Susceptibility to Adjuvant-Induced Arthritis Among Germfree, Specific-Pathogen-Free, and Conventional Rats

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Germfree F344 rats developed severe arthritis with 100% incidence after a single intradermal injection of either squalane containing 0.5 mg of heat-killed Mycobacterium bovis BCG or a water-in-oil emulsion containing 0.2 mg of peptidoglycan derived from Staphylococcus epidermidis. Conventional F344 rats developed less-severe arthritis with 20% incidence for heat-killed BCG and 0% incidence for peptidoglycan. Specific-pathogen-free rats showed an intermediate susceptibility between germfree and conventional rats. Interestingly, both unimmunized specific-pathogen-free and conventional rats, but not unimmunized germfree rats, showed weak delayed-type hypersensitivity reactions to peptidoglycans derived from either S. epidermidis or Lactobacillus plantarum, suggesting that a bacterial flora may furnish a stimulus for induction of cell-mediated immunity to ubiquitous bacterial peptidoglycans. It is thus possible that although a bacterial flora is not necessary for development of adjuvant arthritis, it may have some suppressive effect on the development of the disease in specific-pathogen-free and conventional F344 rats, possibly through modulation of the immune response.

Adjuvant arthritis (AA) can be induced by a single injection of a water-in-oil emulsion containing mycobacterium (14), many other bacteria, and their cell walls (8, 11). A common factor among these bacterial cell walls responsible for promoting the development of AA was proved to be peptidoglycans (PGs) (12), which are found universally in all bacterial cell walls. This disease has been generally believed to be the result of a delayed-type hypersensitivity (DTH) response to components of bacterial cell walls (20), such as PGs (12). The precise mechanisms which underlie its development are still obscure. There is, however, a possibility that bacterial PGs can serve as an adjuvant to assist in the invasion or propagation or both of another infectious agent such as virus, mycoplasma, fungus, etc. (1, 17).

To pursue this matter, it seems very important to investigate whether or not germfree (GF) rats can develop AA. In this regard, Pearson et al. (15) reported that AA could be induced in GF and conventional (CV) rats with equal ease, a finding that suggests bacterial flora is not necessary for development of the disease. In this study Pearson et al. used high-responder rats, i.e., rats in which AA could be induced easily. In the present study we have extended the investigation to measure the effect of a bacterial flora on the development of AA in low-responder rats. In addition, we have determined the occurrence of DTH to PGs in animals developing AA.

MATERIALS AND METHODS

Animals. GF female F344 rats were obtained from Yakult Institute Microbiological Research, Kunitachi, Japan, and were maintained by continued brother and sister mating in plastic flexible isolators on sterile commerical diet and water ad libitum. Feces samples were cultured in thioglycylate broth and cooked meat broth and on potato dextrose agar for indication of contamination during experiments. All bacterial tests were negative before, during, and at the end of the present experiments.

SPF rats which were raised from the GF F344 rats were maintained and bred for several generations in the barrier system to be supplied for experiments. Female SPF rats at three generations were used in the present experiments. Female CV F344 rats were obtained from Charles River Breeding Laboratories, Atsugi City, Japan, in one experiment and from Yakult Institute in another experiment in which GF rats were conventionalized and then maintained and bred for several generations as CV F344 rats. Female Lewis rats were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. All the rats used were 8 to 10 weeks of age at the start of the present experiments.

Bacterial PGs. PGs tested were an oligosaccharide-peptide derived from Staphylococcus epidermidis (SEPS), which consists of three or four disaccharide units (repeating unit of N-acetylglucosaminyl-1,4-N-
acetylmuramic acid) and L-alanyl-D-isoglutaminyl-
mesodiaminopimeryl-D-alanine, and a disaccharide-
peptide derived from Lactobacillus plantarum which
consists of GlcNAc-MurNAc-L-Ala-d-isoGln-meso-
DAP-d-Ala. The preparation of these PGs is described
elsewhere in detail (5, 7, 13).

Induction of AA. PGs were dissolved in sterile
phosphate-buffered saline (PBS; 0.01 M, pH 7.2). A
water-in-oil emulsion was prepared by a dropwise
addition of PBS containing PG to an equal volume
of mineral oil (85% mineral oil and 15% Aracel A) to
make a final concentration of 0.2 mg per 0.05 ml of
the water-in-oil emulsion. Heat-killed Mycobacterium
bovis BCG was obtained through the courtesy of
the Fisheries and Food Central Veterinary, Weybridge,
Surrey, England. Ten milligrams of heat-killed BCG
was triturated well with mortar and pestle and thor-
oughly mixed with 1 ml of squalane instead of mineral
oil (9). To sterilize test materials, the oil vehicle was
autoclaved before preparing the water-in-oil emulsion.
BCG in squalane was also autoclaved.

A water-in-oil emulsion was prepared in the plastic
isolator, injected into GF rats, and then taken out of
the isolator and injected into SPF and CV rats. All the
other procedures were also carried out under the GF
condition. All the rats were injected intradermally
into a footpad with 0.05 ml of the sterile water-in-oil
emulsion.

Evaluation of AA. A visual scoring system was
used according to a previous report (21). In brief, after
inoculation, the rats were examined daily for 6 weeks
to evaluate time of onset day of arthritis and graded
from 0 to 4 for each appendage except for the injected
foot, according to the extent of the erythema, swelling,
and ankylosis of the periarticular tissue, including
the tail. In general, a visual arthritogram score below
4 was evaluated as mild transient arthritis; 5 to 10 was
moderate to severe; and 11 to 16 was very severe. The
concomitant lesions which were usually observed as
er nodule s and skin lesions (20) in the CV rats were
also examined carefully and recorded, but not included
in this visual scoring system.

Skin testing. Skin tests were performed 14 and 21
days after inoculation on a hair-plucked area of the
flank by intradermal injection with 0.1 ml of PBS
containing either 50 μg of PG or 25 μg of purified
protein derivatives (PPD), which were obtained from
Parke, Davis & Co. (47-2031-478). The diameter of
erythema or the induration of the skin test area or
both were carefully recorded at 6 h for immediate
reaction and at 24 and 48 h for DTH reaction. All
procedures were performed in the isolator for the GF
and SPF rats and in the animal room for the CV rats.
Skin test materials were sterilized by filtration through
a membrane filter (Millipore Corp., Bedford, Mass.),
por e size 0.22 μm.

RESULTS

Susceptibility to heat-killed BCG-induced
arthritis among GF, SPF, and CV F344 rats. All the rats were immunized in-
dermally into one footpad with 0.05 ml of squal-
lane containing 0.5 mg of heat-killed BCG. All
of the six GF rats developed severe to very
severe arthritis (Table 1). The clinical signs ap-
peared at 12 to 14 days, came up to a mean score
(11.8) of the highest arthritogram, and gradually
subsided, but still were apparent until rats were
sacrificed 120 days after inoculation. Four out of
six SPF rats also developed the disease with less
severity (mean score of 5.3). The clinical signs
appeared at 12 to 24 days, and most of the
lesions disappeared within 2 months. Only 2 out
of 10 CV rats developed mild disease. Lewis rats
as a positive control developed very severe dis-
ease with 100% incidence in the CV state.

Susceptibility to PG-induced arthritis
among GF, SPF, and CV F344 rats. All the rats
were immunized intradermally into one
footpad with 0.05 ml of water-in-oil emulsion
containing 0.2 mg of SEPS. All of the eight GF
F344 rats developed severe arthritis (Table 2).
The clinical signs appeared at 15 to 19 days
and came up to a mean score of 11.8, and they
were still apparent until rats were sacrificed at 120
days after inoculation. Two out of eight SPF
F344 rats developed very mild disease. CV F344

| Table 1. Susceptibility to BCG-induced arthritis among GF, SPF, and CV F344 rats |
|------------------------|-------|-----------------|-----------------|
| Immunization* | Incidence of AA | Onset day (mean) of AA | Severity of AA |
| F344 | | | |
| GF | 6/6 | 12–14 (13.0) | 11.8 |
| SPF | 4/6 | 12–24 (15.8) | 5.3 |
| CV | 2/10 | 11–14 (12.5) | 8.0 |
| Lewis | | | |
| CV | 6/6 | 9–11 (10.2) | 15.9 |

*All the rats were immunized intradermally into left hind footpad with 0.05 ml of squalane containing 0.5 mg of BCG.
Mean onset day was calculated by averaging the onset day of each rat per group.
This severity was calculated as an arithmetic mean of the highest score of each rat per group.

| Table 2. Susceptibility to PG-induced arthritis among GF, SPF, and CV F344 rats |
|------------------------|-------|-----------------|-----------------|
| Immunization* | Incidence of AA | Onset day (mean) of AA | Severity of AA |
| F344 | | | |
| GF | 8/8 | 15–19 (16.8) | 11.8 |
| SPF | 2/8 | 15–29 (22.0) | 3.5 |
| CV | 0/8 | | |
| Lewis | | | |
| CV | 8/8 | 11–12 (11.5) | 14.2 |

*All the rats were immunized intradermally into the left hind footpad with 0.05 ml of water-in-oil emulsion containing 0.2 mg of PG derived from S. epidermidis (oligomer of PG).
* See footnotes b and c of Table 1.
rats did not develop the disease, whereas CV Lewis rats developed severe disease with 100% incidence, and the clinical signs appeared at 11 to 12 days after inoculation.

**DTH to PGs among GF, SPF, and CV F344 rats after inoculation.** All the rats were skin tested with PPD and PGs 14 days after inoculation with either heat-killed BCG or oligosaccharide-peptide (oligomer of PG) derived from SEPS. After immunization with heat-killed BCG, three out of six GF rats developed weak DTH to both PPD and oligomer of PG, which were 9.5 ± 0.87 mm and 9.9 ± 1.7 mm, respectively, and the rest of GF rats did not develop any hypersensitivity (Table 3). The four normal GF rats did not show any hypersensitivity to both PPD and oligomer of PG. All of the six SPF rats developed DTH to both PPD and oligomer of PG, which were 11.5 ± 0.5 mm and 10.5 ± 0.7 mm, respectively.

After immunization with oligomer of PG, all of the eight GF rats developed DTH to monomer (disaccharide peptide) and oligomer of PG, which were 10.5 ± 1.1 mm and 11.5 ± 0.5 mm, respectively (Table 4). The four normal GF rats did not show any hypersensitivity. All of the eight SPF rats developed stronger DTHs than those of GF rats. Eight CV rats also developed DTHs similar to those of SPF rats. Interestingly, 10 normal unimmunized CV rats and six normal unimmunized SPF rats showed very weak but definite DTH to peptidoglycans. No immediate reaction was observed in any rats tested.

**TABLE 3. DTH to PPD and PGs in GF and SPF F344 rats immunized with BCG**

<table>
<thead>
<tr>
<th>Immunization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>48-h skin reaction&lt;sup&gt;b&lt;/sup&gt; (average ± standard error [mm])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence</td>
</tr>
<tr>
<td>GF</td>
<td></td>
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<tr>
<td>BCG</td>
<td>3/6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>None&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4/4</td>
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<tr>
<td>SPF</td>
<td>6/6</td>
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<tr>
<td>None&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ND</td>
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</tbody>
</table>

<sup>a</sup> All the rats were immunized intradermally into left hind footpad with 0.05 ml of squalane containing 0.5 mg of heat-killed BCG.<br>
<sup>b</sup> Skin tests were performed 14 days after immunization. Neg, Negative; ND, not done.<br>
<sup>c</sup> Oligosaccharide-peptide derived from *S. epidermidis* (see text).<br>
<sup>d</sup> Number of rats with positive skin tests/number of rats immunized with heat-killed BCG.<br>
<sup>e</sup> The negatives were not included in this calculated mean.<br>
<sup>f</sup> Unimmunized control.

**DISCUSSION**

The present study confirmed the report of Pearson et al. (15), in which GF rats developed AA and a bacterial flora was not necessary for development of arthritis. Although Pearson et al. (15) found little or no difference between GF and CV rats for development of AA, we chose a rather less susceptible strain of F344 rats for induction of AA and demonstrated that (i) GF rats developed severe arthritis with 100% incidence after immunization with either heat-killed BCG or an oligomer of PG derived from *S. epidermidis* and (ii) SPF and CV F344 rats developed very mild arthritis with very low incidence. The time of onset was slightly delayed in SPF and CV rats in comparison with that of GF rats. These observations were in contrast to those obtained by Pearson et al. (15). This discrepancy may result from the different susceptibility to AA in the different strains of rats used (12), because Pearson et al. (15) used relatively high-responder rats instead of the low-responder rats used here.

A genetic background between GF and SPF rats tested here must be expected to be identical because the rats have been raised from GF rats and maintained for several generations by brother and sister mating. It is thus possible that the different susceptibility to AA between GF and SPF rats results from a bacterial flora which may have a suppressive effect on the development of AA in SPF and CV F344 rats. Kayashima et al. (6) suggested a role of suppressor T cells in the low-responder rats for development

**TABLE 4. DTH to PGs in GF, SPF, and CV F344 rats immunized with oligomer of PG**

<table>
<thead>
<tr>
<th>Immunization&lt;sup&gt;a&lt;/sup&gt;</th>
<th>48-h skin reaction&lt;sup&gt;b&lt;/sup&gt; (average ± standard error [mm])</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Incidence</td>
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<tr>
<td>GF</td>
<td></td>
</tr>
<tr>
<td>Oligomer of PG</td>
<td>8/8</td>
</tr>
<tr>
<td>None&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4/4</td>
</tr>
<tr>
<td>SPF</td>
<td></td>
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<tr>
<td>Oligomer of PG</td>
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<td>None&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>CV</td>
<td></td>
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<tr>
<td>Oligomer of PG</td>
<td>8/8</td>
</tr>
<tr>
<td>None&lt;sup&gt;f&lt;/sup&gt;</td>
<td>10/10</td>
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</table>

<sup>a</sup> All the rats were immunized intradermally into left hind footpad with 0.05 ml of water-in-oil emulsion containing 0.2 mg of oligomer of PG.<br>
<sup>b</sup> Skin tests were performed 14 days after immunization. Neg, Negative.<br>
<sup>c</sup> Disaccharide-peptide derived from *L. plantarum* (see text).<br>
<sup>d</sup> SEPS (see text).<br>
<sup>f</sup> Unimmunized control.
of AA. The present findings thus suggest that suppressor T cells may be more dominant in the CV state than in the GF state of these low-responder F344 rats. It seems that a bacterial flora may modulate the development of the immune apparatus such as T-helper, T-suppressor, or B-cells or macrophages (4), which is under genetic control.

The present study also indicates that SPF rats developed stronger DTHs to PPD and PGs than those of GF rats. After immunization with heat-killed BCG, half of the GF rats did not show any hypersensitivity and the rest of them showed poor DTH. These observations may be associated with an immaturity of the immune system in GF animals (19) or with retardation in their immune response (18). On the other hand, oligomer of PG induced DTHs to PGs in all the GF rats comparable to those of CV rats (Table 4), suggesting that PG may be a very potent immunogen in the rats. Furthermore, it was an unexpected finding that normal unimmunized SPF and CV rats showed weak but definite DTHs to PGs, although normal unimmunized GF rats did not show any hypersensitivities to PGs. This is the first observation of the existence of DTH reactions to bacterial PGs in unimmunized rats. This is, however, not surprising because there are several reports about naturally occurring cell-mediated immunity to purified glycerol-teichoic acid antigen in guinea pigs (3) and about naturally occurring cellular and humoral immunity to teichoic acid in rats (2). Since PGs are found universally in all bacterial cell walls (16), it is quite possible that bacterial flora may stimulate or generate the observed DTH and may subsequently modulate the immune response to PGs and eventually participate in the decreased susceptibility to AA in CV and SPF F344 rats.

The comparison of the disease susceptibility between GF and CV rats, including SPF and GF rats, would provide very useful tools either in studying the pathogenesis of AA or in elucidating the modulation of the immune system by a bacterial flora.

LITERATURE CITED