Enhancement by Ampicillin of Antibody Responses Induced by a Protein Antigen and a DNA Vaccine Carried by Live-Attenuated Salmonella enterica Serovar Typhi

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Live-attenuated Salmonella species are effective carriers of microbial antigens and DNA vaccines. In a mouse model, the immunoglobulin M (IgM) and total antibody levels directed toward the lipopolysaccharide of Salmonella enterica serovar Typhi were significantly enhanced at day 21 after oral immunization with live-attenuated serovar Typhi (strain Ty21a) when ampicillin was concomitantly administered (P < 0.05 and P < 0.005, respectively). The heat-killed Ty21a-stimulated lymphocyte proliferation indices for the ampicillin group at day 21 were significantly higher than those for the normal saline (NS) group (P < 0.005, P < 0.001, and P < 0.01) for all three doses of antigen (10⁴, 10⁵, and 10⁶ heat-killed Ty21a per well, respectively). The 50% lethal doses for mice from the ampicillin and NS groups immunized with Ty21a with pBR322 after wild-type serovar Typhi challenge on day 24 were 3.4 × 10⁶ and 5.0 × 10⁶ CFU, respectively. The fecal bacterial counts for the ampicillin group at days 1, 3, and 5 were significantly lower than those for the NS group (P < 0.01, P < 0.01, and P < 0.05, respectively), and there was a trend toward recovery of Ty21a in a larger number of mice from the ampicillin group than from the NS group. Furthermore, the IgG2a levels directed toward tetanus toxoid were significantly enhanced at days 7 and 21 after oral immunization with Ty21a that carried the fragment c of tetanus toxoid when ampicillin was concomitantly administered (P < 0.05 and P < 0.005, respectively), and the IgM and total hepatitis B surface antibody levels were significantly enhanced at days 7 (P < 0.005 and P < 0.05, respectively) and 21 (P < 0.01 and P < 0.05, respectively) after oral immunization with Ty21a that carried the DNA vaccine that encodes hepatitis B surface antigen when ampicillin was concomitantly administered.

The present observation may improve the efficacy of the protein antigens and DNA vaccines carried in live-attenuated bacteria, and further experiments should be carried out to determine the best antibiotics and dosage regimen to be used, as well as the best carrier system for individual protein antigens and DNA vaccines.

Mucosal vaccination provides specific advantages for ease of administration, vaccine formulation, and potential to support mass vaccination (7). It has been shown that live-attenuated Salmonella species are effective carriers of microbial antigens and DNA vaccines (3, 5, 13). However, since the efficacy of oral live-attenuated Salmonella enterica serovar Typhi vaccine (Ty21a) in humans is only 70% (14, 15), it can be inferred that use of strain Ty21a as a vaccine carrier for human beings is far from ideal. Moreover, the immunogenetics of mucosal vaccines in people who reside in developing countries are even worse (8, 12). This would further hinder the potential use of Ty21a as a vaccine carrier for global immunization. Therefore, new ways to improve the immunogenicity of Ty21a as well as the protein antigens and DNA vaccines carried in it are mandatory.

Antibiotics have been known to affect immune responses (16, 17, 18). Recently, we have shown that antibiotics, especially ampicillin, enhance the antibody response against the lipopolysaccharide (LPS) of serovar Typhi after intraperitoneal Ty21a immunization in a mouse model (16). In these experiments, the effects of ampicillin on the immunogenicity of oral Ty21a and the protein antigen and DNA vaccine carried in it were studied. We examined the effects of ampicillin on the serum antibody response against LPS of serovar Typhi, the heat-killed Ty21a-stimulated lymphocyte proliferation index (LPI), and the survival of mice upon wild-type S. typhi challenge after oral Ty21a immunization. We also studied the effect of ampicillin on the serum antibody response against tetanus toxoid and hepatitis B surface antigen (HBsAg) in mice administered fragment c of tetanus toxoid and the DNA that encodes HBsAg, each of which was carried in Ty21a, respectively. The possible mechanism of such effects is also discussed.

MATERIALS AND METHODS

Animals. Female BALB/c mice (weight, 18 to 22 g) were used in all experiments. They were housed in cages under standard conditions with regulated day length, temperature, and humidity and were given pelleted food and tap water ad libitum.

Experimental schedule, antibiotic administration, and immunization. The mice were divided randomly into two groups; one group received ampicillin (20 mg/kg of body weight) intraperitoneally, and the other group received 0.25 ml of sterile normal saline (NS). The doses were administered from day −1 to day 20. To determine the effect of ampicillin on the levels of antibodies against LPS of S. enterica serovar Typhi in serum, the heat-killed Ty21a-stimulated LPI, the survival of mice upon wild-type serovar Typhi challenge, and the fecal aerobic bacterial and Ty21a counts after Ty21a administration, 39 mice from the ampicillin group and 39 mice from the NS group were immunized orally with Ty21a (Berna, Berne, Switzerland) that had been transformed with pBR322 (Amerham Pharmacia Biotech, Piscataway, N.J.) (to make the organism ampicillin resistant) by using a gastric tube (2.7 × 10⁶ CFU in 0.3 ml). Fifteen mice from each group were used for measurement of serum antibody levels, LPI, fecal bacterial count, and Ty21a isolation; and the remaining 24 mice in each group were used for wild-type serovar Typhi challenge. For determination of the effect of ampicillin on the antibody response after immunization with the expressed protein antigen carried in Ty21a, 15 mice from the ampicillin group and 15 mice from the NS group were immunized orally with Ty21a transformed with

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The cells were stimulated with phytohemagglutinin at 5 mg/ml, which contained HBsAg under the control of a cytomegalovirus (CMV) promoter (2). 15 mice from the NS group were immunized orally with Ty21a fragment c of tetanus toxoid under the control of a prokaryotic promoter (2). The plates were incubated at 37°C for 24 h. The number of colonies on each plate was quantified, and the fecal counts of the mice were expressed as the number of CFU per gram of stool.

### Table 1: Levels of total antibody and antibody subtypes at days 7 and 21 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS

| Day post-vaccination | Antibody level (mean ± SEM absorbance) | P value |
|----------------------|----------------------------------------|---------|
|                      | Ampicillin group (n = 15) | NS group (n = 15) |         |
| 7                    | Total 0.152 ± 0.072 | 0.043 ± 0.009 | NS<sup>a</sup> |
|                      | IgM 0.422 ± 0.093 | 0.208 ± 0.016 | <0.05 |
|                      | IgG2a 0.004 ± 0.009 | 0.001 ± 0.004 | NS<sup>a</sup> |
| 21                   | Total 0.539 ± 0.111 | 0.112 ± 0.030 | <0.005 |
|                      | IgM 0.375 ± 0.073 | 0.186 ± 0.041 | <0.05 |
|                      | IgG1 0.010 ± 0.002 | 0.010 ± 0.006 | NS<sup>a</sup> |
|                      | IgG2a 0.006 ± 0.004 | 0.002 ± 0.001 | NS<sup>a</sup> |

<sup>a</sup> NS, not significant.

### Table 2: LPI on day 21 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS

| Dose of heat-killed Ty21a antigen (no. of bacteria per well) | LPI (mean ± SEM) |
|-------------------------------------------------------------|------------------|
| Ampicillin group (n = 15)                                   |                 |
| 10<sup>5</sup>                                              | 0.167 ± 0.029   |
| 10<sup>7</sup>                                              | 0.263 ± 0.047   |
| 10<sup>9</sup>                                              | 0.111 ± 0.022   |

<sup>P</sup> value of LPI, <0.05, <0.01, respectively.

### Results

**Antibodies against LPS of serovar Typhimurium in serum.** The levels of total antibody and antibody subtypes against LPS of *S. enterica* serovar Typhimurium in serum at days 7 and 21 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS is summarized in Table 1. The IgM levels of the ampicillin group at days 7 and 21 and the total antibody levels for the ampicillin group at day 21 were significantly higher than the corresponding antibody levels for the NS group (*P* < 0.05, 0.005, and 0.01, respectively). The survival of mice in the ampicillin and NS groups immunized with Ty21a with pBR322 after intraperitoneal challenge with wild-type serovar Typhimurium on day 24 is shown in Table 3. The LD<sub>50</sub> for mice from the ampicillin and NS groups were 3.4 × 10<sup>7</sup> and 5.0 × 10<sup>7</sup> CFU, respectively.

**LPI.** The heat-killed Ty21a-stimulated LPI at day 21 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS is summarized in Table 2. The LPIs for the ampicillin group at day 21 when 10<sup>5</sup>, 10<sup>7</sup>, and 10<sup>9</sup> heat-killed Ty21a per well were used as antigens were significantly higher than those for the NS group (*P* < 0.005, 0.001, and 0.01, respectively).

**LD<sub>50</sub> after wild-type serovar Typhimurium challenge.** The survival of mice in the ampicillin and NS groups immunized with Ty21a with pBR322 after intraperitoneal challenge with wild-type serovar Typhimurium on day 24 is shown in Table 3. The LD<sub>50</sub> for mice from the ampicillin and NS groups were 3.4 × 10<sup>7</sup> and 5.0 × 10<sup>7</sup> CFU, respectively.

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**Figures and Tables:**

- **Figure 1:** Graph showing the absorbance obtained from the serum on that day. The serum antibody level for a particular mouse on a particular day was defined as the absorbance obtained from the serum at the serum dilution for the corresponding type of antibody measurement.
- **Figure 2:** Graph showing the survival of the mice after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS.
- **Figure 3:** Graph showing the LD<sub>50</sub> values for mice from the ampicillin and NS groups immunized with Ty21a with pBR322.
Fecal bacterial counts. The fecal bacterial counts at day –1 and days 1, 3, and 5 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS is summarized in Table 4. The fecal bacterial counts for the ampicillin group at days 1, 3, and 5 were significantly lower than those for the NS group (P < 0.01, P < 0.01, and P < 0.05, respectively).

Fecal Ty21a isolation. The number of mice from which Ty21a was isolated in the feces on days 1, 2, and 3 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS is summarized in Table 5. There was a trend toward the recovery of Ty21a in a larger number of mice from the ampicillin group than from the NS group.

Levels of antibodies against tetanus toxoid in serum. The levels of total antibody and antibody subtypes against tetanus toxoid in serum at days 7 and 21 after oral administration of Ty21a with pTETnr15 to mice treated with ampicillin or NS is summarized in Table 6. The IgG2a levels for the ampicillin group at days 7 and 21 were significantly higher than the corresponding antibody levels for the NS group (P < 0.05 and P < 0.005, respectively).

Levels of antibodies against HBsAg in serum. The levels of total antibody and antibody subtypes against HBsAg in serum at days 7 and 21 after oral administration of Ty21a with pRC/CMV-HBs(S) to mice treated with ampicillin or NS is summarized in Table 7. The IgM and total antibody levels for the ampicillin group at days 7 (P < 0.005 and P < 0.05, respectively) and 21 (P < 0.01 and P < 0.05, respectively) were significantly higher than the corresponding antibody levels for the NS group.

DISCUSSION

Ampicillin improved the B-cell response, the antigen-specific T-cell response, and the protective immune response after oral Ty21a immunization. Previously, we showed that antibodies, especially ampicillin, enhanced the humoral immune response of mice immunized intraperitoneally with Ty21a (16). In the present study, we showed that ampicillin enhanced not only the humoral immune response of mice after oral Ty21a immunization but also the heat-killed Ty21a-stimulated LPl for all three doses of heat-killed Ty21a used. Furthermore, the LD<sub>50</sub> for the group of mice that received ampicillin was sevenfold higher than that for the group that received NS.

The immunogenicity of the DNA vaccine or protein antigen carried in Ty21a was also enhanced by administration of ampicillin. In our experiments the HBsAg DNA vaccine and the fragment c of tetanus toxoid were chosen as the DNA vaccine and the protein antigen carried in Ty21a, respectively, because we have previously shown that ampicillin does not affect the antibody response of mice induced by parenteral administration of recombinant HBsAg or tetanus toxoid (16). In the present study, we showed that ampicillin increased the serum IgM response induced by pRC/CVM-HBs(S) carried in Ty21a. This is in line with the evidence that the major immune response induced by a single intraperitoneal dose of recombinant HBsAg vaccine is IgM. The IgG response occurred only after administration of a booster dose (16). On the other hand, ampicillin enhanced the IgG2a response induced by tetanus toxoid fragment c carried in Ty21a. This is probably because the tetanus toxoid fragment carried in Ty21a is presented by the antigen-presenting cells in a manner different from that when it is given through the subcutaneous route. When given subcutaneously, tetanus toxoid was presented mainly through the major histocompatibility complex class II pathway, inducing mainly antibody responses of the Th2 type (IgM and IgG1). However, when the tetanus toxoid fragment is carried in Ty21a, it is presented through intracellular major histocompatibility complex class I pathways, shifting the antibody response toward Th1. Therefore, it is not surprising that the major serum antibody subtype level that is upregulated is IgG2a. Furthermore, this is in line with the finding of a previous study, which showed that a strong class I-restricted cytotoxic T-cell response against murine lymphocytic choriomeningitis virus was induced by the chimeric protein formed between the nu

### Table 3. Survival of mice in the ampicillin and NS groups immunized with Ty21a with pBR322 after intraperitoneal challenge with wild-type S. typhi on day 24

| Challenge dose of wild-type S. typhi (CFU) | No. of mice surviving |
|-------------------------------------------|----------------------|
| **Ampicillin group** (n = 6) | **NS group** (n = 6) |
| 5.0 × 10<sup>6</sup> | 6 | 3 |
| 1.3 × 10<sup>7</sup> | 4 | 1 |
| 3.4 × 10<sup>7</sup> | 3 | 0 |
| 8.9 × 10<sup>7</sup> | 1 | 0 |

### Table 4. Fecal bacterial count at day –1 and days 1, 3, and 5 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS

| Day post-vaccination | Bacterial count/of feces (mean ± SEM) |
|----------------------|---------------------------------------|
|                      | **Ampicillin group** (n = 15) | **NS group** (n = 15) |
| −1                   | 9.3 × 10<sup>7</sup> ± 1.1 × 10<sup>8</sup> | 1.4 × 10<sup>8</sup> ± 2.9 × 10<sup>8</sup> | NS<sup>a</sup> |
| 1                    | 3.1 × 10<sup>7</sup> ± 7.5 × 10<sup>6</sup> | 1.2 × 10<sup>7</sup> ± 3.1 × 10<sup>6</sup> | <0.01 |
| 3                    | 2.5 × 10<sup>7</sup> ± 1.2 × 10<sup>6</sup> | 7.1 × 10<sup>6</sup> ± 3.1 × 10<sup>6</sup> | <0.01 |
| 5                    | 1.6 × 10<sup>7</sup> ± 5.0 × 10<sup>6</sup> | 4.0 × 10<sup>6</sup> ± 1.2 × 10<sup>6</sup> | <0.05 |

<sup>a</sup> NS, not significant.

### Table 5. Fecal Ty21a isolation on days 1, 2, and 3 after oral administration of Ty21a with pBR322 to mice treated with ampicillin or NS

| Day post-vaccination | No. of mice with feces positive for Ty21a |
|----------------------|------------------------------------------|
|                      | **Ampicillin group** (n = 15) | **NS group** (n = 15) |
| 1                    | 11 | 8 |
| 2                    | 6 | 4 |
| 3                    | 1 | 0 |

### Table 6. Total antitetanus antibody and antibody subtypes at days 7 and 21 after oral administration of Ty21a with pTETnr15 to mice treated with ampicillin or NS

| Day post-vaccination | Antibody subtype | Serum dilution | Antibody level (mean ± SEM absorbance) | P value |
|----------------------|------------------|----------------|----------------------------------------|---------|
|                      |                  | (n = 15)       | Ampicillin group | NS group |
| 7                    | Total            | 1:25           | 0.196 ± 0.055       | 0.181 ± 0.028 | NS<sup>a</sup> |
|                      | IgM              | 1:25           | 0.137 ± 0.024       | 0.148 ± 0.021 | NS<sup>a</sup> |
|                      | IgG1             | 1:25           | 0.191 ± 0.033       | 0.205 ± 0.024 | NS<sup>a</sup> |
|                      | IgG2a            | 1:25           | 0.350 ± 0.042       | 0.200 ± 0.045 | <0.05   |
| 21                   | Total            | 1:10,000       | 0.452 ± 0.115       | 0.440 ± 0.120 | NS<sup>a</sup> |
|                      | IgM              | 1:25           | 0.496 ± 0.085       | 0.365 ± 0.073 | NS<sup>a</sup> |
|                      | IgG1             | 1:25           | 0.631 ± 0.030       | 0.709 ± 0.057 | NS<sup>a</sup> |
|                      | IgG2a            | 1:25           | 0.634 ± 0.050       | 0.571 ± 0.031 | <0.005 |

<sup>a</sup> NS, not significant.
AMPCILLIN ENHANCES ANTIBODY RESPONSE

TABLE 7. Total anti-HBsAg antibody and antibody subtypes at days 7 and 21 after oral administration of Ty21a with pRe/CMV-HBs(S) to mice treated with ampicillin or NS

| Day post-vaccination | Antibody subtype | Antibody level (mean ± SEM absorbance) | P value |
|----------------------|------------------|----------------------------------------|---------|
|                      |                  | Ampicillin group | NS group |         |
|                      | Total            | 0.189 ± 0.023    | 0.111 ± 0.023 | <0.05 |
|                      | IgM              | 0.324 ± 0.047    | 0.126 ± 0.024 | <0.005|
|                      | IgG1             | 0.008 ± 0.001    | 0.009 ± 0.001 | NS† |
|                      | IgG2a            | 0.001 ± 0.001    | 0.002 ± 0.001 | NS† |
| 21                   | Total            | 0.227 ± 0.038    | 0.128 ± 0.016 | <0.05 |
|                      | IgM              | 0.396 ± 0.057    | 0.198 ± 0.020 | <0.01 |
|                      | IgG1             | 0.006 ± 0.001    | 0.007 ± 0.001 | NS† |
|                      | IgG2a            | 0.001 ± 0.001    | 0.002 ± 0.001 | NS† |

† NS, not significant.

clear protein of the virus and an S. enterica serovar Typhi-
murium effector protein carried in live-attenuated serovar Ty-
phinurium (13).

Ampicillin enhanced the immune response of oral Ty21a probably by giving it a survival advantage against the normal bacterial flora of the intestine. We showed that ampicillin significantly suppressed the normal flora of the intestine, resulting in a relatively higher rate of recovery of Ty21a from the feces on days 1, 2, and 3. This is in line with the M-cell sampling theory about antigen presentation in the mucosa of the gastrointestinal tract. It has been shown that S. enterica serovar Typhi cells adhere selectively to the M cells of mucosa-associated lymphoid tissue, which form the gateway of the mucosal immune system (1, 6). This induces engulfment of the bacteria by “macrophagosis.” The engulfed Salmonella will be presented to the T and B lymphocytes that form large intra-
epithelial pockets around the basolateral membranes of the M cells (11). The normal flora of the murine large intestine, which contains 10^8 bacteria per gram of feces, competes with Ty21a by occupying the mucosal adhesion sites, competing with Ty21a for nutrients, and secreting bacteriocins and bacterio-
clin-like substances. When ampicillin is administered, the norm-
mal flora is transiently suppressed 100-fold, giving Ty21a a survival advantage and a greater chance of presenting itself and the protein antigen or DNA vaccine carried in it to the M cells.

These observations have important applications for both prophylactic and therapeutic vaccinations. The problem of dis-
ease transmission through the use of reusable needles for im-
munization is of great concern in developing countries. Mucos-
al vaccination provides specific advantages in terms of ease of admin-
istration, vaccine formulation, and the potential to sup-
port mass vaccination (7). Besides the induction of an immune re-
response to the microorganism itself, live-attenuated bacteria that carry protein antigens are used to induce an immune response against the protein antigen carried in them (5, 13), and the type of immune response can be further tuned with the help of adjuvants. However, the protective efficacy of oral Ty21a in humans is only about 70%, and this is even worse in developing countries, where people suffer from environmental enteropathy, with their gastrointestinal tracts, especially their upper gastrointestinal tracts, being colonized with many more bacteria than those of people in developed countries (8, 12). Therefore, this concept of antibiotic enhancement of the im-
mune response might have a place in selective prophylactic vaccination programs, such as vaccination of nonresponders to routine immunization. Furthermore, DNA vaccination is consid-
ered a possible way for therapeutic vaccination, including the treatment of cancer. Therefore, the present observations may improve the therapeutic effect of DNA vaccines carried in live-attenuated bacteria, and further experiments should be carried out to determine the best antibiotics and dosage regi-
mens to be used, as well as the best carrier system for indi-
vidual protein antigens and DNA vaccines. However, with concerns about the increasing rate of antibiotic resistance worldwide, the incorporation of an antibiotic as part of a rou-
tine vaccination regimen is probably not warranted.

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