Intestinal Bacterial Flora and Host Defense Mechanisms

Atsushi OZAWA, Nobuhiko OHNISHI, Seiki TAZUME, Yuji AIBA and Keiichi WATANABE*

Department of Microbiology and *Department of Pathology, School of Medicine, Tokai University

The protective mechanisms of intestinal bacterial flora against exogenous infections will be discussed in this paper. Experimental data on protective function of Escherichia coli, Enterococcus faecalis and Bacteroides distasonis comprising intestinal flora against oral infection of Shigella flexneri which causes localized infection are presented. Furthermore, the present investigation deals with non-specific defense mechanism which indicate that protective activity of intestinal flora against parenteral infection of Salmonella typhimurium mainly depend upon the larger number of functional kupffer cells in conventional mice than in that of their germfree counterparts.

INTRODUCTION

The analysis of such complex biological phenomena as the interrelationship between intestinal bacterial flora and host, and between the bacterial species comprising intestinal flora are the principal objectives awaiting resolution in the field of interrelational microbiology.

Many findings concerned with polydimen- sional functions of intestinal flora in the host have been accumulated with progress of gnotobiological investigations. Resistance of host against enteric infections (4,6), the regulation of cholesterol metabolism through deconjugation of the bile acids (17), the regulation of the urea cycle which is the mechanism for detoxication of ammonia in the liver (20), modification of immunological reactions (14), and amplifying effect of glucocorticoid activity (12) are considered as some of the protective functions of intestinal flora.

The present investigations were designed to study the role of intestinal flora against exogenous infections.

MATERIALS AND METHODS

1) Bile acid analysis: The composition and concentration of bile acids derived from germfree and conventional mice were analyzed by the gas-liquid chromatography method. Extrac- tion of bile acids was carried out fundamentally according to the method reported by Suzuki (23), Gundy (7) and Sjövall (22).

2) Animals: The mice used were six-week-old male or female germfree and conventional ICR mice. The germfree mice were reared in plastic isolators. Surveillance for contamination consisted of periodic aerobic and anaerobic culture of feces and tissue for bacteria.

3) Culture preparations and infection: 2.0 × 10^5 viable cells of S. flexneri cultured in GAM broth was orally introduced into germfree mice and gnotobiotic mice with E. faecalis or B. distasonis. Fresh culture derived from ampules of lyophilized S. typhimurium LT-2 strain was employed for each experiment, 8.0 × 10^4 viable cells of S. typhimurium was subcutaneously injected into germfree, gnotobiotic mice with E. coli and conventional mice.

4) Assay of phagocytic activities of the reticuloendothelial system: Carbon clearance tests performed by the method of Biozzi et al (3) were used to determine phagocytic activities of the reticuloendothelial system at 1, 2, 3, 5, 7 and 14 days after S. typhimurium infection. The carbon particles were uniform in size measuring 2,500nm in diameter. Pelikan carbon particles were suspended at a concentration of 16mg/ml in 1% gelatin solution. A carbon suspension of 16mg/ml/100g body

Atsushi OZAWA, Department of Microbiology, School of Medicine, Tokai University, Bohseidai, Isehara, Kanagawa 259-11, Japan
weight was injected intravenously into mice. They were bled from the retro-orbital plexus at 0, 2, 5, 10, 15 min. The blood was determined photometrically in a Hitachi spectrophotometer at a wavelength of 650nm. The phagocytic index, $K$, was determined by the equation $K = (\log C_2 - \log C_1)/t_2 - t_1$, where $C_1$ and $C_2$ represent the blood colloid concentration at time ($t_1$) and time ($t_2$), respectively. The corrected phagocytic index is a measure of phagocytic activity per unit weight of tissue and is given by the equation $x = (\text{body weight}/\text{weight liver and spleen}) \times V'K$.

5) Cytochemical studies: Barka-Anderson’s method for assessment of acid phosphatase activity was employed for histochemical analysis using light microscopy (1). Fahimi’s method for endogenous peroxidase activity was employed in cytochemical studies at the electron microscopy level (5).

RESULTS

Gnotobiotic ICR mice monoassociated with S. flexneri were further associated by oral introduction with E. coli and E. faecalis. The S. flexneri given orally decreased gradually as assessed from fecal viable bacterial counts in the gnotobiotic mice and disappeared in approximately 45 days after S. flexneri infection, but the E. coli and E. faecalis coexisted, maintaining full growth level during the observation period (Fig. 1). The patterns of degradation of bile acids in vitro by the various bacteria employed are shown in Table 1. S. flexneri did not ex-

![Fig. 1 The sequel of fecal bacterial number of S. flexneri in coexistence of E. faecalis and E. coli. Each dot with vertical bar: mean ± S.D. of number of bacteria](image)

<table>
<thead>
<tr>
<th>Bacteria employed</th>
<th>Deconjugation of</th>
<th>Oxidation of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Taurocholic acid</td>
<td>Glycocholic acid</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacteroides distasonis</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
hibit metabolizing activity bile acids in vitro. *E. faecalis* showed deconjugative activity of conjugated bile acids. *E. coli* was not capable of hydrolyzing conjugated bile acids but was able to oxidize cholic acid to 7-ketodeoxycholic acid in vitro. *B. distasonis* showed both deconjugative and oxidative activity of bile acids (Table 1).

The metabolites of bile acids in the small intestine were analyzed quantitatively by gas chromatography. As the results, in gnotobiotic mice with *S. flexneri, E. coli*, and *E. faecalis*, 7-ketodeoxycholic acid was detected at the time of rejection of *S. flexneri* from the intestine (Fig. 2).

On the other hand, *B. distasonis* was introduced orally into gnotobiotic mice monoassociated with *S. flexneri* and in this situation, viable bacterial counts of *S. flexneri* recovered from the feces of the gnotobiotic animals did not decrease during 80 days after superinoculation with *B. distasonis*. At this time, 7-ketodeoxycholic acid was not detected in the intestinal contents in spite of the presence of *B. distasonis* which could produce 7-ketodeoxycholic acid in vitro. However, viable bacterial counts of *S. flexneri* from the feces began to decrease when *E. coli* was additionally introduced orally into gnotobiotic mice monoassociated with *S. flexneri* and *B. distasonis* (Fig. 3).

In the gnotobiotic mice with *S. flexneri, B. distasonis* and *E. coli*, 7-ketodeoxycholic acid was detected alongside *S. flexneri* decreases in viable bacterial counts recovered from the feces. (Fig. 4). There seems to be some correlation between increase of amounts of 7-ketodeoxycholic acid and rejection of *S. flexneri* from the intestine. The experimental results obtained here may indicate 7-ketodeoxycholic acid as one of the factors for the rejection of *S. flexneri* from the intestine. Further investigation will be needed to clarify this point.

*S. typhimurium* was introduced as subcutaneous infections in ICR strains of germfree mice, *E. coli*-monoassociated gnotobiotic mice and ICR conventional mice, and the growth of inoculated organisms were followed serially in the liver and spleen. A significantly higher count of the bacilli were obtained from both liver and spleen of germfree mice in comparison to both the *E. coli*-monoassociated gnotobiotic and conventional mice (Fig. 5). Furthermore, by mortality count, 50% of the germfree mice were dead 7–10 days post *S. typhimurium* infection whereas on the contrary, there were no deaths throughout the observation period in either *S. typhimurium* infection groups in *E. coli*-monoassociated and conventional mice (Fig. 6).

The phagocytic index (K value) obtained by the carbon clearance test during infection of mice with *S. typhimurium* showed a corresponding rise in K value reflecting the progression of infection in germfree mice. In conventional mice, it rose 24 hours post-infection but with the values generally showing rapid recovery to levels before infection soon after. The corrected phagocytic index (α value) showed corresponding rise reflecting the progression of infection in conventional mice, whereas in germfree mice the α value rose 7 days post-infection but with the values soon showing rapid recovery to levels before infection (Fig. 7).

In *E. coli* monoassociated mice, the K value rose 7 days post-infection, and the α value rose 2 days post infection but decreased to the levels before infection at 3 days post infection (Fig. 8).

The correlation between the phagocytic index obtained by the carbon clearance test and the number of viable organisms of *S. typhimurium* recovered from the liver and spleen was statistically analyzed. A positive correlation was found between the K value and the number of viable organisms recovered from liver, spleen and the 3 groups of germfree, *E. coli*-monoassociated and conventional mice, with data obtained indicating higher host response to *S. typhimurium* infection by conventional and *E. coli*-monoassociated groups (Fig. 9).

A comparison of the effects of lipopolysaccharide derived from *E. coli* on glucose consumption by peritoneal macrophages obtained from germfree and conventional mice was undertaken. The reactions of peritoneal macrophages to lipopolysaccharide showed higher response in conventional mice than in germfree groups (Fig. 10).

An electron microscopic analysis of endogenous peroxidase localization was undertaken taking the morphological and functional aspects of kupffer cells into consideration.
Fig. 2 The changing pattern of the concentration of 7-ketodeoxycholic acid and fecal bacterial number of S. flexneri in coexistence of E. faecalis and E. coli in mice intestine. Each dot with vertical bar: mean ± S.D. of number of bacteria.

Fig. 3 The sequel of fecal bacterial number of S. flexneri in coexistence of B. distasonis and E. coli
Fig. 4 The changing pattern of the concentration of 7-KDOCA and fecal bacterial counts of *S. flexneri* in coexistence of *B. distasonis* and *E. coli* in mice intestines. Each dot with vertical bar indicates mean ± S.D. of number of bacteria.

Fig. 5 Bacterial growth in liver and spleen of gnotobiotic and conventional mice *S. typhimurium* infection
○: Liver  ●: Spleen
Fig. 6 Survival time of gnotobiotic and conventional mice infected subcutaneously with S. typhimurium

Fig. 7 Sequential observation on K and α values obtained by carbon clearance test in the gnotobiotic and conventional mice infected with S. typhimurium

*: P<0.05  **: P<0.01
Fig. 8 Sequential observation on K and $\alpha$ values obtained by carbon clearance test in the gnotobiotic mice infected with S. typhimurium

**: $P<0.01$

Fig. 9 Correlation between viable bacterial counts of S. typhimurium derived from liver, spleen and K values in gnotobiotic and conventional mice during infection

*: Correlation coefficient $P$ value $<0.01$
There appeared to be no morphological or functional differences in the kupffer cells of germfree and conventional mice as seen from the uptake of carbon particles and the localization of peroxidase activity on the rough endoplasmic reticulum, among other such criteria (Figs. 11, 12).

A microscopic examination of granuloma formation capacity following S. typhimurium infection in mice revealed granuloma formation to be more strongly suppressed in conventional and E. coli-monoassociated gnotobiotic mice than in their germfree counterparts (Figs. 13, 14, 15).

Electron microscopic analysis of hepatic granuloma from the cytochemical standpoint indicated that the majority of cells comprising granuloma in gnotobiotic mice infected with S. typhimurium were promonocytes carrying azurophilic granules and rough endoplasmic reticulum showing peroxidase activity (Fig. 16). In electron microscopic analysis of hepatic granuloma lesions formed in conventional mice with S. typhimurium infection, the cells constituting granuloma were found to be composed of kupffer cells displaying peroxidase activity on the rough endoplasmic reticulum (Fig. 17).

In E. coli-monoassociated gnotobiotic mice with S. typhimurium, the granulomas were composed of monocytes with peroxidase activity only in the azurophilic granules (Fig. 18).

The above-mentioned experimental results indicate that there are qualitative differences in granuloma formation in reaction to S. typhimurium infection in each of the 3 groups of germfree, E. coli-monoassociated and conventional mice.

Before S. typhimurium infection, a small number of kupffer cells exhibiting acid phosphatase activity were found in germfree mice, whereas the number of such cells in the sinusoid of the liver in conventional mice with intestinal flora was approximately twice that of the germfree animals.

Infection of germfree mice with S. typhimurium resulted in a rise in the number of acid phosphatase activity-positive promonocytes while no rise in acid phosphatase-positive kupffer cells were seen in conventional mice post infection (Fig. 19).
Fig. 11 Carbon particle uptake of Kupffer cell in germfree mouse
Magnification: 14,000×

Fig. 12 Carbon particle uptake of Kupffer cell in conventional mouse
Magnification: 15,000×
Fig. 13  Granulomatous lesions in the liver at 14 days after S. typhimurium infection into germofree mice
Magnification: 60 x

Fig. 14  Granulomatous lesions in the liver at 14 days after S. typhimurium infection into gnotobiotic mice with E. coli
Magnification: 60 x
**Fig. 15** Granulomatous lesions in the liver at 14 days after *S. typhimurium* infection into conventional mice
Magnification: 60×

**Fig. 16** The cellular constituent of granulomatous lesions in the liver of gnotobiotic mice monoinfected with *S. typhimurium*
PM: Promonocyte    Magnification: 9,900×
Fig. 17 The cellular constituent of granulomatous lesions in the liver of conventional mice infected with S. typhimurium
K: Kupffer cell  er: rough endoplasmic reticulum
Magnification: 4,800×

Fig. 18 The cellular constituent of granulomatous lesions in the liver of E. coli monoassociated gnotobiotic mice infected with S. typhimurium
M: Monocyte  Magnification: 7,800×
DISCUSSION AND CONCLUSION

Several studies have dealt with the significance of the indigenous intestinal flora in protective activity against Shigella infections which cause localized infection with exudative inflammation. It has been reported that the oral administration of antibiotics eliminate the intestinal flora and render mice susceptible to infection by S. flexneri, and that the introduction of E. coli into the intestines of the mice resulted in elimination of the enteric pathogens (9). Maier found that antagonism against Shigella was most pronounced in mice associated with E. coli and least pronounced in mice associated with Bacteroides fragilis (15).

It has been suggested that the mechanism by which E. coli inhibited the growth of S. flexneri in continuous flow culture and, by implication, also in the intestine might be based on competition of these bacteria for fermentable carbon sources under the prevailing highly reduced conditions. Furthermore, it has been reported that inhibitory substance, especially colicines elaborated by intestinal bacteria do not play an important role in the resistance of indigenous intestinal flora to superinfection by enteric pathogens (8, 18).

Hentges indicated that inhibition of Shigella multiplication in mixed culture was due to the production of formic and acetic acid by the coliform strains, which under reducing conditions and pH levels present in cultures, were bactericidal for Shigella (9). It has been shown that volatile fatty acids produced by Bacteroides and low pH which develops in the culture media interfered with the multiplication of Shigella (10).

We have shown experimental data regarding the significance of intestinal flora in protection against oral infection of S. flexneri. It is emphasized that several factors, such as metabolites of bile acids (24), secretory IgA produced in the intestine (21), bacterial antagonism between intestinal flora and enteric pathogens (8, 9, 18), and activation of the alternative pathway of complement (19) are involved in the protective mechanisms of gut flora against Shigella infections.

S. typhimurium and S. enteritidis causing...
systemic infections in mice exist as facultative intracellular parasites causing granulomatous inflammation of reticuloendothelial tissue. The effect exerted by intestinal bacterial flora on course of infection and host reaction is S. typhimurium infections in mice is a research theme of special significance in clarifying the dynamic functions of intestinal flora in the host.

The experimental results presented herein are discussed with respect to the protective function of intestinal flora against parenteral infection of S. typhimurium which brings about systemic infection with granulomatous inflammation.

We have emphasized with regards to non-specific defense mechanism that protective activity of intestinal flora against parenteral infection of S. typhimurium are mainly dependent upon the larger number of functional kupffer cells in conventional mice than those in their germfree counterparts.

The lympathic tissues of germfree animals have been reported to respond to antigenic stimulation similarly in quality to conventional animals, although the host reactions may differ in rate and intensity (11).

It has been indicated that quantitatively, the increase in enzyme activities was indeed lower in germfree than in conventional macrophages, but prior contact with microorganisms is obviously not essential for induction or secretion of lysosomal enzymes (16).

The activities of the lysosomal enzymes in the kupffer cells would be important factors in the defense mechanisms against bacterial infections. Kupffer cells from germfree rats were found to have lower lysosomal enzyme activities than cells obtained from conventional rats (2).

On the other hand, it has been reported that kupffer cells from germfree and clean conventional rats showed no significant differences in the specific activities of the lysosomal enzymes (13).

We conclude that the significantly higher assessment of the number of kupffer cells in conventional and E. coli-monoassociated mice as compared to germfree mice was an indication of the qualitative and quantitative differences in cell composition comprising granuloma in reaction to S. typhimurium infection in the 3 groups. This in turn makes possible the attribution to intestinal flora a role in influencing cell differentiation of bone marrow-derived stem cells and that they may thus be participating in the quantitative control of kupffer cell production.

ACKNOWLEDGEMENTS

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

15) Maier BR and Hentges DJ: Experimental Shigela infections in laboratory animals. I. Antagonism by human normal flora components in gnotobiotic mice.


