Immuno-effect of Serum and Nasal Antibody against Experimental Inoculation with Influenza A-Equi-2 Virus

Takeshi KUMANOMIDO* and Yutaka AKIYAMA*

Thirty-four horses were inoculated intranasally with influenza A-Equi-2 virus to establish a useful guide of vaccine for protective efficacy on horses. Determination of infection was evaluated only serologically by a significant rise in hemagglutination-inhibition (HI) antibody two weeks after virus inoculation.

The following results were obtained. Twenty-two horses with a serum HI titer of less than 1:32 were all susceptible to infection. Four of five horses with a serum HI titer of 1:64 and seven horses with a serum HI titer of 1:128 or more were resistant to infection, judging from serological findings.

A serum HI titer of 1:128 or more was thought to be an efficient indicator for vaccination on equine influenza. In this study, the 50% protective dose (PD50) was expressed as a serum HI titer of approximately 1:54.

Detectable nasal antibody was presumed to play an important role against infection. It was impossible, however, to indicate the level of nasal secretory antibody as clearly as that of serum antibody.

Introduction

For viral respiratory disease, there have been many reports stressing the significance of local immunity. Nasal antibody has played a great role in the protection against exposure.1-7 It was found that human beings who had low level or no antibody in serum often escaped infection. Furthermore, in man, live virus vaccine has been developed to confer efficient local immunity to influenza infection, since inactivated virus vaccine induced poor local immunity.5

In horses, the epidemic of influenza has not so frequently been observed as in human beings whom it attacks every year. Equine influenza has mostly occurred unexpectedly among horses with no history of vaccination or infection. It was reported8 that regular vaccination had an efficiency to prevent horses from natural infection.

It will, however, be difficult to decide practically whether a horse is infected or resistant, even if the levels of antibody in serum and nasal secretion are provided. In experimental infection in horses, the critical analysis of vaccination efficacy
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is especially impeded by the fact that it is impossible to induce the same clinical signs as those of natural infection.

This investigation was carried out to determine serologically the efficient levels of serum and nasal antibody against experimentally infected with influenza A-Equi-2 virus in order to draw a useful guide line for vaccination program.

Materials and Methods

Experimental animals Twenty-nine horses (Anglo-Arab and half-bred) were divided into three groups. Group A of 6 horses had no history of exposure to A-Equi-2 virus or vaccination. Group B of 12 horses showed hemagglutination inhibition (HI) antibody titers of less than 1:32 in serum before inoculation. It had, however, previously been vaccinated against, or experimentally infected with, influenza A-Equi-2 virus. Group C of 11 horses exhibited various antibody levels in serum and nasal secretion by experimental infection or vaccination prior to exposure. Five horses of group C were re-inoculated against the same virus seven months after the first inoculation.

Virus inoculation Each horse was infected intranasally with 1.0 ml of alantoic fluid containing approximately 107.5/0.2 ml (EID$_{50}$) of influenza A/Equi 2/Tokyo/71 (Heq2 Neq2) virus.

Serological tests Following virus inoculation, undiluted nasal secretion was collected at intervals by the improved method of Kumanomido and Akiyama. Nasal washings were also collected at intervals and concentrated to approximately one-fortieth of the volume in order to be compared with the titer of undiluted nasal secretion mentioned above. All of them were examined for occult blood with hematest tablets and stored at $-20^\circ$C until use.

HI test Serum dilutions were prepared on a plastic plate with a 0.2 ml system. The antigen used for the HI test had been purchased from the Nippon Institute for Biological Science, Tachikawa, Tokyo. It was prestige material for the preparation of inactivated vaccine.

The virus neutralizing test of the nasal secretion was described previously. The titer of neutralizing antibody was estimated from the presence of hemagglutinin using the method of Behrens-Kärber.

Virus isolation was not performed in this investigation. The evidence of infection was estimated only by serological findings; that is, a fourfold rise in HI antibody titer two weeks after inoculation.

Results

1. Antibody response

Fig. 1 shows the response of antibody in serum HI and nasal neutralizing titer

![Fig. 1. Serum and nasal secretory antibody responses to experimental infection in group A](image)

Ordinate, left: Reciprocal serum HI antibody titer.
right: Nasal neutralizing antibody titer ($-\log 2$).
Abscissa: Time in days after inoculation.
Remarks. --- : Serum HI antibody response.
• : Reciprocal mean of nasal neutralizing antibody response.
The titers ranged from maximum to minimum.
of group A, which had experienced neither infection nor vaccination prior to virus inoculation. The titers of antibody response after virus inoculation showed a tendency to be lower than those induced by natural infection. The HI titer ranged from 1:8 to 1:64 two weeks after virus inoculation and showed a tendency to decrease three months later.

Fig. 2 shows the response of antibody in group B, which had experienced vaccination or infection approximately one year or more before examination. All the animals, except two, of this group had a low HI antibody titer of less than 1:32, without secretory antibody. They were also susceptible to virus inoculation and exhibited a fourfold rise in serum HI antibody titer which ranged from 1:64 to 1:512 two weeks after virus inoculation. The HI titers in this group persisted at almost the same level up to three months after inoculation.

Nasal secretory neutralizing antibody produced following infection presented such responses as parallel to those of HI antibody.

Fig. 3 shows the response of antibody in group C, which had also experienced vaccination or experimental infection.

Fig. 2. Serum and nasal secretory antibody responses to experimental infection in group B
Remarks. See Fig. 1.

Fig. 3. Serum and nasal secretory antibody responses to experimental infection in group C
Remarks.
- : Nasal neutralizing antibody collected by sponge method.
- : (by nasal washing collection)
- : Below the detectable antibody level of 1:0.5 (−log 2).
For other remarks see Fig. 1.
# Immuno-effect against Equine Influenza A-Equi-2

## Table 1. Results of experimental infection with influenza A-Equi-2 virus

<table>
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<tr>
<th>Group</th>
<th>Horse No.</th>
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<th>Post-inoculation</th>
<th>Serological findings**</th>
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Remarks.  
* Reciprocals of serum HI and nasal neutralizing (NT) antibody titer.  
** Significant rise; fourfold rise of serum HI antibody titer (+); no significant rise (±); no response (−).
prior to examination. There were various levels of HI and nasal neutralizing antibody prior to inoculation. In the first inoculation of this group, four horses, Nos. 19, 20, 22 and 25, with a serum HI titer of less than 1:32 were susceptible to infection. One horse, No. 26, which possessed a serum HI titer of 1:64, was also susceptible. The other horses, Nos. 21, 23, 24, 27, 28 and 29, which had a serum HI titer of 1:64 or more, exhibited no responses, proved to be resistant to infection.

In the secondary inoculation of group C, five horses, Nos. 19 to 23, which possessed a serum HI titer of 1:64 or more 210 days after the first inoculation, revealed no responses, demonstrated also to be resistant to infection.

The nasal secretory neutralizing antibody titers of four horses, Nos. 19 to 22, were at a level of approximately 1:18 (−4.17log2) to 1:180 (−7.5log2) two weeks after the first inoculation. They remained almost at the same level 210 days after inoculation. There were no horses, except No. 25, of group C which had no antibody in the nasal secretion collected by the sponge method prior to examination. On the other hand, all the remaining seven horses, except No. 22, which possessed detectable neutralizing antibody in the nasal secretion collected by nasal washing prior to inoculation were resistant to infection.

Table 1 gives the result of experimental infection. It demonstrates the serum and nasal secretory antibody titer pre- and post-inoculation and shows the evidence of infection by serological findings; that is, a fourfold rise in serum HI antibody response.

2. Immuno-effect against infection

Fig. 4 shows the level of HI antibody titer at the time of inoculation. In it, each horse is represented by one square. Whether a horse was susceptible or resistant to infection was determined by the significance of serum antibody response; that is, a fourfold rise in HI antibody response two weeks after virus inoculation.

It was examined about total thirty-four horses which were inoculated influenza A-Equi-2 virus. Twenty-two horses with a serum HI antibody titer of less than 1:32 pre-inoculation were all susceptible to infection. Four of five horses with a serum titer of 1:64 at the time of inoculation and seven horses possessing a serum titer of

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Fig. 4. Immuno-effect of serum HI antibody level at the time of inoculation with influenza A-Equi-2 virus

Remarks. ■ : Represents one horse.

PD_{50} is expressed as the titer of a half maximum on the infection rate.

Ordinate: Reciprocal serum HI antibody titer.
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1:128 or more were also resistant to infection, as judged from serological findings.

In this study, the 50% protective dose (PD₅₀), which was determined by serological findings and expressed as half the maximum of the infection rate, was approximately the HI titer of 1:54 pre-inoculation.

Fig. 5 shows the level of nasal secretory neutralizing antibody titer pre-inoculation. Whether a horse was susceptible or resistant to infection was also determined by a significant rise in serum HI antibody response.

Fifteen horses which had lacked nasal neutralizing antibody pre-inoculation were all infected with this virus. Three horses which possessed detectable neutralizing antibody in nasal secretion collected by the sponge method were also susceptible to infection. Two of five horses which showed a nasal neutralizing antibody titer of approximately 1:2 (−1.5 log₂) and one of three horses which presented a titer of approximately 1:4 (−2.5 log₂) were susceptible to infection. The other six horses which possessed a nasal antibody titer of approximately 1:8 (−3.17 log₂) or more were resistant to infection, as judged from serological findings.

Discussion

This investigation was carried out to set up a useful guide of protective efficacy on horses which have acquired immunity by vaccination, by determining an antibody level at which a horse would be infected or resistant. It is difficult to produce such experimental infection in a horse as giving rise to clinical signs similar to those of natural infection. Therefore, in this report, positive infection was judged only from serological findings; that is, a four-fold rise in serum HI antibody titer after virus inoculation.

Twenty-two horses which possessed an HI antibody titer of less than 1:32 were susceptible to infection. Four of five horses which possessed an HI titer of 1:64 and seven horses which had a titer of 1:128 or higher were resistant to infection. From these results, the HI titer of 1:128 would be needed to provide protective efficacy to infection.

In this investigation, the number of experimental animals was limited. Nevertheless, the 50% protective dose (PD₅₀) of HI antibody was at the level of approximately 1:54. Hobson et al., who had administrated live vaccine to human beings indicated that the PD₅₀ of the influenza A₂ and Hong Kong strain was approximately in the order of 1:36.

According to Bryans et al. there was no evidence of infection in horses with a serum titer of 1:40 or more during an
epidemic of influenza A-Equi-1. Rouse and Ditchfield\(^9\) performed further investigation on experimental infection of ponies with influenza A-Equi-2 virus. They found that no ponies with a serum HI titer of 1:80 or more had been positive for infection. In their investigation, the PD\(_{50}\) was an HI titer of 1:56 in twenty-five ponies.

The critical determination of infection was based on virus excretion and presence of illness. The serological finding, however, as described by Tremonti et al.,\(^{14}\) was presumed to be the most sensitive indicator of infection. In this respect, it seems that in the horse a serum HI antibody titer of 1:128 may be a level high enough to indicate protective efficacy on vaccination. The results mentioned above are almost the same as those described by Rouse and Ditchfield.\(^9\)

Concerning influenza virus, further investigation is needed on a variant type which may appear. In England, Powell et al.\(^8\) reported that horses vaccinated regularly were resistant to infection, whereas the epidemic strain was somewhat different in hemagglutinin from the A-Equi-1 subtype strain. In this respect, the protective efficacy of vaccination would be estimated in advance.

With regard to the viral respiratory disease in man, Smith et al.\(^4\) mentioned from their experimental infection with parainfluenza type I virus that either protection or infection was closely related to the presence of nasal antibody. On the other hand, the level of antibody in serum was not so clearly indicated as the level of it in nasal secretion for protective efficacy. In their study,\(^4\) however, no virus isolation was demonstrated in any individual who possessed an antibody titer of 1:128 or high. Furthermore, Tremonti et al.\(^{14}\) reproduced experimental infection with parainfluenza type II virus. They found that there was an interrelationship between the antibody level in serum and that in nasal secretion in the prevention of infection to some extent, and that some of the human beings who possessed a low neutralizing antibody titer of 1:8 in serum had been infected at challenge in spite of the presence of nasal antibody. They also pointed out that some of human beings who possessed a high neutralizing antibody titer of 1:16 or greater in serum had been infected in the absence of antibody in nasal secretion. From these results, it is presumed that a sufficiently high level of antibody in serum may have played an efficient role in the prevention of infection.

Influenza virus usually damages the respiratory mucosa, according to Hers.\(^{15}\) It is reasonable to assume that antibody in nasal secretion may have a direct effect to protect human beings and animals from infection. The mechanism of nasal secretion, however, has not been clarified. It is impossible to overlook the importance of serum antibody, which may be concerned indirectly with the protection.

The level of nasal antibody was higher when determined by the sponge collection method than when determined by the nasal washing collection method. This is probably because that nasal secretion by the sponge collection was constrained to discharge to some extent.

The antibody level of nasal secretion determined by the washing collection method, however, was irregular, whereas that determined by the sponge collection
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method was found to remain constant even almost seven months after experimental infection with the live virus influenza A-Equi-2.

No horses were negative for antibody in nasal secretion in this investigation. Notwithstanding, they possessed a high serum antibody titer of 1:128 or more.

In this investigation, the level of detectable nasal antibody was thought to play a great role against infection. A serum HI antibody titer of 1:128 or more was also thought to be high enough for a useful guide of serological findings for equine influenza vaccination. These results were similar to those reported by Rouse and Ditchfield.9)

Acknowledgments

The authors wish to thank Miss E. Kobori, of the Tochigi Branch Laboratory, Equine Health Laboratory, Japan Racing Association, for her technical assistance.

Literature Cited
ウマインフルエンザ A-Equi-2 ウィルスの実験感染による血清および鼻汁抗体の免疫効果について

熊塚御堂 憲*・秋山 綾*

ウマインフルエンザ免疫馬における感染防禦効果を調べる目的でインフルエンザ A-Equi-2 型ウイルスをのべ 34 例の馬に鼻腔内噴霧した。

ウィルス感染による反応は血清学的に回復期血清の赤血球凝集抑制（H1）抗体の上昇によって判定した。

その結果、ウィルス感染時に 1:32 H1価以下の抗体を保有する 22 例はすべて感染し、いずれも回復期血清において有意な抗体の上昇が認められた。1:64 H1価の抗体保有馬 5 例中 4 例および 1:128 H1価以上の抗体保有馬 7 例は血清学的所見において感染を証明出来なかった。

このことからウマインフルエンザワクチン接種計画において血清H1価 1:128 は有効な指標であると考えられる。

証明される程度の鼻汁抗体は感染防禦に対して大きな役割を果たしていると考えられるが、鼻汁分泌抗体レベルは血清抗体レベルほど明確に示することは出来なかった。

昭和50年5月31日受付
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