant difference ($^{*}P < 0.05$ compared with *Pb* ANKA-infected mice). Results are expressed as means ± SD of three mice. Experiments were performed three times with similar results.

Pb ANKA and *Pb* XAT showed severe anemia and died in the late phase of infection.

3.3. Non-lethal malaria parasites alter the immune response involved in development of ECM

It has been shown that some murine malaria parasites which are more virulent can competitively suppress less virulent parasites during co-infection (Van Baalen and Sabelis, 1995). To confirm the existence of *Pb* XAT during co-infection, we determined the proportion of crisis-form parasites. Crisis-form parasites are known to be morphologically and physiologically moribund malaria parasites (Jensen et al., 1987) and observed in the descending phase of the first peak of parasitemia during *Pb* XAT infection but not *Pb* NK65 infection (Waki et al., 1985). A morphological examination showed that crisis-form parasites were observed in *Pb* XAT singly infected mice but not in *Pb* ANKA-singly infected mice on day 6 p.i. (Fig. 3B). Importantly, mice co-infected with *Pb* XAT showed the same proportion of crisis-form parasites as *Pb* XAT singly infected mice on day 6 p.i. (Fig. 3B). These results indicate the existence of *Pb* XAT during co-infection and suggest that *Pb* XAT may alter the immune response involved in development of ECM.

3.4. Production of inflammatory cytokines is suppressed during co-infection

Inflammatory cytokines such as TNF-α and IFN-γ have been shown to be associated with development of ECM caused by *Pb* ANKA (Yañez et al., 1996; Amani et al., 2000; Rudin et al., 1997; Engwerda et al., 2002). To examine whether production of these cytokines is suppressed by co-infection with *Pb* XAT, we determined the levels of cytokines in plasma from uninfected and infected mice. *Pb* ANKA-singly infected mice showed significantly higher levels of TNF-α and IFN-γ than uninfected mice on day 6 p.i. (Fig. 4). Although the levels of TNF-α and IFN-γ in plasma from co-infected mice or *Pb* XAT singly infected mice also increased on day 6 p.i., their levels were significantly lower than those in *Pb* ANKA-singly infected mice. These results suggest that co-infection with *Pb* XAT may suppress the production of inflammatory cytokines which are involved in development of ECM.

3.5. The severity of Pb ANKA infection in IL-10-deficient mice is not reduced, even when they are co-infected with non-lethal malaria parasites

In a previous study, we found that IL-10 plays a role in suppression of the exacerbation of symptoms in mice co-infected with *Pb* NK65 and *Pb* XAT (Niikura et al., 2008). To examine whether IL-10 is associated with the reduction in severity induced by co-infection with *Pb* ANKA and *Pb* XAT, we determined the mortality, parasitemia and body weight of IL-10-deficient mice co-infected with *Pb* ANKA and *Pb* XAT. All wild-type mice co-infected with *Pb* ANKA and *Pb* XAT survived to day 21 p.i. (Fig. 5D), confirming the data obtained in Fig. 1. IL-10-deficient mice co-infected with *Pb* ANKA and *Pb* XAT began to die from day 7 p.i. and all mice died by day 11 p.i. (Fig. 5D). Their body weights were significantly lower than co-in-

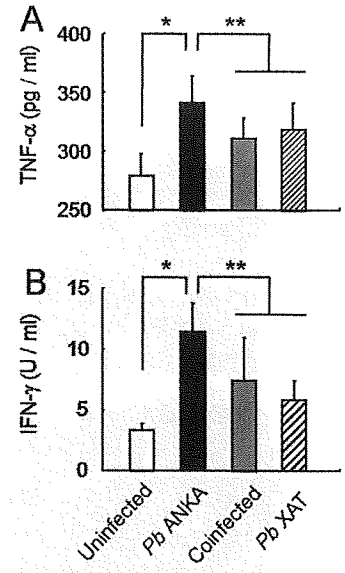

Fig. 4. Co-infection with *Plasmodium berghei* (*Pb*) XAT strain suppresses the production of inflammatory cytokines which are associated with development of experimental cerebral malaria (ECM). Mice were infected with malaria parasites as described in the legend to Fig. 1. Plasma was collected from uninfected and infected mice on day 6 p.i. with *Pb* ANKA. Levels of TNF-α or IFN-γ were determined by ELISA. (A) Levels of TNF-α in plasma. (B) Levels of IFN-γ in plasma. Asterisks indicate a statistically significant difference ($^{*}P < 0.05$ compared with uninfected mice; $^{**}P < 0.05$ compared with *Pb* ANKA-infected mice). Results are expressed as means ± SD of three mice. Experiments were performed three times with similar results.

fected wild-type mice (Fig. 5F) although their parasitemias had almost the same pattern as those of wild-type mice at day 10 p.i. (Fig. 5E). On the other hand, the mortality, parasitemia and body weight were not significantly different between singly infected wild-type mice and IL-10-deficient mice (Fig. 5A–C and G–I). These results suggest that IL-10 may be involved in the suppressive effect of co-infection with *Pb* XAT on the outcome of lethal *Pb* ANKA infection.

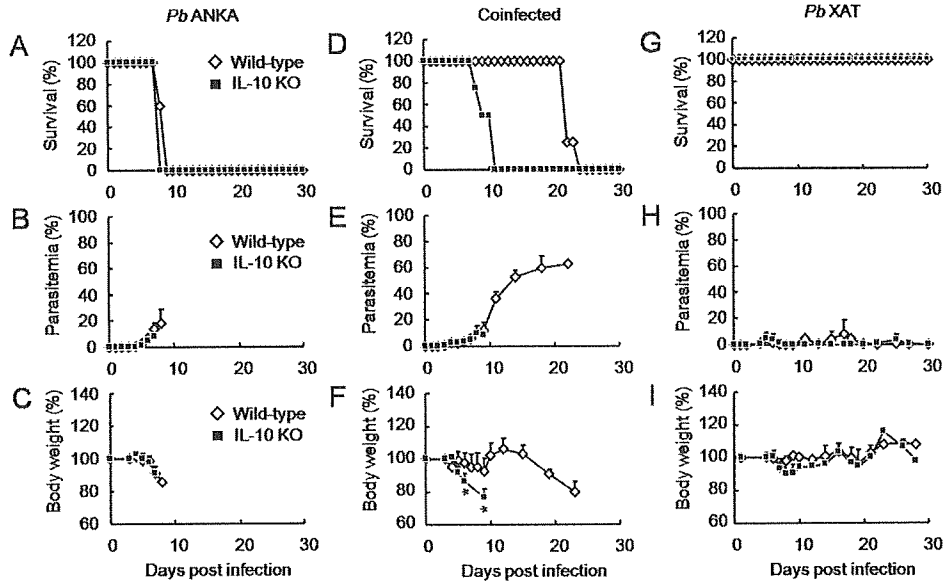

Fig. 5. IL-10 may be involved in the suppressive effect of co-infection with *Plasmodium berghei* (*Pb*) XAT strain on the outcome of lethal *Pb* ANKA strain infection. IL-10-deficient mice (IL-10 KO) and age-matched wild-type mice were infected with *Pb* ANKA. In the co-infected group mice were infected with *Pb* XAT was 1 day before inoculation with *Pb* ANKA. Shown are results of infection with *Pb* ANKA (A–C), co-infection with *Pb* ANKA and *Pb* XAT (D–F) and infection with *Pb* XAT only (G–I). (A, D and G) Survival rates. (B, E and H) Course of parasitemia. (C, F and I) Body weight. Asterisks indicate a statistically significant difference ($^*P < 0.05$ compared with wild-type mice). Results are expressed as means ± SD of three mice. Experiments were performed three times with similar results.

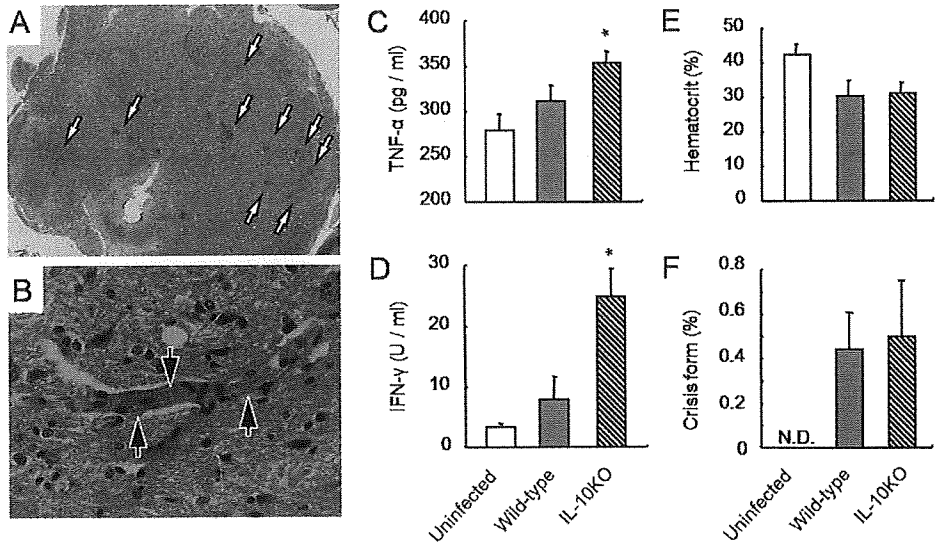

Fig. 6. IL-10 plays a crucial role in suppression of experimental cerebral malaria (ECM) during co-infection with *Plasmodium berghei* (*Pb*) XAT strain. IL-10-deficient mice (IL-10 KO) and age-matched wild-type mice were co-infected with *Pb* ANKA strain and *Pb* XAT. Mice in the co-infection group were infected with *Pb* XAT 1 day before infection with *Pb* ANKA. Brains were obtained from infected IL-10-deficient mice after development of ECM on day 8 p.i. with *Pb* ANKA, and histological analyses were performed (A and B). Plasma (C and D) and blood (E and F) were collected from uninfected and infected mice on day 6 p.i. with *Pb* ANKA. (A) Analysis of hemorrhages in cerebellum. H & E, magnification 20×. Open arrows indicate hemorrhages. (B) Analyses of hemorrhages and microvessels containing parasitized red blood cells (pRBC) and leukocytes. H & E, magnification 400×. Closed arrows indicate pRBCs. (C) Levels of TNF-α in plasma. (D) Levels of IFN-γ in plasma. (E) Levels of hematocrit. (F) Levels of crisis-form parasites. N.D., not detected. Asterisks indicate a statistically significant difference ($^*P < 0.05$ compared with co-infected wild-type mice). Results are expressed as means ± SD of three mice. Experiments were performed three times with similar results and the representative data are shown.

3.6. The prevention of ECM by co-infection with Pb XAT does not occur in IL-10 deficient mice

To investigate whether the co-infected IL-10-deficient mice develop ECM, we performed histological examinations of brains. In brains from co-infected IL-10-deficient mice, hemorrhages containing pRBCs were observed (number of hemorrhages, 13.0 ± 6.92/4 mm^2 in cerebellum, Fig. 6A). Moreover, accumulations of pRBCs and leukocytes were observed within cerebral microvessels (Fig. 6B). The plasma from co-infected IL-10-deficient mice contained significantly higher levels of TNF-α and IFN-γ compared with those of co-infected wild-type mice on day 6 p.i. (Fig. 6C and D). Co-infected IL-10-deficient mice showed the same levels of hematocrit or crisis-form parasites as co-infected wild-type mice on day 6 p.i. (Fig. 6E and F). These results suggest that IL-10 may suppress the high levels of TNF-α and IFN-γ and mediate the suppression of ECM during co-infection with *Pb* XAT.

3.7. Early IL-10 response may be important for the prevention of pathogenesis

To examine when IL-10 is induced and whether its levels differ between singly infected and co-infected mice, we determined the levels of cytokine mRNA in spleens from singly infected or co-infected mice (Fig. 7). Although *Pb* ANKA-singly infected mice showed only weak expression of IL-10 mRNA on day 3 p.i., mice co-infected with *Pb* ANKA and *Pb* XAT showed strong IL-10 mRNA expression, which was comparable to that observed in mice during *Pb* XAT single infection (Fig. 7A). In contrast, strong IL-10 mRNA expression was detected in the spleens of *Pb* ANKA-singly infected mice and co-infected mice, compared with that observed in *Pb* XAT singly infected mice on day 6 p.i. (Fig. 7A). These results suggest that the enhanced levels of IL-10 in spleens on day 3 p.i. may be involved in suppression of pathogenesis during co-infection.

Next, we examined IL-10R-expressing cells during infection by flow cytometry. The numbers of $CD3^-$ $IL\text{-}10R^+$ cells in spleens from *Pb* ANKA-infected mice were much lower than those in co-infected or *Pb* XAT-infected mice on day 6 p.i. (Fig. 7B). In contrast, the $CD3^+$ $IL\text{-}10R^+$ cell population significantly increased in *Pb* ANKA-infected mice by day 6 p.i. compared with co-infected or *Pb* XAT-infected mice (Fig. 7B and Table 1). On day 3 p.i., neither $CD3^-$ $IL\text{-}10R^+$ cells nor $CD3^+$ $IL\text{-}10R^+$ cells were significantly different between singly infected mice and co-infected mice (Table 1). On the other hand, $CD3^+$ $IL\text{-}10R^+$ cells from IL-10-deficient mice were significantly expanded (Fig. 7C) and the number of the cells were much higher than those from co-infected wild-type mice and *Pb* ANKA-singly infected mice (Table 1). These results suggest that $CD3^+$ $IL\text{-}10R^+$ cells may be involved in development of pathogenesis such as ECM.

4. Discussion

In the present study, we investigated the influence of co-infection with non-lethal *Pb* XAT on the outcome of lethal *Pb* ANKA infection which caused high parasitemia and ECM in mice. Although several groups have examined the effect of the simultaneous presence of different *Plasmodium* species and/or strains during *Pb* ANKA infection (Snounou et al., 1992; Mitchell et al., 2005; Voza et al., 2005), there is little understanding of the mechanisms by which ECM is suppressed during co-infection. We found here that co-infection with *Pb* XAT suppressed high parasitemia during early infection (Fig. 1) and prevented the development of ECM (Fig. 2) caused by *Pb* ANKA infection. Since the higher proportion of crisis-form parasites in the descending period after the first peak of parasitemia, such as day 6 p.i., was observed in *Pb* XAT singly infected and co-infected mice but not in *Pb* ANKA-singly infected mice (Fig. 3B), we considered that *Pb* XAT parasites must be present during the co-infection, and these results drove us to examine whether early immune responses, which had mediated development of ECM, were modified by the presence of *Pb* XAT.

From our results demonstrating that the production of inflammatory cytokines was suppressed in co-infected mice compared with that in *Pb* ANKA-singly infected mice (Fig. 4), we postulated that IL-10 might be associated with the suppression of ECM induced by co-infection with *Pb* ANKA and *Pb* XAT. As expected, the suppressive effect of co-infection with non-lethal *Pb* XAT on mortality, severe body weight loss and ECM during *Pb* ANKA infection was abrogated in IL-10-deficient mice (Figs. 5 and 6). In plasma from co-infected IL-10-deficient mice, the levels of TNF-α and IFN-γ were significantly higher than those in wild-type mice co-infected with *Pb* XAT on day 6 p.i. (Fig. 6). IL-10 inhibits inflammatory cytokines such as IFN-γ, TNF-α (Mosmann and Moore, 1991) and IL-12 (Xu et al., 2001). In malaria, IL-10 as well as TGF-β have been shown to be critical for host survival during *Pb* ANKA (Tan et al., 2000; Kossodo et al., 1997) and *Plasmodium chabaudi* AS strain (Li et al., 2003) infection. Moreover, our previous study suggested that IL-10 plays a role in the reduction in liver injury by co-infection with non-lethal malaria parasites (Niikura et al., 2008). Altogether, our results suggest that IL-10 plays a crucial role in prevention of ECM resulting from co-infection with *Pb* ANKA and *Pb* XAT.

Table 1
Numbers of $CD3^+IL\text{-}10R^+$ cells and CD3-$IL\text{-}10R^+$ cells ($\times 10^4$) in spleens of uninfected and *Plasmodium berghei* (*Pb*)-infected mice.

		Days p.i.		
		0	3	6
$CD3^+IL\text{-}10R^+$ cells	Uninfected	7.38 ± 1.31		
	Pb ANKA		16.08 ± 3.52	235.71 ± 67.15[a]
	Coinfected (wild-type)		20.13 ± 2.72	98.59 ± 10.41
	Pb XAT		25.67 ± 0.21	101.12 ± 4.66
	Coinfected (IL-10KO)		N.T.	690.34 ± 109.74[b,c]
$CD3^+IL\text{-}10R^+$ cells	Uninfected	36.86 ± 4.27		
	Pb ANKA		118.79 ± 35.87	81.69 ± 15.04[a]
	Coinfected (wild-type)		107.62 ± 12.89	114.09 ± 12.77
	Pb XAT		141.34 ± 6.19	122.69 ± 2.16
	Coinfected (IL-10KO)		N.T.	550.88 ± 150.30[b]

KO, knock-out; N.T., not tested. Results are expressed as means ± SD of three mice. Experiments were performed three times with similar results.
[a] $P < 0.05$ compared with co-infected wild-type mice or *Pb* XAT strain-infected mice on day 6 p.i.
[b] $P < 0.05$ compared with co-infected wild-type mice on day 6 p.i. with *Pb* ANKA strain parasites.
[c] $P < 0.05$ compared with *Pb* ANKA-infected mice on day 6 p.i.

The development of ECM is associated with early immune responses (Mitchell et al., 2005). As shown in Fig. 7A, high expression of IL-10 mRNA was observed in spleens from co-infected mice or *Pb* XAT-infected mice on day 3 p.i., compared with that in *Pb* ANKA-infected mice. On the other hand, the expansion of $CD3^+$ $IL\text{-}10R^+$ cells on day 6 p.i. was suppressed in spleens from *Pb* XAT-infected mice or co-infected wild-type mice, compared with that from *Pb* ANKA-infected mice (Table 1). These results suggest that the presence of *Pb* XAT may suppress the expansion of $CD3^+$ $IL\text{-}10R^+$ cells by inducing IL-10 production in early infection and subsequently prevents development of ECM in mice. In fact, high levels of $CD3^+$ $IL\text{-}10R^+$ cell expansion was observed in co-infected IL-10-deficient mice which developed ECM and died earlier. On the other hand, the population of $CD3^-$ $IL\text{-}10R^+$ cells decreased in spleens from *Pb* ANKA-infected mice on day 6 p.i., compared with that of *Pb* XAT-infected mice or co-infected mice (Table 1). These results initially led us to postulate that $CD3^-$ $IL\text{-}10R^+$ cells would be associated with prevention of ECM. However, it was negated by the data showing that the population of $CD3^-$ $IL\text{-}10R^+$ cells from IL-10-deficient mice was not decreased but was instead much higher than that from co-infected wild-type mice. At the least, it is possible that the reduction of the $CD3^-$ $IL\text{-}10R^+$ cell population induced by *Pb* ANKA infection may be prevented by presence of *Pb* XAT.

High levels of IL-10-mRNA were also observed on day 6 p.i. in spleens from co-infected mice and levels were comparable to those of *Pb* ANKA-singly infected mice (Fig. 7A). IL-10 has been shown to down-regulate antigen presenting cell function by decreasing cytokine production and major histocompatibility complex class II expression through binding to IL-10R (O'Farrell et al. 1998; Fiorentino et al. 1991a,b). As co-infected wild-type mice showed high levels of parasitemia in the late phase of infection (Fig. 5E), it is postulated that enhanced levels of IL-10-mRNA on day 6 p.i. observed in co-infected mice might suppress the acquired immunity which should be induced later and protect hosts from malaria parasites. Although IL-10 plays an important role for prevention of ECM, it may also have undesired effects, such as suppressing the clearance of malaria parasites during infection.

In the present study, we showed that co-infection with *Pb* XAT, which is the same species as *Pb* ANKA, protects hosts from ECM caused by lethal *Pb* ANKA infection in an IL-10-dependent manner. In contrast, IL-10-deficient mice co-infected with *P. yoelii yoelii* and *Pb* ANKA did not show any ECM symptoms (unpublished data). It is also reported that the suppression of ECM by co-infection with *P. yoelii yoelii* was not altered by neutralization of IL-10 (Voza et al., 2005). The mechanism of the prevention of ECM by co-infection with *P. yoelii yoelii*, which is a different species from *Pb* ANKA, would be different from that in co-infection with *Pb* XAT. The development of ECM and liver injury by lethal murine malaria parasites has been shown to be induced by excessive inflammation (Waki et al., 1992; Yañez et al., 1996; Rudin et al., 1997; Yoshimoto et al., 1998; Amani et al., 2000). Co-infection with *Pb* XAT may provide a good experimental model for understanding immunopathology such as ECM and liver injury caused by lethal murine malaria parasites. Results obtained from in vivo models of co-infection with murine malaria parasites will contribute to understanding the suppression of lethal pathogenesis during *Plasmodium* spp. infection.

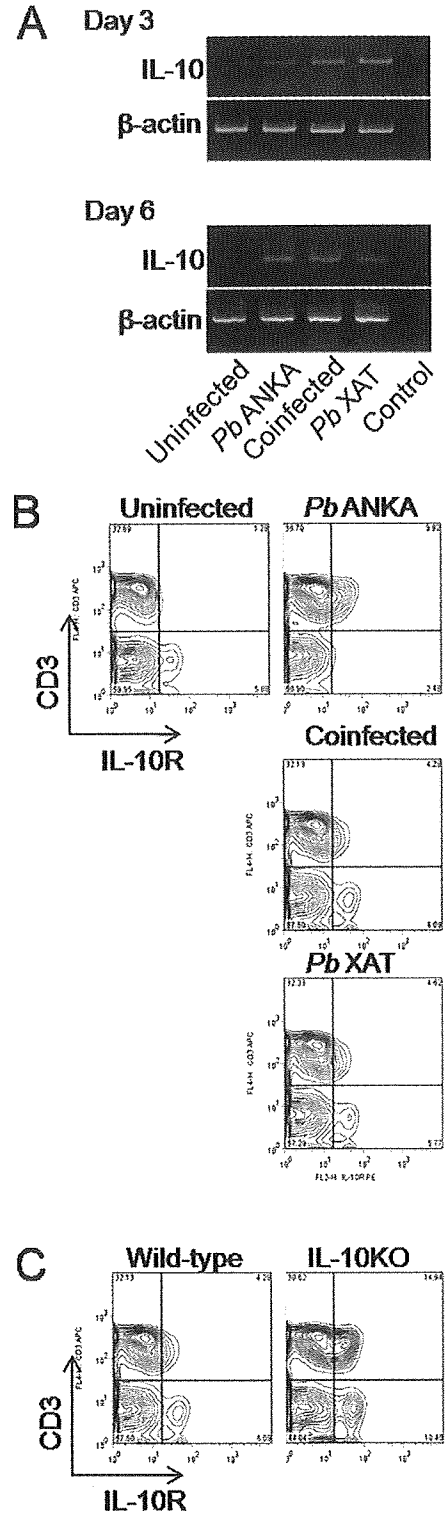

Fig. 7. Analyses of splenic IL-10 mRNA expression and IL-10 receptor (R)-expressing cells in spleen. Total RNA was isolated from spleens of infected and co-infected mice on days 3 and 6 p.i. with *Pb* ANKA and subjected to reverse transcription (RT)-PCR using cytokine-specific primers. The samples without RNA template were used as controls (A). Spleen cells were harvested from singly infected and co-infected mice (B) or co-infected wild-type and co-infected IL-10-deficient (IL-10 KO) mice (C) on day 6 and were subjected to flow cytometry for CD3 and IL-10R expression. (A) Expression of IL-10 mRNA in spleens on days 3 and 6. (B) Analysis of IL-10R-expressing cells in spleens from uninfected or infected mice. (C) Analysis of IL-10R-expressing cells in spleens from co-infected wild-type mice or IL-10-deficient mice. Experiments were performed three times with similar results and the representative data are shown.

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Reference

Amani, V., Vigário, A.M., Belnoue, E., Marussig, M., Fonseca, L., Mazier, D., Rénia, L., 2000. Involvement of IFN-γ receptor-mediated signaling in pathology and anti-malarial immunity induced by *Plasmodium berghei* infection. Eur. J. Immunol. 30, 1646–1655.

Breman, J.G., 2001. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am. J. Trop. Med. Hyg. 64, 1–11.

Coltel, N., Combes, V., Hunt, N.H., Grau, G.E., 2004. Cerebral malaria – a neurovascular pathology with many riddles still to be solved. Curr. Neurovasc. Res. 1, 91–110.

Engwerda, C.R., Belnoue, E., Grüner, A.C., Rénia, L., 2005. Experimental models of cerebral malaria. Curr. Top. Microbiol. Immunol. 297, 103–143.

Engwerda, C.R., Mynott, T.L., Sawhney, S., De Souza, J.B., Bickle, Q.D., Kaye, P.M., 2002. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. J. Exp. Med. 195, 1371–1377.

Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., O'Garra, A., 1991a. IL-10 inhibits cytokine production by activated macrophages. J. Immunol. 147, 3815–3822.

Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W., O'Garra, A., 1991b. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J. Immunol. 146, 3444–3451.

Hunt, N.H., Grau, G.E., 2003. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends Immunol. 24, 491–499.

Imwong, M., Pukrittayakamee, S., Grüner, A.C., Rénia, L., Letourneur, F., Looareesuwan, S., White, N.J., Snounou, G., 2005. Practical PCR genotyping protocols for *Plasmodium vivax* using *Pvcs* and *Pvmsp1*. Malar J. 4, 20.

Jensen, J.B., Vande Waa, J.A., Karadsheh, A.J., 1987. Tumor necrosis factor does not induce *Plasmodium falciparum* crisis forms. Infect. Immun. 55, 1722–1724.

Kobayashi, F., Niikura, M., Waki, S., Matsui, T., Fujino, T., Tsuruhara, T., Kamiya, S., 2007. *Plasmodium berghei* XAT: contribution of γδT cells to host defense against infection with blood-stage nonlethal malaria parasite. Exp. Parasitol. 117, 368–375.

Kossodo, S., Monso, C., Juillard, P., Velu, T., Goldman, M., Grau, G.E., 1997. Interleukin-10 modulates susceptibility in experimental cerebral malaria. Immunology 91, 536–540.

Li, C., Sanni, L.A., Omer, F., Riley, E., Langhorne, J., 2003. Pathology of *Plasmodium chabaudi chabaudi* infection and mortality in IL-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor β antibodies. Infect. Immun. 71, 4850–4856.

Luxemburger, C., Ricci, F., Nosten, F., Raimond, D., Bathet, S., White, N.J., 1997. The epidemiology of severe malaria in an area of low transmission in Thailand. Trans. R. Soc. Trop. Med. Hyg. 91, 256–262.

Mason, D.P., McKenzie, F.E., 1999. Blood-stage dynamics and clinical implications of mixed *Plasmodium vivax–Plasmodium falciparum* infections. Am. J. Trop. Med. Hyg. 61, 367–374.

Mason, D.P., McKenzie, F.E., Bossert, W.H., 1999. The blood-stage dynamics of mixed *Plasmodium malariae–Plasmodium falciparum* infections. J. Theor. Biol. 198, 549–566.

McQueen, P.G., McKenzie, F.E., 2006. Competition for red blood cells can enhance *Plasmodium vivax* parasitemia in mixed-species malaria infections. Am. J. Trop. Med. Hyg. 75, 112–125.

Miller, L.H., Baruch, D.I., Marsh, K., Doumbo, O.K., 2002. The pathogenic basis of malaria. Nature 415, 673–679.

Mitchell, A.J., Hansen, A.M., Hee, L., Ball, H.J., Potter, S.M., Walker, J.C., Hunt, N.H., 2005. Early cytokine production is associated with protection from murine cerebral malaria. Infect. Immun. 73, 5645–5653.

Mosmann, T.R., Moore, K.W., 1991. The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol. Today 12, A49–53.

Niikura, M., Kamiya, S., Kita, K., Kobayashi, F., 2008. Coinfection with nonlethal murine malaria parasites suppresses pathogenesis caused by *Plasmodium berghei* NK65. J. Immunol. 180, 6877–6884.

O'Farrell, A.M., Liu, Y., Moore, K.W., Mui, A.L., 1998. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. EMBO J. 17, 1006–1018.

Rudin, W., Eugster, H.P., Bordmann, G., Bonato, J., Müller, M., Yamage, M., Ryffel, B., 1997. Resistance to cerebral malaria in tumor necrosis factor-α/β-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response. Am. J. Pathol. 150, 257–266.

Schofield, L., Grau, G.E., 2005. Immunological processes in malaria pathogenesis. Nat. Rev. Immunol. 5, 722–735.

Siribal, S., Nakasiri, S., Looareesuwan, S., Chavalitshewinkoon-Petmitr, P., 2004. Identification of human malaria parasites and detection of mixed infection in Thai patients by nested PCR. Southeast Asian J. Trop. Med. Public Health 35, 5–9.

Snounou, G., Bourne, T., Jarra, W., Viriyakosol, S., Wood, J.C., Brown, K.N., 1992. Assessment of parasite population dynamics in mixed infections of rodent plasmodia. Parasitology 105, 363–374.

Snow, R.W., Craig, M.H., Deichmann, U., le Sueur, D., 1999. A preliminary continental risk map for malaria mortality among African children. Parasitol. Today 15, 99–104.

Tan, R.S., Kara, A.U., Feng, C., Asano, Y., Sinniah, R., 2000. Differential IL-10 expression in interferon regulatory factor-1 deficient mice during *Plasmodium berghei* blood-stage infection. Parasite Immunol. 22, 425–435.

Van Baalen, M., Sabelis, M.W., 1995. The dynamics of multiple infection and the evolution of virulence. Am. Nat. 146, 881–910.

Voza, T., Vigário, A.M., Belnoue, E., Grüner, A.C., Deschemin, J.C., Kayibanda, M., Delmas, F., Janse, C.J., Franke-Fayard, B., Waters, A.P., Landau, I., Snounou, G., Rénia, L., 2005. Species-specific inhibition of cerebral malaria in mice coinfected with *Plasmodium spp.* Infect. Immun. 73, 4777–4786.

Waki, S., Nakazawa, S., Taverne, J., Targett, G.A., Playfair, J.H., 1985. Immunity to an attenuated variant of *Plasmodium berghei*: role of some non-specific factors. Parasitology 91, 263–272.

Waki, S., Tamura, J., Imanaka, M., Ishikawa, S., Suzuki, M., 1982. *Plasmodium berghei*: isolation and maintenance of an irradiation attenuated strain in the nude mouse. Exp. Parasitol. 53, 335–340.

Waki, S., Uehara, S., Kanbe, K., Ono, K., Suzuki, M., Nariuchi, H., 1992. The role of T cells in pathogenesis and protective immunity to murine malaria. Immunology 75, 646–651.

Xu, X., Sumita, K., Feng, C., Xiong, X., Shen, H., Maruyama, S., Kanoh, M., Asano, Y., 2001. Down-regulation of IL-12 p40 gene in *Plasmodium berghei*-infected mice. J. Immunol. 167, 235–241.

Yañez, D.M., Manning, D.D., Cooley, A.J., Weidanz, W.P., van der Heyde, H.C., 1996. Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. J. Immunol. 157, 1620–1624.

Yoshimoto, T., Takahama, Y., Wang, C.R., Yoneto, T., Waki, S., Nariuchi, H., 1998. A pathogenic role of IL-12 in blood-stage murine malaria lethal strain *Plasmodium berghei* NK65 infection. J. Immunol. 160, 5500–5505.

In Vitro Antibacterial Activity of Phx-3 against *Helicobacter pylori*

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Phx-3, one of the phenoxazine derivatives, is reported to have inhibitory effect on *Mycobacterium* species and *Chlamydia pneumoniae* but not *Escherichia coli*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes*. The bactericidal activities of Phx-3 against *Helicobacter pylori* strains have not been assessed. Then, we measured minimum inhibitory concentration of Phx-3 for *Helicobacter* strains and assessed the morphological and biochemical effects of Phx-3 on *H. pylori*. In present study, it has shown that *H. pylori* strains including clarithromycin resistant strain and *Helicobacter musterae* were killed effectively by the treatment with Phx-3. Furthermore, severe morphological changes such as membrane blebbing and formation of hollows in *H. pylori* were detected. In addition, induction of heat shock protein 60 was observed. Taken together, Phx-3 has antibacterial activity against *Helicobacter pylori*.

Key words *Helicobacter pylori*; phenoxazine; bactericidal agent

Phonoxyazine derivatives which are produced by microorganisms[1—3] and found in insect[4] possess various biological activities such as anti-tumor[2] and anti-microbial activities.[1,5—8] Although biological activity of actinomycin was elucidated, other phenoxyazine derivatives have not been examined well. We have been studied the anti-bacterial effects of Phx-1 (2-amino-4,4alpha-dihydro-4alpha-7-dimethyl-3*H*-phenoxazine-3-one), Phx-2 (3-amino-1,4alpha-dihydro-4alpha-8-dimethyl-2*H*-phenoxazine-2-one) and Phx-3 (2-amino-phenoxazine-3-one), which are produced by the reaction of *o*-aminophenol or its derivatives with bovine hemoglobin.[7] Additionally, the chemical structure of Phx-3 (Fig. 1) is identical to questiomycin A, which was isolated from the culture fluids of *Streptomyces* by Anzai *et al.*[1] as a compound with bactericidal activity against *Mycobacterium tuberculosis*. Subsequent studies showed that Phx-3 also inhibits the growth of non-tuberculosis *Mycobacterium* species.[7] In contrast, some other Gram-negative and Gram-positive bacteria were resistant to as much as 100 μg/ml of Phx-3[7] except for facultative intracellular bacteria *Chlamydia pneumoniae*.[8]

Helicobacter pylori, which infects approximately 50% of the population worldwide, is a causative agent for chronic gastroduodenal ulcer, and possibly gastric cancer. Triple therapy consisting of amoxicillin, clarithromycin (CAM) and a proton pump inhibitor has proven to be effective for the eradication of *H. pylori* so far.[9] As in the case of other bacteria, however, there has been an increase in the appearance of drug-resistant strains of *H. pylori*. It has been reported that failure of eradication of *H. pylori* infection with triple therapy was approximately 20% of infected patients in Japan.[10] With the increase in the frequency of CAM-resistant *H. pylori*, there is rising concern about the potential decline in the eradication rate of this infection.[11] Therefore, it is important to develop new drugs to combat this bacterium. In the present study for further investigation of possibility of phenoxazine derivatives as bactericidal compound, we examined the effects of Phx-3 on *H. pylori*.

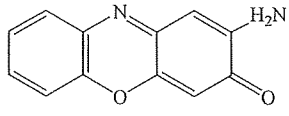

Fig. 1. Chemical Structure of Phx-3 as Identified by Shimizu *et al.*[7]
The structure, 2-amino-phenoxazine-3-one is identical to that of questiomycin A.[2]

MATERIALS AND METHODS

Bacteria and Cultivation We used three standard strains of *H. pylori* (American Type Culture Collection [ATCC] 43504, ATCC 43579, and The National Collection of Type Cultures NCTC [NCTC] 11638 and three clinical isolates (TK 1029, 1047, and 1402). Brucella broth (Difco Laboratories, Inc., Detroit, MI, U.S.A.) supplemented with 7% horse serum was used for the liquid culture of *H. pylori* strains. For the plate culture, 1.5% of bacto agar (Difco Laboratories, Inc. Detroit, MI, U.S.A.) was supplemented to the medium. Bacteria were incubated at 37 °C under microaerobic atmosphere produced by the AnaeroPack®-MicroAero (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Chemicals Phx-1, Phx-2 and Phx-3 were synthesized from 2-amino-5-methylphenol, 2-amino-4-methylphenol or *o*-aminophenol, respectively, with bovine hemoglobin according to the method described in.[12] Phx-3 was dissolved in methanol as a stock solution at the concentration of 25 mM and was stored at −20 °C until use. The chemical structure of Phx-3 is illustrated in Fig. 1.

Measurements of the Minimal Inhibitory Concentration (MIC) for *H. pylori* We evaluated the ability of Phx-3 to inhibit the growth of *H. pylori* by determining the MICs as described by the guidelines of the Clinical and Laboratory Standards Institute for Clinical Laboratory Standards.[13] Briefly, the 72-h old subcultures diluted and adjusted to the 1×10^7 to 1×10^8 colony forming units per ml were replicated

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directly on the Phx-3-containing agar dilution plates. The MICs were determined after 3 d-cultivation. The sensitivities of these strains to CAM were measured by the E-test® according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

Electro Microscopy The bacterial cells were collected and prefixed in an aqueous solution of glutaraldehyde in 50 mM phosphate buffer (pH 7.4) at 4 °C. Following washing, the bacterial cells were fixed with OsO_4 in Veronal-acetate buffer (pH 6.0) for 16 h at room temperature. After treatment with 0.5% uranyl acetate, the samples were subsequently dehydrated in a graded series of alcohol. Micrographs with scanning electron microscope were observed with model JSM-5600LV (Japan Electron Optics Laboratory, Tokyo, Japan).

Western Blotting *H. pylori* ATCC 43504 strain was cultured for 8 h, and 12 μg/ml of Phx-3 was added. Following the 6-h, bacteria cells were harvested by centrifugation at 8500×***g*** for 10 min and washed with phosphate buffered saline (PBS). The bacterial cells were disrupted by ultrasonic waves (Sonifier, Bronson Ultrasonics Co., Danbury CT, U.S.A.). Cell debris was removed by centrifugation at 8500×***g*** for 20 min. The cell lysates were developed with 12.5% acrylamide gel and transfer to the polyvinylidene difluoride (PVDF) membrane. Following to the reaction with primary and secondary antibodies, reacted proteins with primary antibodies were detected with DAB (3,3′-diaminobenzidine). The anti-Omp19 (Biodesign International, Saco, ME, U.S.A.) and H20[14] monoclonal antibodies were used for the detection of Omp19 and HSP60, respectively.

RESULTS AND DISCUSSION

We assessed the anti-microbial effect of Phx-3 on *H. pylori* ATCC 43504, ATCC 43579, NCTC 11638, TK 1029, 1047, and 1402. None of *H. pylori* strains were able to grow in the presence of 1 μg/ml or more of Phx-3 (data not shown). Among examined strains, *H. pylori* TK 1047 was considered CAM-resistant according to the CLSI guidelines as the MIC of CAM against this strain was 1.5 μg/ml for CAM. Therefore, Phx-3 was able to prevent the growth of CAM resistant strain as well as sensitive strains.

In addition, we found that the MIC of Phx-3 against *Helicobacter musterae* ATCC 43772 was 0.5 μg/ml, which was similar to the MICs of Phx-3 for the *H. pylori* strains. Therefore, it appears that Phx-3 inhibits the growth of *Helicobacter* species including *H. pylori*.

To further examine the antibacterial effect of Phx-3 on *H. pylori*, we counted the number of colonies of bacteria in the culture at selected time points after the addition of various concentrations of Phx-3. As shown in Fig. 2, Phx-3 killed *H. pylori* at the concentrations equal to or higher than the MIC. After 48 h, Phx-3 reduced the numbers of bacteria to below the limit of detection (Fig. 2). Unlike other bacteria to which Phx-3 has weak antibacterial activity,[7] our results suggest that *Helicobacter* species are highly susceptible to Phx-3.

Anti-*H. pylori* agents have been shown to cause a variety of morphological changes.[15–17] We therefore examined the morphological changes of *H. pylori* ATCC 43504 cells after a 6-h culture in the presence or absence of 6 μg/ml of Phx-3 using scanning electron microscopy. The electron micrographs shown in Fig. 3 revealed that Phx-3 caused severe morphological changes, including membrane blebbing, the formation of hollows and the formation of spherical cells. Interestingly, no coccoid cells were observed until 8 h after treatment with Phx-3. Because the membrane-like structures were attached to the surface of the bacterial cells treated with Phx-3, the exfoliation of outer membrane was suspected.

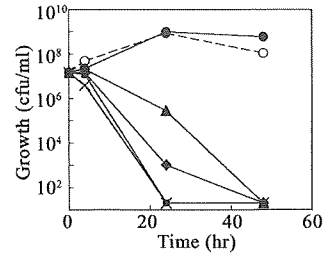

Fig. 2. Bactericidal Effect of Phx-3 on *H. pylori* ATCC 43504

The cells were treated with Phx-3 at 0.5 μg/ml (solid circles), 1 μg/ml (solid triangles), 2 μg/ml (solid diamonds), 4 μg/ml (solid squares) or 8 μg/ml (crosses). The cellular viability was determined at 0, 3, 24, and 48 h by the viable cell counting on Brucella agar supplemented with 7% horse serum. The number of bacteria in the culture in the absence of Phx-3 was also measured (open circles). All experiments were performed in duplicate.

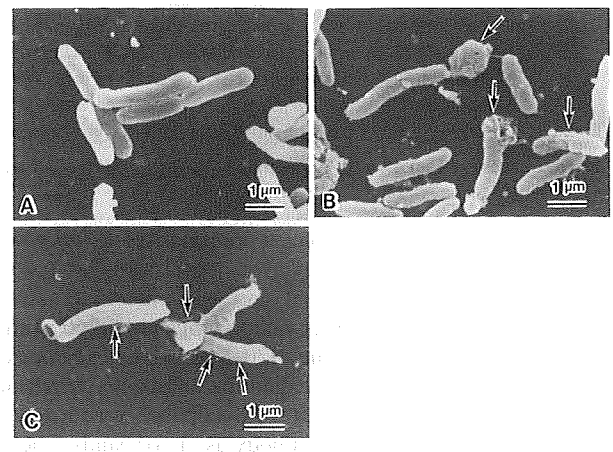

Fig. 3. Scanning Electron Microscopy of *H. pylori* Treated with (B and C) or without (A) Treatment of 6 μg/ml Phx-3

Formation of spherical cells and membrane blebbing were observed (arrows) in Phx-3-treated cells. Bars represent 1 μm.

We next examined the localization of outer membrane protein in the *H. pylori* cells. The lysates were separated on polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (Fig. 4C). The Omp19 protein was detected using monoclonal antibody against Omp19. We detected no differences in the amounts of Opm19 proteins between Phx-3 treated and un-treated *H. pylori* (Fig. 4A). The result showed that the outer membrane was not exfolitated by the addition of Phx-3 contrary to our expectation. We also compared the amount of HSP60, general stress protein of *H. pylori* after treatment with Phx-3. It was shown that Phx-3 treated cells produced more heat shock protein 60 than untreated cells (Fig. 4B). The damages of the bacterial cells caused by Phx-3 may be generated in not only the surface of the cells but also whole cells. As shown in Fig. 4B (lane 2), H20 monoclonal antibody used in this study to detect HSP60 of *H. pylori* reacted with unidentified proteins of *H. pylori*.

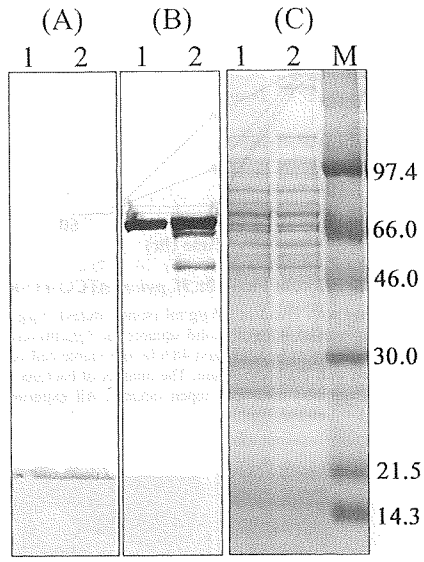

Fig. 4. Whole Cell Lysates of *H. pylori* ATCC 43504 with or without Treatment of Phx-3 Were Examined by Polyacrylamide Gel Electrophoresis, followed by Western Blotting with Anti-Omp19 (A) and Anti-HSP-60 Antibodies (B) or Staining with Coomassie Brilliant Blue (C)

Lane 1, *H. pylori* ATCC43504; lane 2 *H. pylori* ATCC43504 treated with Phx-3; lane M: protein molecular weight markers. Numbers at the left of lane M indicate the molecular weights of the markers.

Yamaguchi and coworkers[14] purified *H. pylori* HSP60 using affinity chromatography with H20 monoclonal antibody. After loading of the purified HSP60 fraction in Western blot analysis, several proteins except for the HSP60 were still detected with H20 monoclonal antibody as minor bands. These results suggest that they may be degraded HSP60 protein or cross-reacting antigen induced by various stresses.

Motility of *H. pylori* during colonization in the gastric mucosa is an important factor for the pathogenesis following *H. pylori* infection.[18] We therefore assessed the possibility that Phx-3 affects the motility of *H. pylori* by measuring s arming motility on the soft agar medium containing 0.4% agar. We found that pretreatment or simultaneous treatment with Phx-3 at concentrations below the MIC did not alter the swarming of *H. pylori* (data not shown).

The three-dimensional structure of chemical substances sometimes provides clues to their mechanism of action. Phx-3 is a planar molecule with 2-amino and 3-ketone residues. These features provide structural rigidity, which could allow Phx-3 to intercalate into the double-stranded DNA. Phx-3 could form hydrogen bonds with deoxyguanosine between the GpC pairs. We therefore extracted the genomic DNA of *H. pylori* and examined the DNA patterns by agarose gel electrophoresis. By staining with ethidium bromide, we did not detect any changes until 8 h after the treatment with Phx-3 (data not shown).

Furthermore, we examined the bactericidal effect of Phx-1 and Phx-2 on *H. pylori* strains, and the MICs of these compounds against tested nine *H. pylori* strains and *H. mustelae* strain were between 50 μg/ml and 100 μg/ml, and 20 μg/ml and 100 μg/ml, respectively. These results indicate no bactericidal activities of Phx-1 and Phx-2 against *Helicobacter*. Difference of structures between Phx1 or Phx-2, and Phx-3 was the presence of methyl residues which give to the extension to three-dimensional change. It is possible that the planar structure of Phx-3 is important to its bactericidal activity. Bendic and Volanschi evaluated the intermolecular interactions between the drug-nucleic acid complexes by modeling based on molecular mechanics optimization.[19] Their molecular modeling of questiomycin A (Phx-3) suggested that it can bind in the minor groove after intercalation in double-stranded DNA. The bactericidal activity of Phx-3 might be therefore due to interaction with the genomic DNA of *H. pylori*.

It is not clear why Phx-3 shows the specificity of the bactericidal activity for *Helicobacter*. Specie specific intrinsic drug resistance is thought to be determined by several factors such as size of porin(s), efflux pump, modification of drug, affinity of active point and surface charge. Exner *et al.*[20] identified four pore forming outer membrane proteins related to porin, and the channel size of porin in *H. pylori* was relatively small. This could contribute to the resistance of *H. pylori* to hydrophilic antibiotics. Among them, the efflux of the toxic drugs is an important factor controlling the intrinsic sensitivity. *H. pylori* is highly sensitive to many hydrophilic and hydrophobic agents despite relatively low susceptibilities to the polycation polymyxin B and cationic antimicrobial peptides.[21] Bina *et al.*[22] proposed that reasonable uptake of hydrophobic substances coupled with nonfunctioning efflux systems could explain the relatively high susceptibility of *H. pylori* to hydrophobic antibiotics. Phx-3 might therefore behave like other hydrophobic antibiotics with respect to uptake into and efflux from bacterial cells. After accumulation of Phx-3, the molecules may intercalate into the double-stranded DNA and inhibit transcription.

In summary, we demonstrated the specific bactericidal activity of Phx-3 against *H. pylori in vitro*. This specific sensitivity of *H. pylori* seems to be due to the genus specific structure of cell-surface. And, possible mechanism of bactericidal activity of Phx-3 is interaction with bacterial DNA and inhibition of its transcription. On the other hand, effects of Phx-3 on the eukaryote cells were previously assessed by Yamaguchi and colleagues.[8] They described that concentrations of less than 10 μM of Phx-3 did not show cytotoxicity to HEp-2 and THP-1 cells.

These results obtained provide important information for development of novel anti-*H. pylori* drugs.

REFERENCES

1) Anzai K., Isono K., Okuma K., Suzuki S., *J. Antibiot.*, **13**, 125—132 (1960).
2) Brockmann H., Muxfeldt H., *Chem. Ber.*, **91**, 1242—1265 (1958).
3) Hollstein U., *Chem. Rev.*, **74**, 625—652 (1974).
4) Butenandt A., Biekert E., Kubler H., Linzen B., *Physiol. Chem.*, **319**, 238—256 (1960).
5) Iwata A., Yamaguchi T., Sato K., Izumi R., Tomoda A., *Tohoku J. Exp. Med.*, **200**, 161—165 (2003).
6) Holmes S. C., Gait M. J., *Nucleosides Nucleotides Nucleic Acids*, **22**, 1259—1262 (2003).
7) Shimizu S., Suzuki M., Tomoda A., Arai S., Taguchi H., Hanawa T., Kamiya S., *Tohoku J. Exp. Med.*, **203**, 47—52 (2004).
8) Uruma T., Yamaguchi H., Fukuda M., Kawakami H., Goto H., Kishimoto T., Yamamoto Y., Tomoda A., Kamiya S., *J. Med. Microbiol.*, **54**, 1143—1149 (2005).
9) Talley J. N., Lam S. K., Wong B. C., Xia H. H., *Expert Opin. Pharmacother.*, **3**, 1301—1311 (2002).
10) Asaka M., Sugiyama T., Kato M., Satoh K., Kuwayama H., Fukuda Y.,

Fujioka T., Takemoto T., Kimura K., Shimoyama T., Shimizu K., Kobayashi S., *Helicobacter*, **6**, 254—261 (2001).
11) Adamek R. J., Suerbaum S., Pfaffenbach B., Opferkuch W., *Am. J. Gastroenterol.*, **93**, 386—389 (1998).
12) Iwata A., Yamaguchi T., Sato K., Yoshitake N., Tomoda A., *Biol. Pharm. Bull.*, **28**, 905—907 (2005).
13) Clinical and Laboratory Standards Institute, "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard M7-A5," 6th ed., CLSI, Wayne, PA, 2000.
14) Yamaguchi H., Osaki T., Taguchi H., Hanawa T., Yamamoto T., Kamiya S., *J. Med. Microbiol.*, **46**, 819—824 (1997).
15) Akada J., Shirai M., Fujii K., Okita K., Nakazawa T., *Antimicrob. Agents Chemother.*, **43**, 1072—1076 (1999).
16) Kamoda O., Anzai, K., Mizoguchi J., Shiojiri M., Yanagi T., Nishino T., Kamiya S., *Antimicrob. Agents Chemother.*, **50**, 3062—3069 (2006).
17) Nakao M., Malfertheiner P., *Helicobacter*, **3**, 21—27 (1998).
18) Suerbaum S., *Trends Microbiol.*, **3**, 168—171 (1995).
19) Bendic C., Volanschi E., *Internet Electronic J. Mol. Design*, **5**, 320—330 (2006).
20) Exner M. M., Doig P., Trust T. J., Hancock R. E., *Infect. Immun.*, **63**, 1567—1572 (1995).
21) Wang G. E., Taylor D. E., "*Helicobacter pylori* Infection and Immunity," ed. by Yamamoto Y., Friedman H., Hoffman P., Kluwer Academic/Plenum Publishers, New York, 2002.
22) Bina J. E., Alm R. A., Uria-Nickelsen M., Thomas S. R., Trust T. J., Hancock R. E. W., *Antimicrob. Agents Chemother.*, **44**, 248—254 (2000).

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Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers

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ABSTRACT

Background: Japanese longevity is the highest in the world. This is partly explained by low occurrence of cardiovascular diseases, which in turn is attributed to the Japanese traditional diet (JTD). Recent research demonstrates that nitric oxide (NO), a key regulator of vascular integrity, can be generated from nitrate (NO_3^-), abundantly found in vegetables. It can reduce blood pressure (BP) via its serial reduction to nitrite (NO_2^-) and to bioactive NO. Interestingly, JTD is extremely rich in nitrate and the daily consumption is higher than in any other known diet.

Objective and design: In a randomized, cross-over trial we examined the effect of a 10-day period of JTD on blood pressure in 25 healthy volunteers. Traditional Japanese vegetables were encouraged to be consumed and avoided during the control period. Daily nitrate intake was calculated.

Results: Nitrate naturally provided by the JTD was 18.8 mg/kg/bw/day, exceeding the Acceptable Daily Intake by five times (ADI, 3.7 mg/kg/bw).

Plasma and salivary levels of nitrate and nitrite were higher at the end of the JTD period. Diastolic BP decreased on average 4.5 mm Hg during JTD compared to the control diet ($P = 0.0066$) while systolic BP was not affected. This effect was evident in normotensive subjects and similar to that seen in the recent studies.

Conclusions: An ordinary nitrate rich diet may positively affect blood pressure. Our findings further support the importance of the role of dietary nitrate on BP regulation suggesting one possible explanation of healthy aspects of traditional Japanese food.

Background

At an age when the average European person is predicted to die – 77 years for men and 81 for women – inhabitants of Okinawa in Japan usually have many more years of good health. Not only do the Japanese live longer, they age successfully, are lean, energetic and have low occurrence of chronic illnesses like heart disease and cancer [1]. This has partly been attributed to Japanese traditional diet rich in vegetables and fish. A typical Japanese meal consists of a rice dish complimented with soybean products, fish, seafood, and a variety of vegetables. Among the vegetables eaten every day, there are a variety of green leafy vegetables, mushrooms and seaweed. Interestingly, the population who lives longest (Okinawans), has the highest consumption of kombu (seaweed) in Japan [2]. Furthermore, the vegetable diet pattern in Japanese is associated with a significantly lower blood pressure, and serum triacylglycerides [3]. Specific foods that could reduce cardiovascular diseases have recently been identified [4–6] but more research is obviously required to identify what particular components in fruit and vegetables are associated with this decrease.

NO is a key regulator in vascular integrity. Recently a fundamentally different pathway for NO generation in addition to the classical NO synthase-dependent pathway has been described. NO can be generated from inorganic nitrate and nitrite, abundantly found in green leafy vegetables [7,8]. In humans, after absorption in the upper gastrointestinal tract, approximately 25% of circulating nitrate is actively taken up by the salivary glands and is concentrated up to 20-fold in saliva. Once in the oral cavity, commensal bacteria on the dorsal surface of the tongue reduce nitrate to nitrite by the action of nitrate reductase enzymes [8–10]. Swallowed nitrite is then reduced to NO and other bioactive nitrogen oxides in the acidic environment of the stomach. Nitrite that survives the acid conversion can enter the systemic circulation and increase its storage pool in blood and tissues. Studies in humans show increased plasma nitrite concentrations after oral ingestion of nitrate and use of an antibacterial mouthwash after

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consumption of dietary nitrate attenuates the rise in plasma nitrite, showing the importance of the oral bacteria in the nitrate conversion to nitrite [11].

However, beyond this "prokaryotic pathway" of nitrite generation in blood and tissue an "eukaryotic pathway" has also been recently described by Lundberg's group: also mammalian cells are capable of nitrate reduction to nitrite via the involvement of a nitrate reducing enzyme, XOR [12]. Nitrite accumulation in blood and tissues represent a biological pool for NO generation since several different mammalian enzymes and metalloproteins possess nitrite reductase activity such as xanthine oxidoreductase (XOR), aldehyde oxidase (AO), heme proteins and mitochondrial respiratory chain enzymes [11–13].

The measurable biological effects of nitrate derived NO include rapid local vasodilatation and acute reduction in blood pressure [5,13]. It also enhances gastroprotection [14–16], plays a role in mitochondrial respiration [17], cardiac function [18] and exerts antiapoptotic effects [19].

Significant physiologic benefits may be associated with the dietary nitrate. The content of inorganic nitrate in certain vegetables and fruits can provide a physiological substrate for reduction to nitrite and NO that produces vasodilatation, decreases blood pressure and supports cardiovascular function [20–22].

The Dietary Approaches to Stop Hypertension (DASH) studies found that diets rich in vegetables can lower blood pressure to levels similar to those achieved with single hypotensive medications [23,24]. This protection has been attributed to the high content of antioxidants, yet large clinical trials have failed to provide evidence in support of this theory [25,26]. The strongest protection against coronary heart disease was associated with the consumption of green leafy vegetables (e.g., spinach, lettuce) [27]. These vegetables commonly have a high inorganic nitrate content [28]. Interestingly, the BP reduction described after ingestion of beetroot juice decreased BP only if saliva was continuously swallowed, demonstrating the critical involvement of an enterosalivary circulation of nitrate for its bioactivation [5]. Other foods rich in nitrate–nitrite are mushrooms and seaweed. Asian population, especially Japanese, consume a diverse range of mushrooms and seaweed on a daily basis. Overall, the traditional Japanese diet contains a great number of green, leafy vegetables, making it exceptionally rich in nitrate, and the daily consumption higher than in any other known diet. We therefore aimed to examine if the Japanese traditional food, reflected in ingestion of dietary nitrate, affects plasma nitrate/nitrite and arterial blood pressure.

Experimental procedures

The 25 participants of the study were physically active, healthy Japanese volunteers (10 men and 15 women; mean age 36 ± 10 years, BMI < 18.5). They gave informed consent and the study was granted full ethics approval by the Local Research Ethics Committee at Kyorin University School of Medicine and was registered at clinicaltrials.gov, NCT 00928824. The study had a randomized cross-over design with two dietary intervention periods during which the subjects received either Japanese or control (non-Japanese) diet. The exclusion criteria were any serious illnesses, infectious diseases and use of systemic medication. Study subjects had an overnight fast on the morning of saliva and blood collection and blood pressure recording. There were two smoking participants and they were instructed not to change their smoking habits during the study period.

Common Japanese vegetables, identified as the daily source of nitrate [29,30], were encouraged to consume during the consequent 10 days. To avoid the concentration differences in nitrate/nitrite in locally produced foods, the participants were provided with fresh vegetables and staple foods from the same store twice a week during both intervention periods. During the control period, participants were instructed to avoid these vegetables. Instead, the study subjects received and followed the instructions how to replace traditional Japanese meals; for example, by having cornflakes, muesli, yogurt or sandwiches for breakfast. The control diet was designed to eliminate the risk of any major differences between diets in total protein, carbohydrates, saturated and unsaturated fat. The control diet was controlled for nitrate and nitrite sources and those were excluded from the consumption. The mean total intake of energy was approximately 1900 kcal per day. Each participant was asked to recall and fill in all food and drink items consumed daily during the study periods of 10 days, including detailed information about recipe ingredients. Daily nitrate/nitrite intake calculations were based on the dietary recall, body weight information and referred nitrate/nitrite concentrations of ingested foods [29]. Nitrate contents in various vegetables and Japanese foods (examples are shown in Table 1) were based on the calculations by Tsuji et al. (assessed by ion exchange HPLC–UV chromatography) [29]. Blood pressure was measured manually in a sitting position by the blinded physician according to a standard protocol. There was no wash-out period between the study periods. Measurements were taken before breakfast three times: at baseline, at the shift of diets in the middle and at the end of the study. Blood samples (3 mL) were collected and treated for plasma nitrate and nitrite measurement according to procedure previously described in detail [31]. Blood samples were centrifuged immediately at 2200g for 10 min at 4 °C and the plasma stored at −80 °C until measurement of nitrite and nitrate concentration. Saliva samples were collected at the same time in tubes containing EDTA (final concentration 2 mM) and stored at −80 °C for later nitrate and nitrite determination. Samples were analyzed for nitrite and nitrate concentrations by ion chromatography (ENO 20 Analyzer; Eicom, Kyoto, Japan). Sample concentrations reflect the mean value from triplicate analyses.

The data were analyzed using the Graph Pad Prism Software. Group differences were tested with Mann–Whitney (independent groups) and Wilcoxon's signed rank (paired measurements) tests. In all cases, $P < 0.05$ was considered statistically significant.

Results

Healthy Japanese individuals participating in the study followed proposed dietary schemes without significantly loosing or gaining body weight (mean [±SD], 58.7 ± 9.3 at the start of the trial, 58.9 ± 9.1 kg after the non-Japanese and 58.7 ± 9.3 after the Japanese diet). The trial subjects did not express any inconvenience fol-

Table 1
Example of nitrate levels (NO_3^-, mg/kg) in some typical Japanese foods included in daily diets, based on dietary recall [29].

Vegetable	$NO3^-$, mg/kg
Ta cai	5670 ± 1270
Chin gin cai	3150 ± 1760
Garland chrisantemum	4410 ± 1455
Osaka shirona	2500 ± 753
Spinach	3560 ± 552
Burdock	2350 ± 438
Sayaingen beans	945 ± 141
Chinese cabbage	1040 ± 289
Winter mushrooms	983 ± 93
Honghimeji mushrooms	1836 ± 48
Shiitake mushrooms	454 ± 38
Purple laver	2825 ± 2200
Laver	3990 ± 3940
Nozavana pickles	2170 ± 35
Water dropwort	504 ± 187

lowing the Japanese diet; on the contrary, it was associated with an old-fashioned Japanese diet consumed at participants' parents/grandparents homes. At the same time it was difficult to follow the control diet. Therefore, a dietary expertise was used to adjust the control diet and to ensure its nitrate levels to be within the ADI range. Individually consumed daily nitrate intake was approximated to a mean concentration of 18.8 mg/kg of body weight/day during the Japanese diet study phase. Nitrate, naturally derived from Japanese diet exceeded five times the Acceptable Daily Intake (ADI = 3.7 mg/kg/body weight).

After 10 days of Japanese diet, the circulating plasma nitrate levels were higher than after period of control diet (mean [±SD], 43.2 ± 17.4 and 153.9 ± 149 μM, respectively; $P < 0.001$), as were plasma nitrite levels (131.5 ± 75.34 and 203.5 ± 102.3 nM, respectively; $P = 0.0063$) (Fig. 1). Fasting salivary nitrate levels were (median (range), 569.6 (14.4–5778) μM after Japanese diet ($P = 0.0008$) and 199.7(0.1–703.7) μM after control; nitrite levels were 134.2 (1.2–1411) μM at the end of the Japanese diet, and 71.9 (0.4–453.2) μM at the end of the control phase $P < 0.0018$.

The mean diastolic blood pressure was 4.5 mm Hg lower after Japanese diet compared with non-Japanese diet, 71.3 ± 7.9 and 75.8 ± 7.8, $P = 0.0066$) (Fig. 2). There were no significant differences in systolic blood pressure (data not shown).

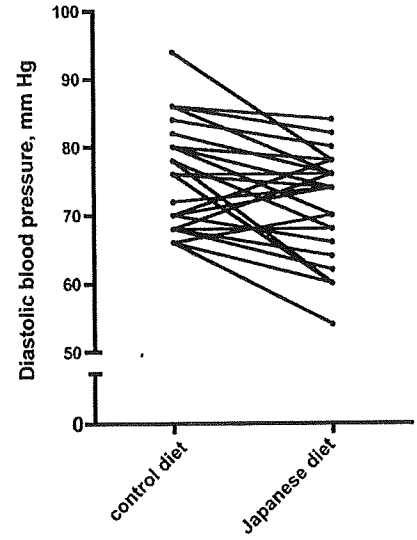

Fig. 2. Effects of 10-Day Japanese traditional foods or control diet on diastolic blood in 25 healthy volunteers. The mean diastolic pressure was by 4.5 mm Hg lower during Japanese traditional food intake ($P = 0.0066$).

Discussion

Until recently, it has been commonly agreed that NO in vivo could only be synthesised by NOS with nitrite and nitrate as inert biological end-products of NO metabolism. However, it was demonstrated in 1994, that nitrite derived from dietary nitrate was a substrate for NOS-independent generation of NO in the acidic condition of the human stomach [8,9]. Despite the demonstration of a pharmacological role for nitrite in vascular and immune function, the potential health aspects of food sources of nitrates and nitrites have not received much attention [22].

The WHO reported in 2002 that the harmful effects of chronic hypertension stand for the ca 11% of all following diseases. Identifying dietary components that might protect against cardiovascular diseases will therefore be important for public health worldwide. Nitrate has been highlighted to be such a component [5,14]: administration of sodium nitrate (0.1 mmol/kg/d) to healthy volunteers over 3 days reduced diastolic BP by 3.7 mm Hg [13] and Webb an co-workers showed similar effects with a vegetable juice rich in nitrate [5]. In the present study, ordinary Japanese diet increased intravascular stores of nitrite probably

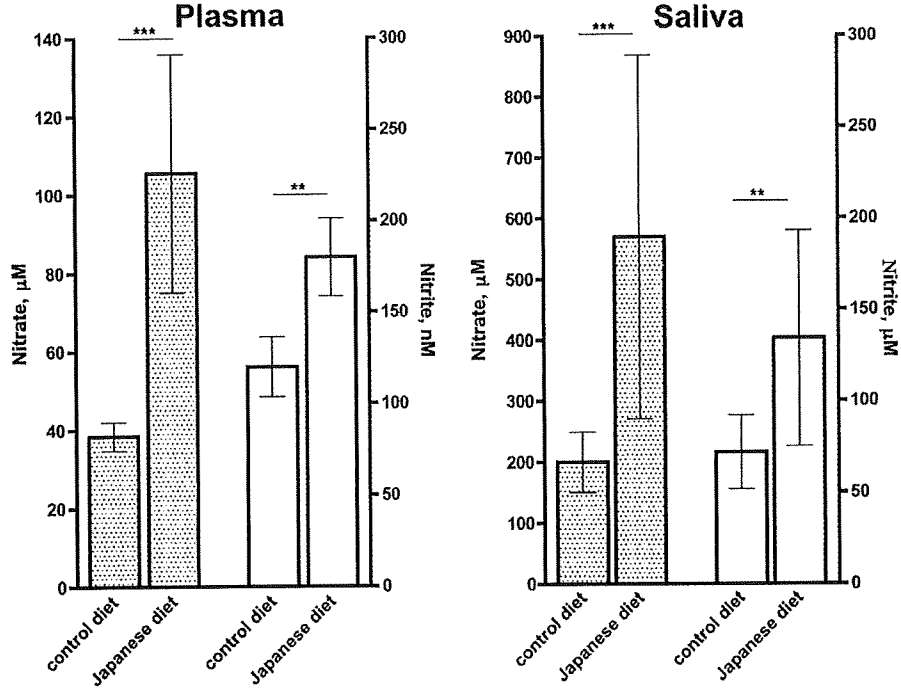

Fig. 1. Effects of 10-Day Japanese traditional foods or control food on salivary and plasma nitrate and nitrite in 25 healthy volunteers. After overnight-fasting (10 h), plasma nitrate and nitrite concentrations were higher during Japanese traditional diet ($P < 0.001$ and $P < 0.05$).

due to bioconversion. As a result, the BP decreased, because the nitrite was further converted to a potent vasodilator, NO [32]. Blood pressure decrease in normotensive Japanese volunteers was similar to that seen in the Webb and Larsen studies [5,13,23] and suggests that NO provided in the form of dietary nitrate, found in the Japanese traditional diet, would likely have a cardioprotective effect.

It is argued that the BP lowering effect of Japanese foods could be attributed to antioxidants, vitamins, polyphenols and high K^+ content of fruit and vegetables [33], although recent large scale clinical trials have failed to provide evidence in support of this hypothesis [25,26]. At the same time, nitrite reduction to NO is greatly enhanced by reducing compounds such as vitamin C and polyphenols, both of which are abundant in the Japanese foods and in the DASH diet.

Further, Webb an co-workers elegantly showed that the lowering effect of vegetable juice on BP was independent of K^+ levels, since the rise in plasma K^+ was unaffected by spitting, while nitrate effect on BP were abolished by these procedure [5]. Moreover, since dietary sodium nitrate supplementation in the present study has similar effects as shown by Larsen et al. [13], this convincingly suggests that it is nitrate and not antioxidant, polyphenols or potassium that is responsible for the BP effect.

In the present study the amount of nitrate naturally provided by the Japanese diet exceeded the ADI by four times and could therefore be questioned. Although seemingly high, these levels were easy to reach when the participants ate vegetables that corresponded to a typical traditional Japanese diet. Green leafy vegetables present in Japanese food (chingensai, komatsuna and garland chrisantemum etc.) contain on average a similar amount of nitrate as European spinach, and Japanese are high consumers of a variety of mushrooms and seaweed, also rich in nitrate/nitrite (Table 1). The variety and amounts of nitrate rich vegetables eaten every day in the traditional Japanese diet is much greater than in a European diet: almost all the foods shown in Table 1 were included in the daily diet, which corresponded an ordinary Japanese diet. Altogether, these eating habits explain the high daily intake of nitrate. Nitrate intake from dietary sources in our study is similar with the recent report from Bryan's group, who has calculated that the DASH diet could result in the consumption of up to 1222 mg nitrate per day thereby exceeding by 550% the WHO's ADI for nitrate in adults [34]. The concentration of nitrate in a single vegetable species varies depending on the soil and growth as well as storage and transport conditions [35]. In our study, we handled the possible variation in concentrations by providing the participants with the foods from the same store.

Some nitrogenous compounds, such as nitrate, since long have been and still are considered potential human health hazards; especially when given to infants, nitrates in bacterially contaminated well water could be reduced to nitrite and cause the condition known as methemoglobinemia [20]. However, the exposure studies on children and adults have not confirmed that nitrate intake is associated with methemoglobinemia [36,37], and alternative explanations for methemoglobinemia in infants has been suggested such as gastroenteritis and associated iNOS-mediated production of nitric oxide induced by bacteria contaminated water [38]. The data supporting the toxicity of nitrates and nitrites for healthy adolescent and adult populations is questionable, as is the scientific basis for exposure regulations for nitrate and nitrite [20,21,39]. Another issue, especially in Japan, is that ingested nitrites may react with secondary amines or *N*-alkylamides to generate carcinogenic *N*-nitroso compounds (NOCs) [40] and the prevalence of gastric cancer in the Japanese population is very high. Although shown in animal models [41], the proof in humans has not been substantiated. Furthermore, the nitrites in foods may be "neutralized" if accompanied by vitamin C, an antioxidant that inhibits the nitrosation effect of nitrites on secondary amines [42]. Clearly, more research is needed to address whether nitrate and nitrite intake is associated with increased cancer risk.

Today we are facing a paradigm shift, and the recent description of the vasoprotective, blood pressure-lowering, and antiplatelet aggregation properties of nitrite suggests that a re-examination of these health effects would be beneficial [5,43,44]. Of the recent studies describes increased plasma nitrite and nitrate concentrations of natives in the high-altitude of Tibet as a natural physiology not associated with harmful physiological effects [45]. The DASH diet study led to the public dietary health recommendations in the United States [46].

We are aware of our study's limitations and the findings therefore should be generalized cautiously. First, the sample size was small. Second, the compliance was difficult to access, since the dietary recalls do not always guarantee accuracy due the risk of underreporting. Despite of this, the strength of the present study is that it is an ordinary diet and could therefore be recommended as a preventive strategy if further confirmed in longer studies also including subjects with cardiometabolic risk factors.

We conclude that Japanese traditional diet, rich in nitrate reduces diastolic blood pressure in healthy volunteers. Our results show effects of an easy to follow, diverse diet and suggest a possible explanation of healthy aspects of Japanese food. By highlighting the daily nitrate and nitrite contents of vegetables our study strengthens the existing evidence to advise vegetable consumption for health benefits. Time might have come to re-evaluate the ADI recommendations regarding nitrate consumption.

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References

[1] Y. Yamori, A. Miura, K. Taira, Implications from and for food cultures for cardiovascular diseases: Japanese food particularly Okinawan diets., Asia Pac. J. Clin. Nutr. 10 (2001) 144–145.
[2] H. Sho, History and characteristics of Okinawan longevity food, Asia Pac. J. Clin. Nutr. 10 (2001) 159–164.
[3] K. Nakamoto, S. Watanabe, H. Kudo, A. Tanaka, Nutritional characteristics of middle-aged Japanese vegetarians, J. Atheroscler. Thromb. 15 (2008) 122–129.
[4] F. Visioli, P. Bogani, S. Grande, C. Galli, Mediterranean food, health building human evidence, J. Physiol. Pharmacol. 56 (Suppl. 1) (2005) 37–49.
[5] A.J. Webb, N. Patel, S. Loukogeorgakis, M. Okorie, Z. Aboud, S. Misra, R. Rashid, P. Miall, J. Deanfield, N. Benjamin, R. MacAllister, A.J. Hobbs, A. Ahluwalia, Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite, Hypertension 51 (2008) 784–790.
[6] M.A. Martinez-Gonzalez, M. Bes-Rastrollo, L. Serra-Majem, D. Lairon, R. Estruch, A. Trichopoulou, Mediterranean food pattern, the primary prevention of chronic disease recent developments, Nutr. Rev. 67 (Suppl. 1) (2009) S111–S116.
[7] J.O. Lundberg, E. Weitzberg, M.T. Gladwin, The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics, Nat. Rev. Drug Discov. 7 (2008) 156–167.
[8] J.O. Lundberg, E. Weitzberg, J.M. Lundberg, K. Alving, Intragastric nitric oxide production in humans: measurements in expelled air, Gut 35 (1994) 1543–1546.
[9] N. Benjamin, F. O'Driscoll, H. Dougall, C. Duncan, L. Smith, M. Golden, H. McKenzie, Stomach NO synthesis, Nature 368 (1994) 502.
[10] J.O. Lundberg, M. Govoni, Inorganic nitrate is a possible source for systemic generation of nitric oxide, Free Radic. Biol. Med. 37 (2004) 395–400.
[11] M. Govoni, E.A. Jansson, E. Weitzberg, J.O. Lundberg, The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash, Nitric Oxide 19 (2008) 333–337.
[12] E.A. Jansson, L. Huang, R. Malkey, M. Govoni, C. Nihlen, A. Olsson, M. Stensdotter, J. Petersson, L. Holm, E. Weitzberg, J.O. Lundberg, A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis, Nat. Chem. Biol. 4 (2008) 411–417.

[13] F.J. Larsen, B. Ekblom, K. Sahlin, J.O. Lundberg, E. Weitzberg, Effects of dietary nitrate on blood pressure in healthy volunteers, N. Engl. J. Med. 355 (2006) 2792–2793.
[14] J. Petersson, M. Phillipson, E.A. Jansson, A. Patzak, J.O. Lundberg, L. Holm, Dietary nitrate increases gastric mucosal blood flow and mucosal defense, Am. J. Physiol. Gastrointest. Liver Physiol. 292 (2007) G718–G724.
[15] E.A. Jansson, J. Petersson, C. Reinders, T. Sobko, H. Bjorne, M. Phillipson, E. Weitzberg, L. Holm, J.O. Lundberg, Protection from nonsteroidal anti-inflammatory drug (NSAID)-induced gastric ulcers by dietary nitrate, Free Radic. Biol. Med. 42 (2007) 510–518.
[16] M. Miyoshi, E. Kasahara, A.M. Park, K. Hiramoto, Y. Minamiyama, S. Takemura, E.F. Sato, M. Inoue, Dietary nitrate inhibits stress-induced gastric mucosal injury in the rat, Free Radic. Res. 37 (2003) 85–90.
[17] H. Nohl, K. Staniek, B. Sobhian, S. Bahrami, H. Redl, A.V. Kozlov, Mitochondria recycle nitrite back to the bioregulator nitric monoxide, Acta Biochim. Pol. 47 (2000) 913–921.
[18] T. Rassaf, U. Flogel, C. Drexhage, U. Hendgen-Cotta, M. Kelm, J. Schrader, Nitrite reductase function of deoxymyoglobin: oxygen sensor and regulator of cardiac energetics and function, Circ. Res. 100 (2007) 1749–1754.
[19] F.M. Gonzalez, S. Shiva, P.S. Vincent, L.A. Ringwood, L.Y. Hsu, Y.Y. Hon, A.H. Aletras, R.O. Cannon 3rd, M.T. Gladwin, A.E. Arai, Nitrite anion provides potent cytoprotective and antiapoptotic effects as adjunctive therapy to reperfusion for acute myocardial infarction, Circulation 117 (2008) 2986–2994.
[20] G.M. McKnight, C.W. Duncan, C. Leifert, M.H. Golden, Dietary nitrate in man: friend or foe?, Br J. Nutr. 81 (1999) 349–358.
[21] J.L. L'Hirondel, Nitrate and Man: Toxic, Harmless or Beneficial, CABI Publishing, Wallingford, UK, 2002.
[22] J.O. Lundberg, M. Feelisch, H. Bjorne, E.A. Jansson, E. Weitzberg, Cardioprotective effects of vegetables: is nitrate the answer?, Nitric Oxide 15 (2006) 359–362
[23] L.J. Appel, T.J. Moore, E. Obarzanek, W.M. Vollmer, L.P. Svetkey, F.M. Sacks, G.A. Bray, T.M. Vogt, J.A. Cutler, M.M. Windhauser, P.H. Lin, N. Karanja, A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group., N. Engl. J. Med. 336 (1997) 1117–1124.
[24] F.M. Sacks, L.P. Svetkey, W.M. Vollmer, L.J. Appel, G.A. Bray, D. Harsha, E. Obarzanek, P.R. Conlin, E.R. Miller 3rd, D.G. Simons-Morton, N. Karanja, P.H. Lin, Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group., N. Engl. J. Med. 344 (2001) 3–10.
[25] D.P. Vivekananthan, M.S. Penn, S.K. Sapp, A. Hsu, E.J. Topol, Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials, Lancet 361 (2003) 2017–2023.
[26] G. Bjelakovic, D. Nikolova, L.L. Gluud, R.G. Simonetti, C. Gluud, Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis, JAMA 297 (2007) 842–857.
[27] K.J. Joshipura, F.B. Hu, J.E. Manson, M.J. Stampfer, E.B. Rimm, F.E. Speizer, G. Colditz, A. Ascherio, B. Rosner, D. Spiegelman, W.C. Willett, The effect of fruit and vegetable intake on risk for coronary heart disease, Ann. Int. Med. 134 (2001) 1106–1114.
[28] G. Ysart, P. Miller, G. Barrett, D. Farrington, P. Lawrance, N. Harrison, Dietary exposures to nitrate in the UK, Food Addit. Contam. 16 (1999) 521–532.
[29] K.M.S. Tsuji, Y. Morita, T. Shibata, N. Kaneta, K. Wakabayashi, S. Uchibori-Hase, S. Ide, K. Fujiwara, H. Suzuki, Y. Ito, Naturally occurring of nitrite and nitrate existing in various raw and processed foods, J. Food Hyg. Soc. Jpn. 34 (1993) 294–302.
[30] S.M. Yada T, M. Ishibashi, T. Osawa, Z. Oshiro, Y. Ito, A study of daily intake of group B food additives in fiscal year 1995–1996 in Japan, Jpn. J. Food Chem. 5 (1998) 178–190.
[31] M. Feelisch, T. Rassaf, S. Mnaimneh, N. Singh, N.S. Bryan, D. Jourd'Heuil, M. Kelm, Concomitant *S*-, *N*-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo, FASEB J. 16 (2002) 1775–1785.
[32] K. Cosby, K.S. Partovi, J.H. Crawford, R.P. Patel, C.D. Reiter, S. Martyr, B.K. Yang, M.A. Waclawiw, G. Zalos, X. Xu, K.T. Huang, H. Shields, D.B. Kim-Shapiro, A.N. Schechter, R.O. Cannon, M.T. Gladwin, Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation, Nat. Med. 9 (2003) 1498–1505.
[33] H.J. Adrogue, N.E. Madias, Sodium and potassium in the pathogenesis of hypertension, N. Engl. J. Med. 356 (2007) 1966–1978.
[34] N.G. Hord, Y. Tang, N.S. Bryan, Food sources of nitrates and nitrites: the physiologic context for potential health benefits, Am. J. Clin. Nutr. (2009).
[35] Z.H. Wang, S.X. Li, Nitrate Accumulation and its Regulation by Nutrient Management in Vegetables, China Agricultural University Press, Beijing, China, 2008.
[36] M. Cornblath, A.F. Hartmann, Methemoglobinemia in young infants, J. Pediatr. 33 (1948) 421–425.
[37] J.M. Kortboyer, M.J .Zeilmaker, The Oral Bioavailability of Sodium Nitrite Investigated in Healthy Adult Volunteers, National Institute of Public Health and the Environment, Bilthoven, Netherlands, 1997.
[38] D.S. Powlson, T.M. Addiscott, N. Benjamin, K.G. Cassman, T.M. de Kok, H. van Grinsven, J.L. L'Hirondel, A.A. Avery, C. van Kessel, When does nitrate become a risk for humans?, J Environ. Qual. 37 (2008) 291–295.
[39] A. EFS, Nitrate in vegetables: scientific opinion of the panel on contaminants in the food chain, EFS J. 689 (2008) 1–79.
[40] T.T. Mensinga, G.J. Speijers, J. Meulenbelt, Health implications of exposure to environmental nitrogenous compounds, Toxicol. Rev. 22 (2003) 41–51.
[41] R.H. Adamson, Induction of hepatocellular carcinoma in nonhuman primates by chemical carcinogens, Cancer Detect. Prev. 14 (1989) 215–219.
[42] G.S. Rao, J.C. Osborn, M.R. Adatia, Drug-nitrite interactions in human saliva: effects of food constituents on carcinogenic N-nitrosamine formation, J. Dent. Res. 61 (1982) 768–771.
[43] N.S. Bryan, J.W. Calvert, J.W. Elrod, S. Gundewar, S.Y. Ji, D.J. Lefer, Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury, Proc. Natl. Acad. Sci. USA 104 (2007) 19144–19149.
[44] N.S. Bryan, J.W. Calvert, S. Gundewar, D.J. Lefer, Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice, Free Radic. Biol. Med. 45 (2008) 468–474.
[45] S.C. Erzurum, S. Ghosh, A.J. Janocha, W. Xu, S. Bauer, N.S. Bryan, J. Tejero, C. Hemann, R. Hille, D.J. Stuehr, M. Feelisch, C.M. Beall, Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans, Proc. Natl. Acad. Sci. USA 104 (2007) 17593–17598.
[46] L.J. Appel, M.W. Brands, S.R. Daniels, N. Karanja, P.J. Elmer, F.M. Sacks, Dietary approaches to prevent and treat hypertension: a scientific statement from the American Heart Association, Hypertension 47 (2006) 296–308.

DNAマイクロアレイ法による鉄欠乏性貧血関連 *Helicobacter pylori* 遺伝子の検討

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背景

Helicobacter pylori は成人期の慢性胃炎や胃・十二腸潰瘍の発症に関連し、小児期の胃・十二腸潰瘍との関連性もメタ分析[1]および日本の多施設研究[2]により立証された。さらに、*H. pylori* の胃癌発症への関与も大規模疫学研究、動物モデル、さらには除菌による介入試験[3]により明らかとなった。胃粘膜の萎縮が *H. pylori* による胃癌発症の重要な因子として認識されているが、その発生率は低いものの、胃癌の発生リスクが高い日本[4]やコロンビア[5]の *H. pylori* 感染小児においても認められる。分子生物学的な胃癌の発症機序の解明と予防戦略の確立は、成人領域における主要な研究テーマとなっている。

一方、最近になって、多くの症例報告や大規模な疫学研究により、*H. pylori* が鉄欠乏性貧血 (IDA) ないし鉄欠乏状態に関連することが指摘され[6,7]、研究者の大きな関心を集めている。報告されている症例の多くは小児ないし若年者であり、小児期の *H. pylori* 感染症研究における主要なテーマとなりつつある。

しかしながら、*H. pylori* によるIDAの発症機序は不明であり、今日までにいくつかの仮説が提出されている。第一の仮説は、*H. pylori* により惹起される粘膜病変、出血性胃炎や潰瘍出血による失血性貧血である[7]。この *H. pylori* 関連の粘膜病変を原因とするIDA症例が存在することは疑いない。しかし、*H. pylori* によるIDAが高頻度にみられる小児において、出血性粘膜病変がみられることは少なく、*H. pylori* による胃粘膜病変の多くは非出血性の結節性胃炎である[2]。少なくとも、消化管出血を小児期の *H. pylori* 関連IDAの主因とするには無理があると考える。第二の仮説は、胃酸分泌低下による鉄イオンの吸収障害に原因を求めるものである。我々が食物から摂取する鉄の80%は非ヘムの第二鉄イオンであるが、消化管からの鉄イオンの吸収には、主に胃酸とアスコルビン酸による第一鉄イオンへの還元が必要とされる[8]。したがって、*H. pylori* による萎縮性胃炎を有する成人例においては、低酸症により鉄イオンの吸収が低下している可能性がある[9]。実際、成人のIDA例の多くは全胃炎であり、非IDA例では胃前庭部優位の胃炎であったとの報告もある[9]。一方、小児においても中等度以上の胃粘膜萎縮が *H. pylori* 感染者の一部に認められるが、胃体部の萎縮は極めて低率である[4]。そして、*H. pylori* 感染小児の胃酸分泌能は、非感染のコントロール群と比較して低下していない[10]。実際、*H. pylori* 感染小児において、除菌の前後における鉄イオンの吸収に変化はみられなかった[11]。以上のことを考慮すると、「*H. pylori* 感染—胃酸分泌の低下—鉄イオンの吸収障害—IDA発症」説を小児に適応することは困難である。

最近、有力な仮説として、*H. pylori* による宿主であるヒトからの競合的な鉄の摂取・利用が提唱されている。現在までに、ニッケルイオン関連遺伝子も含めると20近い鉄代謝・輸送に関連する遺伝子が知られており、IDAの発症機序の解明に向けた研究も報告されている。まず、フェリチン様蛋白Pfrをコードする *H. pylori* 遺伝子 *pfr* が検討されたが、IDA群とコントロール群間で有意な遺伝子変異は検出されなかった[12]。次に、*H.*

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pylori 遺伝子として鉄イオン輸送蛋白をコードする *feoB* 遺伝子も検討されたが、IDA との関連に関して有意な結論には至っていない[13]。

現時点で、IDA の発症機序に関して、*H. pylori* の全遺伝子を網羅的に検討した報告はない。また、鉄代謝・輸送に関与する *H. pylori* 遺伝子に関する我々の知識は極めて乏しい。今回、*H. pylori* に IDA 責任遺伝子が存在するとの仮説を立て、DNA マイクロアレイ法を用いて検討を行った。

方法

IDA 株およびコントロール株

IDA を呈した小児 4 例（平均 14.5 歳）から分離培養された *H. pylori* 株を対象とした。全例に上部消化管内視鏡検査を施行し、胃・十二指腸潰瘍を含む出血性病変がないことを確認した。便潜血反応はすべての症例で陰性であった。全例において体重および身長は各年齢における正常範囲内であり、偏食傾向は認めなかった。IDA 群の血清ヘモグロビン、鉄およびフェリチンの平均値はそれぞれ 6.6 g/dl、11 μg/dl、1.6 ng/ml であった。

コントロール株として、年齢および性をマッチさせた IDA のない小児からの 4 株を検討した。上部消化管内視鏡検査では、全例が慢性胃炎であった。血清ヘモグロビン、鉄およびフェリチンの平均値はそれぞれ 14.5 g/dl、112 μg/dl、25.1 ng/ml であった。

マイクロアレイ法

既報[14]より、鉄関連遺伝子の発現の検討には exponential phase の培養 *H. pylori* が妥当と考えられたため、exponential phase の total RNA を用いて全遺伝子の発現量を IDA 群とコントロール群間で比較した。対象とした 8 株を通常の培養条件下で 15-17 時間、液体培養 (exponential phase、OD600 = 0.4-0.65) した後、QUIAGEN を用いて total RNA を抽出した。抽出した total RNA の質的確認を 1% アガロースゲル電気泳動で行い、良好な泳動パターンを示した試料のみをマイクロアレイの実験に供した。

マイクロアレイ法には、各株から得られた total RNA 10 μg を用いた。そして、HP26695 4-plex array (NimbleChip, Roche NimbleGen) を使い 42℃ で、16 時間ハイブリダイゼーションを行った後、GenPix 4000B microarray scanner を用いて mRNA 量を測定した。

鉄による発現調節の検討

両群の各 2 株を対象とした。鉄欠乏条件（deferoxamine mesylate 50μM 添加）下で液体培養 (exponential phase) を行った後、上述のマイクロアレイ法を行い、mRNA 量を測定した。そして、通常の培養条件下での結果と比較して、鉄による全遺伝子の発現調節を検討した。

VacA の cytotoxic assay

本研究におけるマイクロアレイ法の妥当性を検証するため、通常の培養条件下における細胞空胞化毒素 VacA の毒素活性とマイクロアレイ法による *vacA* 遺伝子の発現量を比較した。VacA の毒素活性の測定には、両群の 8 株から得られた培養上清を用いた。各希釈系列の培養上清で AGS 細胞を培養して、顕微鏡下で細胞空胞化を観察して半定量的に VacA 蛋白量を測定した。さらに、抗 VacA 抗体を用いてウエスタンブロット法を行い、cytotoxic assay の結果との相関も検討した。

病理組織学的検討

IDA 群およびコントロール群の胃粘膜生検試料（胃前庭部および胃体部）を対象として、シドニー分類[15]に基づき Inflammation、Activity、*H. pylori* density、および Atrophy の各病理学的パラメーターを比較検討した。

統計学的解析

マイクロアレイ法のデータ解析には、IDA 群とコントロール群間で 3.5 倍以上の発現差を有意とした。また、3.5 倍以上の発現差を示した遺伝子において、t - 検定 (Bonferroni 補正法) による有意差検定を併用した。

結果

A) 本研究におけるマイクロアレイ法の妥当性

遺伝子発現の相関

全遺伝子の発現において、IDA 群とコントロール群

は極めて良好な相関を示した (r = 0.962)。

Cytotoxic assay と *vacA* 遺伝子発現との相関

マイクロアレイ法による *vacA* 遺伝子の発現量は、cytotoxic assay 法による半定量的 VacA 蛋白量と相関を示した。また、cytotoxic assay 法の結果はウエスタンブロット法の結果と一致した。

鉄による鉄関連遺伝子の発現調節

通常の培養条件下における遺伝子発現と比較して、両群ともに鉄欠乏条件での *pfr* 遺伝子の発現は down-regulation (0.1-0.3 倍)、鉄イオン輸送蛋白遺伝子 *fecA1* と *frpB1* は up-regulation (4.0-17.3 倍) を示した。これらの結果は既報の結果[14]と一致した。

以上の結果は、本研究におけるマイクロアレイ法による遺伝子発現のデータの妥当性を支持するものと考えられた。

B) IDA 責任遺伝子の検討

鉄関連遺伝子の発現

pfr、*fur*、*fecA1/2/3*、*feoB*、および *frpB1/3* など主要な鉄関連遺伝子は、両群間で有意の発現差を示さなかった。一方、コントロール株に比べて、*ceuE1* (0.02 倍) および *frpB2* (0.22 倍) は IDA 株で低い発現を示した。

IDA 群で高発現を示した遺伝子

通常の培養条件下において、コントロール株に対して IDA 株で有意に高い発現を示したのは、*HP0682* (84.0 倍)、*hopO* (10.9 倍)、*hsdM* (10.6 倍)、*hopP* (3.7 倍) など 29 の遺伝子であった。このうち、*HP0682* を含めた 19 の遺伝子の機能は不明であった。

一方、*vacA* (3.2 倍)、*cag3* (2.5 倍)、*babB* (2.4 倍) などの病原性関連遺伝子は、3.5 倍未満の発現差ではあったが、統計学的に有意に高い発現を示した。

IDA 群で低発現を示した遺伝子

通常の培養条件下において、コントロール株に対して IDA 株で有意に低い発現を示したのは、上述した *ceuE1* および *frpB2* を含めて、*iceA* (0.15 倍) など 12 の遺伝子であった。このうち、6 つの遺伝子は機能が不明であった。

C) 病理組織学的検討

IDA 群およびコントロール群共に、病理学的に慢性活動性胃炎を示した。一方、胃前庭部および胃体部の各生検試料において、Inflammation、Activity、*H. pylori* density、および Atrophy の各パラメーターの程度に有意差は認めなかった。

考察

当初、フェリチン遺伝子 *pfr*、鉄関連遺伝子の発現を鉄イオン依存的に調節するレギュレーター遺伝子 *fur*、あるいは第一および第二鉄イオンの輸送蛋白遺伝子 *feoB* および *fecA1/2/3* などに着目していた。しかし、*frpB1/3* なども含めて、多くの鉄関連遺伝子の発現は両群間で有意差を認めなかった。この結果は、IDA 群とコントロール群間で有意な *pfr* 遺伝子変異は検出されなかったとする研究[12]と一致する。また、*feoB* 遺伝子においては IDA との関連性に明確な結論が出ていなかったが[13]、これを含めて、本研究の結果は多くの鉄関連遺伝子の IDA 発症への関与は否定的であることを示している。有意な低発現を示した *frpB2* および *ceuE1* 遺伝子の関与については、その発現蛋白の機能解明も含めて、今後の検討を待つ必要がある。

一方で、責任遺伝子候補として、IDA 株で高い発現を示した *HP0682* や *hopO/P* など、低発現の *ceuE1* や *frpB2* などの遺伝子が絞り込まれた。中でも、外膜蛋白遺伝子 *hopO* と *hopP* が、鉄の取り込みに関与するヒトラクトフェリン結合蛋白の分子量 (70 kDa)[16] にほぼ一致したことは特記に値する。以前の研究[17]において、*H. pylori* はヒトラクトフェリンを介して、宿主から鉄イオンを取り込んでいることが指摘されている。さらに、*H. pylori* は 70 kDa のラクトフェリン結合蛋白をその外膜に発現し、この外膜蛋白はヒトラクトフェリンに高い親和性を示し、また鉄イオンによる down-regulation を受けることも判明している[16]。本研究の結果を考え合わせると、*H. pylori* による IDA の発症機序を考える上で極めて興味深い。

一方、*vacA*、*cag3*、*babB*、*iceA* などが有意な発現差

を示した。中でも、*vacA* 遺伝子の発現は鉄欠乏状態で up-regulation を受けることが知られている[18,19]。したがって、何らかの機序で、これら病原性関連遺伝子が *H. pylori* の鉄イオンの取り込みに関与している可能性がある。

おわりに

以前、特に発展途上国において、*H. pylori* が成長障害に関連するとの指摘があった。結論に至っていなかったが、*H. pylori* が高率に IDA ないし鉄欠乏状態を起こすことが判明し、両者の関連性についての議論が高まっている。すなわち、食料難と相俟った「*H. pylori* – IDA – 成長障害」悪循環説[20]が浮上し、今後の大きな研究テーマとなることが予想される。本研究の成果と今後の展開は、小児の *H. pylori* 感染症の病態解明だけでなく、成長障害の改善・発育向上に関する *H. pylori* 感染症の対策・戦略の確立に大きく寄与すると考える。

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文献

1) Macarthur C, Saunders N, Feldman W: *Helicobacter pylori*, gastroduodenal disease, and recurrent abdominal pain in children. JAMA 1995; 273: 729-734

2) Kato S, Nishino Y, Ozawa K, et al.: The prevalence of *Helicobacter pylori* in Japanese children with gastritis or peptic ulcer disease. J Gastroenterol 2004; 39: 734-873

3) Fukase K, Kato M, Kikuchi S, et al.: Effect of eradication of *Helicobacter pylori* on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomized controlled trial. Lancet 2009; 372: 392-397

4) Kato S, Nakajima S, Nishino Y, et al.: Association between gastric atrophy and *Helicobacter pylori* infection in Japanese children: a retrospective multicenter study. Dig Dis Sci 2006; 51: 99-104

5) Ricuarte O, Gutierrez O, Cardona H, et al. Atrophic gastritis in young children and adolescents. J Clin Pathol 2005; 58: 1189-1193

6) Barabino A: *Helicobacter pylori*-related iron deficiency anemia: a review. *Helicobacter* 2002; 7: 71-75

7) Dubois S, Kearney DJ: Iron-deficiency anemia and *Helicobacter pylori* infection: a review of the evidence. Am J Gastroenterol 2005; 100: 453-459

8) Lombard M, Chua E, O' Toole P: Regulation of intestinal non-haem iron absorption. Gut 1997; 40: 435-439

9) Annibale B, Capurso G, Lahner E, et al.: Concomitant alterations in intragastric pH and ascorbic acid concentration in patients with *Helicobacter pylori* gastritis and associated iron deficiency anemia. Gut 2003; 52: 496-501

10) Kato S, Ozawa K, Koike T, et al. Effect of *Helicobacter pylori* infection on gastric acid secretion and meal-stimulated serum gastrin in children. *Helicobacter* 2004; 9: 100-105

11) Sarker SA, Davidsson L, Mahmud H, et al.: *Helicobacter pylori* infection, iron absorption, and gastric acid secretion in Bangladeshi children. Am J Clin Nutr 2004; 80: 149-153

12) Choe YH, Hwang TS, Kim HJ, et al.: A possible relation of the *Helicobacter pylori pfr* gene to iron deficiency anemia? *Helicobacter* 2002; 7: 237-244

13) Jeon BH, Oh YJ, Lee NG, et al.: Polymorphism of the *Helicobacter pylori feoB* gene in Korea: a possible relation with iron-deficiency anemia? *Helicobacter* 2004; 9: 330-334

14) Thompson LJ, Merrell DS, Neilan BA, et al. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. Infect Immun 2003; 71: 2643-2655

15) Dixon MF, Genta RM, Yardley JH, et al.: Classification and grading of gastritis. The updated Sydney system. Am J Surg Pathol 1996; 20: 1161-1181

16) Dhaenens L, Szczebara F, Husson MO: Identification, characterization, and immunogenicity of the lactoferrin-binding protein from *Helicobacter pylori*. Infect Immun 1997; 65: 514-518

17) Husson MO, Legrand D, Spik G, et al.: Iron acquisition by *Helicobacter pylori*: importance of human lactoferrin. Infect Immun 1993; 61: 2694-2607

18) Szczebara FL, Dhaenens L, Armand S, et al. Regulation of the transcription of genes encoding different virulence factors in *Helicobacter pylori* by free iron. FEMS Microbiol Lett 1999; 175: 165-170

19) Merrell DS, Thompson LJ, Kim CC, et al.: Growth phase-dependent response of *Helicobacter pylori* to iron starvation. Infect Immun 2003; 71: 6510-6525

20) Windle HJ, Kelleher D, Crabtree JE. Childhood *Helicobacter pylori* infection and growth impairment in developing countries: a vicious cycle? Pediatrics 2007; 119: e754-e759

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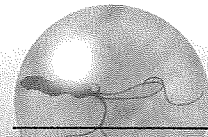

特集

Helicobacter pylori と他の細菌・ウイルスの混合感染

Helicobacter pylori とマイコプラズマの重複感染

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慢性胃炎の胃粘膜組織からマイコプラズマ（*Mycoplasma*）が検出され，マイコプラズマ感染が *Helicobacter pylori*（*H. pylori*）感染とともに胃粘膜の炎症を誘導している可能性が報告された．また，胃癌組織からブタ由来の *Mycoplasma hyorhinis* が高率に検出されたとの報告がなされている．喘息発作や動脈硬化症におけるマイコプラズマと *H. pylori* の感染が検討されたが，*H. pylori* 感染はこれらの疾患の病態に影響を与えているものとは考えられなかった．*H. pylori* と慢性気管支炎や結核との関連性が報告されているが，マイコプラズマ感染とのリンクについては不明である．*H. pylori* とマイコプラズマとの慢性重複感染が生体にどんな効果を与えているかについての検討が今後期待される．

KEY WORDS

Helicobacter pylori，マイコプラズマ，dual infection（重複感染）

はじめに

マイコプラズマ（*Mycoplasma*）は細胞壁を欠くグラム陰性菌で，最小の細菌である．マイコプラズマのうち *Mycoplasma pneumoniae* はヒトの原発性異型肺炎（マイコプラズマ肺炎）を引き起こす病原細菌である．海外の報告では，ヒト胃癌組織よりマイコプラズマが高率に検出されるほか，ヒト慢性胃炎組織からも各種のマイコプラズマが PCR 法により検出されている．胃内に棲息する *Helicobacter pylori*（*H. pylori*）とマイコプラズマとの重複感染に関する報告ならびに自験例を紹介し，両者の感染効果を考察する．

1．マイコプラズマの性状

マイコプラズマは直径 0.3～0.8 μm の球型または西洋梨型を示すグラム陰性菌で，細菌濾過器を通過し得る最小の細菌である．細胞壁を保有しないため多形性である．細胞表面は蛋白と脂質の 3 層膜により構成される．固形培地での発育は表層から内部へ中心部がくい込むように増殖する．マイコプラズマは桑の実状，目玉焼き状，乳首状などのコロニーを形成する（図❶）．増殖にはステロールを要求する．ヒトのマイコプラズマである *Mycoplasma pneumoniae* のゲノムサイズは 816,394 bp であり，細胞寄生性を示さない病原体としては最小となる．

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