Immunofluorescent Examination of the Nasal Mucous Membrane of Mouse in Prophylactic Trials against Sendai Virus

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Sendai virus infection was examined by the immunofluorescent method in the nasal mucous membrane (NMM) of the mice having received each of five kinds of prophylactic treatment by direct or contact exposure. Administration of interferon or poly I:C via the nasal route decreased in number the viral-antigen-positive cells in NMM in the postexposure period, and the latter treatment protected the lower respiratory organs from contagion. Immunoprophylaxis of the entire respiratory organs against contagion was nearly complete with 6 dosages of UV-inactivated vaccine via the nasal route, but not by administration of alginate adjuvant UV-vaccine via the percutaneous route or ether-inactivated vaccine via the nasal route. However, the adjuvant vaccine protected the lower respiratory organs against contagion. Except for one successful UV-vaccine group, the test-positive cells in NMM of the five vaccinated groups and control increased markedly till postexposure day (PED) 5 and 10 by direct and contact infection, respectively, and thereafter decreased slowly. Viral antigen of the trachea and lungs disappeared on PED 7 and 12 with the development of serum antibody after infection by the two methods. This investigation of viral-antigen-positive cells in the entire respiratory organs, particularly in NMM differentiated in topical immunity and the viral infection between the two regions.—Key words: Immunofluorescence, Nasal mucosa, Sendai virus.


Since Sendai virus was isolated from mouse lungs in 1958, studies on experimental infection with the virus of the trachea and lungs of the mouse have stressed the etiopathogenetic standpoint [1-3, 6, 9, 10].

Ecology of Sendai virus on the individual mouse level would not be understood without examination of the nasal mucous membrane (NMM) for the virus is transmitted by inhalation. Such topical infection has not sufficiently been investigated because of the anatomically complex structure.

Sendai virus infection of the respiratory organs was examined in this study, especially in NMM of the mouse. Several prophylactic trials by the immunofluorescent method were undertaken as the basic research for the study of the nasal immunity.

MATERIALS AND METHODS

Virus: Sendai virus, the Nagoya strain, was inoculated into the chorioallantoic cavity of 10-day-old chicken eggs and allowed to multiply for 3 days. The infective titer of the fluid was $10^{6.6}$ EID$_{50}$ per ml.

Interferon: Mouse interferon prepared by centrifugation and acid treatment from the brains of the mice inoculated with Japanese encephalitis virus, was obtained by the courtesy of Dr. Shimokata of the Faculty of Medicine, Nagoya University. The dose of 125 U (0.025 ml) was administered twice via the nasal route on day 3 and 1 before contact exposure.

Poly I:C (P-L Biochemical product contained 10% poly I:C by weight): A dose of 0.0625 mg (0.025 ml) of the product in a 0.25% suspension was given twice at a
days' interval via the nasal route, and the mice were exposed 2 days post-administration.

**Ether-inactivated vaccine:** The virus-infected allantoic fluid was mixed thoroughly with an equal volume of ether, and the inoculum was prepared by centrifugation and aeration. Its HA titer was 1:1,024. The inoculum was administered six times each with a dose of 0.025 ml at 2 or 3 days' intervals for 21 days via the nasal route, and viral exposure was effected one week after the final dosage.

**UV-inactivated vaccine:** Three milliliters of the viral suspension dialyzed against physiological saline was irradiated in a 9-cm Petri dish for 15 min at a distance of 30 cm from a UV lamp (15 watt), and ethyl mercury thiosalicylate was in 0.1%. Inactivation of the virus was confirmed by the HA test of the chorioallantoic fluid from the hatching eggs inoculated with the UV-rayed fluid. Its HA titer was 1:256. One group of 3-week-old mice was given 0.025 ml of the inoculum 6 times at 2 or 3 days' intervals via the nasal route for 21 days and exposed one week after vaccination. Another group of the same age was inoculated with a mixture of UV-inactivated vaccine with an equal volume of 4% sodium alginate adjuvant (Algivant: Colab laboratory, Inc.) once via the I.P. route and twice via the S.C. route in a dose of 0.2 ml and 0.1 ml, respectively, for 21 days, and exposed 1 week after vaccination.

**Specimens and fluorescent staining:** Two mice from each of the groups receiving the preceding inocula and two from the untreated control group were sacrificed intermittently from postexposure day (PED) 1, 3 or 5 to 24 depending on the severity of arteria axillaris for blood-collecting. After removing the facial skin, the ridge of the nasal bone was excised with scissors. NMM was througly scraped off with an ophthalmologic forceps, and smeared evenly three areas of 1.5 cm² on slide glasses together with various bones in the nasal cavity. The smears were dried in an incubator for 1 hr. Twenty to thirty sections of the trachea and lungs were cut longitudinally and intermittently on a freezing microtome to a thickness of 8 μm. These were fixed with absolute ethanol for 10 min at 21-23°C, followed by staining with a 10⁻² dilution of an immune rabbit serum having 2,000 HI titer against Sendai virus for 40 min and with fluorescein isothiocyanate conjugates of anti-rabbit-IgG goat serum for 50 min at the same temperature. Later, the specimens were mounted in buffered glycerin (pH 9.5), and examined at 200-times magnification under a fluorescence microscope with a dark-field condenser and by UV excitation. The specificity was confirmed with smears of NMM or sections of the trachea and lungs infected or uninfected with Sendai virus. Total viral-antigen-positive cells in NMM of two mice were counted in every examination and summed up with respect to every mouse group.

**Mice:** Four-week-old-mice (SPF, ICR, male) were used in this experiment, except for two UV-vaccine-administered groups immunized at 3-week old. They were kept isolated and given sterile food and water.

**Challenge:** a) Direct nasal challenge. The mice were anaesthetized by inhalation of ether and a dose of 10⁵.8 EID₅₀ (0.025 ml) of the virus was each inoculated intranasally with a microsyringe. b) Contact infection. The preceding infected mice were used as infectors; the two infected mice were intermingled with five uninfected mice in the contact infection trials.

**Detection of Sendai virus antibody:** The HI titers of the blood samples taken from the mice having received various treatments were determined by the microtitre technique before and after challenge exposure.

**RESULTS**

1. Nasal administration of interferon or poly I:C.
The results of contagion in the mice administered interferon via the nasal route are shown in Fig. 1. The viral-antigen-positive cells in NMM increased markedly in the mice killed on PED 12 and 10, and declined sharply on PED 15 and 12 in the treated group and untreated control, respectively. The former group showed a delayed peak in the number of the antigen-positive cells, and the peak counts were lower than that of the control. The viral antigen in the lower respiratory organs appeared from PED 5 to 10 in both groups, and did not manifest any evident difference in the detection rates and quantity of the cells between the groups. Development of serum HI titers of the treated group was a little late (on PED 18) than that of control mice (Table 4).

Results of contact infection in the poly I:C-administered mice are shown in Fig. 2. The observation period of the experimental group was from PED 3 to 24. The fluctuation in the number of antigen-positive cells in NMM was similar to that in the interferon group, but the peak in the number of antigen-positive cells was on PED 9, which accorded with that of control. The total number of the antigen-positive cells in NMM of the treated group was lower than that in the control group. The rates of the test-positive trachea and lungs PED 5 to 10 were 1/18 and 0/18 in the treated group and 5/18 and 3/18 in the control group, respectively. Serum HI titers
of the treated group developed later (on PED 15) than did the control mice (Table 4). Next, heavy viral infection with direct nasal challenge was attempted to the poly I:C-administered mouse group. The treated group showed a rapid increase in number of the positive cells in NMM at an early stage of infection (Fig. 2). However, the antigen-positive cells in NMM of the control mice were more numerous by 15,000 cells than those of the treated group. Besides, the treated group displayed fewer antigen-positive cells in the lower respiratory organs PED 1 and a smaller number of antigen-positive cells in the lungs, despite there was no difference in the detection rate from the control group. Serum HI titer developed PED 7 in all groups (Table 4). A few viral-antigen-positive cells were seen in the tracheas and/or the bronchi in two mice PED 7, and in the bronchus and/or bronchiolus in a mouse PED 10.

2. Administration of UV- or ether-inactivated vaccine.

Contact exposure: The results with each mouse group dosed with three inocula are shown in Table 1. The mouse group having received UV-inactivated vaccine via the nasal route was almost completely protected from infection in both NMM and the lower respiratory organs (Tables 1 and 3). The serum HI titer of the group reached 1:32-64 postvaccination, but no higher titer during PED. With control mice, the titer (1:8-256) developed on PED 12.

The ether-vaccine group, however, was not given sufficient protection in NMM (Table 1) nor developed serum of HI titer. The titer developed PED 12 also in the control group. No significant difference was seen in the infection of the lower respiratory organs among the groups (Tables 3 and 4).

The mouse group receiving UV-vaccine with alginate adjuvant via the percutaneous (I.P. and S.C.) route was provided a little prophylactic effect on NMM and the tracheas and complete protection of the lungs against contact infection (Tables 1 and 3). High HI titers of serum (1:32-64) developed in this group by vaccination, and produced higher titers (1:64-512) from PED 12 following contact exposure (Table 4).

The common findings in the three contact-exposed mouse groups including control, except for the group given nasal immunization with UV-vaccine, are as follows: the antigen-positive cells in the NMM rapidly increased in number till PED 10 and thereafter gradually decreased in a marked degree. The antigen in the lower respiratory organs disappeared PED 12 with the development of serum antibody (Table 4).

Direct heavy challenge to vaccinated

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Route</th>
<th>PED&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>17,20</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-inactivated vaccine</td>
<td>i.p.</td>
<td>0</td>
<td>447&lt;sup&gt;c)&lt;/sup&gt;</td>
<td>1,050</td>
<td>1,859</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>3,364</td>
<td></td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>8</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Ethereal-inactivated vaccine</td>
<td>i.n.</td>
<td>0</td>
<td>488</td>
<td>921</td>
<td>2,850</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>4,281</td>
<td></td>
</tr>
<tr>
<td>Control&lt;sup&gt;d)&lt;/sup&gt;</td>
<td>i.n.</td>
<td>0</td>
<td>381</td>
<td>859</td>
<td>3,798</td>
<td>30</td>
<td>2</td>
<td>0</td>
<td>5,070</td>
<td></td>
</tr>
</tbody>
</table>

a) Postexposure day.
b) Administration of vaccine with alginate adjuvant.
c) Total number of viral-antigen-positive cells in NMM of two mice.
d) Nonvaccinated.
NASAL INFECTION OF SENDAI VIRUS

Table 2. Numbers of Sendai viral-antigen-positive cells in the NMM of mice in prophylactic trials by direct viral administration

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Route</th>
<th>PED&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>UV-inactivated vaccine</td>
<td>i.p.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>638&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i.n.</td>
<td>763</td>
</tr>
<tr>
<td>Ether-inactivated vaccine</td>
<td>i.n.</td>
<td>934</td>
</tr>
<tr>
<td>Control&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td>1,078</td>
</tr>
</tbody>
</table>

<sup>a</sup> Postexposure day.
<sup>b</sup> Administration of vaccine with alginate adjuvant.
<sup>c</sup> Total number of viral-antigen-positive cells in NMM of two mice.
<sup>d</sup> Nonvaccinated.

Table 3. Appearances of Sendai viral antigen in the lower respiratory organs of mice in prophylactic trials

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Contact infection</th>
<th>Direct challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ether&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>i.p., s.c.</td>
<td>i.n.</td>
</tr>
<tr>
<td>Larynx</td>
<td>2/16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1/16</td>
</tr>
<tr>
<td></td>
<td>3/16</td>
<td>0/16</td>
</tr>
<tr>
<td>Lungs</td>
<td>0/16</td>
<td>1/16</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mouse group administered UV-inactivated vaccine.
<sup>b</sup> Mouse group administered ether-inactivated vaccine.
<sup>c</sup> Administration of UV-vaccine with alginate adjuvant via the I.P. and S.C. routes.
<sup>d</sup> Intranasal administration of vaccine.
<sup>e</sup> Number of mice with viral antigen/Number of mice examined.

**groups:** Heavy viral exposure of the three vaccinated mouse groups and control caused a marked increase in number of the antigen-positive cells in NMM up to PED 5, and showed a remarkable decrease subsequently (Table 2). Efficacy of the vaccination seemed to appear in NMM compared with unvaccinated control mice, but virtually no efficacy in the lower respiratory organs that became test-positive from PED 1 to 5-10 (Tables 3 and 4). UV-vaccine given via the nasal route also gave the highest protection among them shown by the least number of antigen-positive cells in NMM.

The serum HI titer became positive PED 8 in the ether-vaccine group and control. In the group given intranasally UV-vaccine, the titer increased (1:256-512) from PED 7 to 18 and was far higher than that of the contact-exposed UV-vaccine groups. The titers of the alginate adjuvant UV-vaccine group increased notably from 1:64-128 to 1:256-1,024 from PED 8 to 18 by the heavy viral challenge (Table 4). Most antigen-positive cells in the lower respiratory organs disappeared with the sudden rise in the titer as shown in contact infection.

**DISCUSSION**

Rise and fall in the number of viral-antigen-positive cells in NMM was a characteristic indication of Sendai virus infection, although differences among the infected lower respiratory organs were difficult to identify.
Table 4. Relation between the development of serum HI titers and disappearance of Sendai viral-antigen-positive cells in the trachea and/or lungs in mouse protection test

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Route</th>
<th>Contact infection</th>
<th>Direct challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 to 10 PED&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 to 20 PED</td>
</tr>
<tr>
<td>Interferon</td>
<td>i.n.</td>
<td>6/6&lt;sup&gt;b&lt;/sup&gt; (1:2-4)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0/8 (1:4-64)</td>
</tr>
<tr>
<td>Poly I/C</td>
<td>i.n.</td>
<td>4/6 (1:2-4)</td>
<td>14/10 (1:4-128)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>UV-vaccine</td>
<td>i.p./s.c.</td>
<td>4/6 (1:32-64)</td>
<td>0/8 (1:64-512)</td>
</tr>
<tr>
<td>UV-vaccine</td>
<td>i.n.</td>
<td>0/6 (1:32-64)</td>
<td>1/8 (1:32-64)</td>
</tr>
<tr>
<td>Ether-vaccine</td>
<td>i.n.</td>
<td>5/6 (1:4)</td>
<td>1/8 (1:8-256)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>6/6 (1:4)</td>
<td>0/8 (1:8-256)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Postexposure day
<sup>b</sup> Number of mice with viral antigen in the trachea and/or lungs/number of mice examined.
<sup>c</sup> Reciprocal number of serum HI titer against Sendai virus postexposure period.
<sup>d</sup> Number of mice with viral antigen in the trachea and/or lungs/number of mice examined.
<sup>e</sup> Observation period was 24 days.
<sup>f</sup> Traces of viral antigen in the tracheas and/or lungs on PED 7 and 9.
<sup>g</sup> One or two viral-antigen-positive cells in the larynx and lungs on PED 12.
<sup>h</sup> Only one antigen-positive cell in the tracheal mucous membrane of each mouse on PED 7, 10 (UV-vaccine) and 12 (Ether-vaccine).
<sup>i</sup> Alginate adjuvant was used together with UV-vaccine.
<sup>j</sup> Not examined.

by the immunofluorescent method.

UV-inactivated vaccine given through different routes gave antipodal results: non-prophylaxis in NMM by percutaneous vaccination and reinforcement of the topical immunity by nasal vaccination, with development of serum antibody titers in both groups. Many workers have reported similar findings on immunization with inactivated influenza virus and rhinovirus [11, 13].

The present author reported previously that persistent infection of NMM with Sendai virus became more conspicuous with the mice dosed subcutaneously with immunosuppressants (cortisone and cyclophosphamide). [7, 8]. Judging from these experimental findings, observation of the appearance and fluctuations of Sendai viral-antigen-positive cells in the NMM provides an expedient method for rapid diagnosis and estimation of nasal immunity in mice.

Most patients developed simultaneously secretory antibody in NMM and the serum antibody after influenza virus infection. The protective effect of each antibody is unclear, and their synergic protective action is supposed at present [5].

Shore et al. [12] postulated that the inhibition of influenza virus by subcutaneous vaccination is due to a high ratio of IgG to IgA in the lower respiratory organs. It is generally accepted that the efficacy depends on the development of serum antibody to more than 1:32-64 of HI titers.

Blandford and Heath [3] reported disappearance of the viral antigen by coating the secretory antibody in Sendai virus-infected tracheas of mice. They [4] also recognized that the rate of increase in number of IgA-containing cells in the bronchial submucosa of Sendai virus-infected mice was not as high as that of IgG-containing cells.

In the present results, a characteristic of Sendai virus infection of NMM of mice was observed separately from the lower respiratory organs greatly influenced by antibody production in the blood. Nonspecific resistance and immunity in NMM seem to be difficult to obtain at the present time. It is desirable that the vaccination against Sendai
virus should be conducted aiming at prophylaxis in the entire respiratory organs. Development of the antibody to an efficacious level in both NMM and blood must therefore be induced through nasal vaccination, if an inactivated Sendai virus is applied for vaccine (unpublished observation).

REFERENCES


