

Studies with inactivated equine influenza vaccine

2. Protection against experimental infection with influenza virus

A/equine/Newmarket/79 (H3N8)

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SUMMARY

Forty ponies immunized with inactivated virus vaccine containing A/equine/Miami/63 (H3N8) virus and six unvaccinated, seronegative ponies were experimentally challenged with a representative of recent equine H3N8 virus isolates, A/equine/Newmarket/79. All unvaccinated ponies became infected as judged by virus excretion, febrile responses and antibody responses, but only two of the vaccinated ponies were fully protected. Pre-challenge antibody levels to A/Newmarket/79 virus detected by single radial haemolysis (SRH) correlated well with the degree of clinical protection but the levels required for complete protection (SRH zones > 65 mm²) were high. The importance of these results in relation to conventional vaccination procedures against equine influenza is discussed.

INTRODUCTION

In 1979 a widespread outbreak of equine influenza, caused by an H3N8 strain, occurred in Europe (Klingeborn, Rockborn & Dinter, 1980; van Oirschot *et al.* 1981). During the outbreak some horses became infected despite being vaccinated with A/equine/Miami/63 (H3N8) virus antigens 3–12 months previously (Burrows *et al.* 1982, Mumford, unpublished observations). Isolates of the H3N8 subtype recovered from horses during 1979 have been shown to be variants of the reference influenza virus strain A/equine/Miami/63 (Hinshaw *et al.* 1982) and antigenically close to A/equine/Fontainebleau/1/79 designated as a new prototype of H3N8 strains (WHO, 1982).

In the present study, ponies vaccinated with A/equine/Miami/63 (H3N8) antigen, were experimentally challenged with a recent virus isolate, A/equine/Newmarket/79 (H3N8). The relationship between pre-challenge antibody to A/Newmarket/79 virus detected by single radial haemolysis (SRH) and protection was assessed by measurement of the antibody response to infection, pyrexia and virus excretion.

MATERIALS AND METHODS

Challenge virus

The equine influenza virus used for the challenge study was isolated from a vaccinated thoroughbred horse in Newmarket during the 1979 outbreak of equine influenza in Britain. This isolate was antigenically close to A/Fontainebleu/1/79 (H3N8) designated as the new prototype of H3N8 strains (A. Douglas, personal communication). The challenge inoculum of A/equine/Newmarket/79 was the fourth egg passage and contained 10^8 EID₅₀/ml of allantoic fluid; 5 ml of undiluted virus were administered intranasally to each pony. This amount of virus, has previously been shown to be an effective challenge for horses (Rouse & Ditchfield, 1970; Blaskovic *et al.* 1966).

Vaccines

The vaccines used in this study have been described in the accompanying paper (Wood *et al.* 1983*b*). They were conventional bivalent, whole virus inactivated vaccines containing graded concentrations of A/equine/Prague/56 (H7N7) and A/equine/Miami/63 (H3N8) viruses, of potencies ranging from 5 to 50 μ g of haemagglutinin (HA) per strain per dose.

Serological tests

The HI and SRH tests and the analysis of strain specific (SS) and cross reactive (CR) antibody to H3N8 strains in pre- and post-challenge sera have been described previously (Wood *et al.* 1983*b*).

Study design

This challenge study was carried out in Welsh mountain yearling ponies. Details of the serological responses of these ponies to graded doses of vaccines are presented in an accompanying paper (Wood *et al.* 1983*b*). One group of 20 ponies received a single dose of vaccine and a second group of 20 received two doses of vaccine administered with a 4-week interval between the two doses. Each group of 20 ponies was further divided into four subgroups which received different vaccines containing 5, 15 or 50 μ g HA per dose or an adjuvanted vaccine (adjuvant code name PD) containing 15 μ g HA per dose (Wood *et al.* 1983*a*). During the month prior to challenge one pony from each sub-group was given an additional dose of aqueous vaccine containing sufficient antigen to boost antibody titres and ensure that some individuals in the group had high levels of antibody at the time of challenge. Six unvaccinated seronegative ponies were included in the study as controls.

The ponies were challenged intranasally with A/equine/Newmarket/79 virus 22 weeks after the first dose of vaccine. Serum samples collected on the day of challenge and 18 days later were tested for SRH and HI antibody. Rectal temperatures were measured on the day of challenge and 2, 4, 5, 6, 7 and 10 days after challenge at approximately the same time each afternoon to minimize fluctuations due to normal diurnal variation. Nasopharyngeal swabs were taken 2, 3, 4, 5, 6, 7 and 10 days after challenge.

Virus isolation

The swabs were maintained in virus transport medium (Burrows, 1968) (VTM) on ice during transport from the isolation facilities to the laboratory. All swabs were frozen at -70°C prior to virus isolation attempts. Exudate from each swab was diluted 1:10 and 1:100 in VTM and 0.1 ml aliquots from undiluted and diluted samples were inoculated into the allantoic cavity of 10-day-old embryonated hens' eggs (3 eggs per sample). Allantoic fluid from the three eggs was harvested and pooled after 48 hours incubation at 35°C before testing for haemagglutinating activity.

*Analysis of results**Pony groups*

At the time of challenge, 22 weeks after the first dose of vaccine, antibody levels had declined such that differences between the original vaccine groups were small. For this reason, results of the challenge study were analysed in relation to pre-challenge antibody levels (Table 1) rather than vaccine groups described in the previous paper (Wood *et al.* 1983*b*). The ponies were divided into three groups based on the level of SRH antibody to A/Newmarket/79 present in the pre-challenge serum (Table 1). Group 1 contained six seronegative (no detectable SRH zone with undiluted serum) unvaccinated control ponies; group 2 contained 24 seronegative ponies, 16 had received one dose, seven had received two doses and one had received three doses of vaccine. Group 3 contained 16 ponies with detectable (SRH zone $\geq 4\text{ mm}^2$) pre-challenge SRH antibody levels, one of those ponies had received one dose, 12 had received two doses and three had received three doses of vaccine.

Temperature responses

Temperatures $\geq 38.9^{\circ}\text{C}$ were regarded as abnormal for the purpose of calculating the number of significant temperatures and duration of febrile responses. For each group the following expressions were calculated:

mean significant temperature response

$$= \frac{\text{the sum of the means of significant temperatures for each pony}}{\text{no. of ponies with significant temperatures}},$$

mean duration of temperature response

$$= \frac{\text{no. of days with significant temperature for each pony}}{\text{no. of ponies with significant temperatures}}.$$

Virus excretion

Duration of virus excretion was calculated from virus recovered after a single passage of swab extract in the allantoic cavity of 10-day-old fertile hens' eggs. The level of virus excretion was estimated on the basis of presence of virus in neat, 1:10 and 1:100 dilutions of swab extract.

Statistical analysis

The significance of the differences between febrile responses and virus excretion in the pony groups was assessed by Student's *t*-test. In comparisons of SRH

antibody levels to A/Miami/63 and A/Newmarket/79 viruses, the significance of differences was assessed by comparing the ratios of SRH titres to 1 and analysing the log ratios using Student's *t*-test.

RESULTS

Protection against experimental infection with an H3N8 variant A/equine/Newmarket/79 provided by bivalent vaccines containing the prototype strain A/equine/Miami/63 (H3N8) was assessed by measurement of SRH antibody responses, rectal temperatures and virus excretion following challenge.

Febrile responses

Table 1 shows febrile responses to challenge with the groups of ponies arranged according to pre-challenge SRH antibody levels to A/Newmarket/79 virus. All six seronegative control ponies (group 1) developed febrile responses, whereas 22 out of 24 vaccinated seronegative ponies (group 2) and 10 out of 16 vaccinated seropositive ponies (group 3) did so. There were no significant differences in the means of either the magnitude or the duration of febrile responses when vaccinated seronegative ponies (group 2) were compared with seronegative control ponies (group 1). Where pre-challenge antibody was detectable (group 3), the duration but not the magnitude of febrile responses was significantly ($P < 0.01$) lower than that in seronegative vaccinated ponies (group 2).

Virus excretion

All six control ponies (group 1), 23 out of 24 group 2 ponies and 14 out of 16 group 3 ponies excreted virus after challenge (Table 1). The mean duration of virus excretion in unvaccinated seronegative ponies (group 1) was significantly ($P < 0.01$) longer than that in the vaccinated seronegative ponies (group 2). Similarly, the mean duration of virus excretion in vaccinated seronegative ponies (group 2) was significantly ($P < 0.01$) longer than that in the vaccinated seropositive ponies (group 3). The amount of virus excreted did not differ between the two groups of vaccinated ponies (2 and 3) but there was significantly ($P < 0.01$) more virus excreted by the controls (group 1) than by the seronegative vaccinated ponies (group 2). Virus titres of $\geq 10^{2.5}$ EID₅₀/0.1 ml were detected in nasal secretions from all six seronegative control ponies for at least 4 days whereas similar levels of virus excretion were detected in only 11 out of 24 seronegative vaccinated ponies and for no more than 2 days.

Serological responses

(a) SRH antibody levels

Table 2 shows SRH antibody responses to challenge in the three groups of ponies.

All six control ponies (group 1) developed significant SRH antibody rises to both A/Miami/63 and A/Newmarket/79 strains after challenge with A/Newmarket/79 virus. Similarly, all 24 seronegative vaccinated ponies (group 2) developed significant antibody responses to both virus strains. In group 3, 14 out of 16 seropositive vaccinated ponies responded to both strains. The two ponies in which no significant antibody response could be demonstrated, had pre-challenge SRH

Table 1. *Temperature responses and virus excretion of ponies after challenge with A/Newmarket/79 virus*

Description	Pony group		Temperature responses			Virus excretion	
	No.	Pre-challenge New/79 SRH antibody (mm ²)	No. of significant responses (%)	Mean duration (days)	Mean significant temperature (°C)	No. excreting virus (%)	Mean duration (days)
(1) Unvaccinated	6	< 4*	6 (100)	3.0	39.4	6 (100)	5.0
(2) Vaccinated	24	< 4	22 (92)	3.5	39.6	23 (96)	3.3
(3) Vaccinated	16	≥ 4	10 (62)	2.1	39.4	14 (87)	2.1

* Minimum detectable SRH zone size = 4 mm².
 † Significant temperature response is an increase in temperature to 38.9 °C or above.

Table 2. *SRH antibody responses of ponies to challenge with A/Newmarket/79 virus*

Description	Pony group		New/79 SRH antibody		Miami/63 SRH antibody	
	No.	Pre-challenge New/79 SRH antibody (mm ²)	Pre-challenge Mean titre (mm ²)	No. of responses†	Pre-challenge Mean titre (mm ²)	No. of Responses†
1 Unvaccinated	6	< 4*	0	6	0	6
2 Vaccinated	24	< 4	0	24	3.4	24
3 Vaccinated	16	≥ 4	26.2	14	41.1	14

* Minimum detectable SRH zone size = 4 mm².
 † Significant antibody response is an increase of 50% in SRH zone area.

Table 3. *Specificity of antibody to equine-2 influenza virus in ponies before and after challenge with A/Newmarket/79 virus*

Vaccination status of ponies	No. of sera with stated antibody specificity					CR + MSS Clinical infection
	Pre-challenge*	Post-challenge				
		CR + MSS†	CR only	CR + MSS	CR + MSS‡	
Vaccinated	23/23	14/23	7/23	1/23	1/23	21/23§
Un-vaccinated	Seronegative	0/4	0/4	4/4	0/4	4/4

* Tested 17 weeks before challenge.

† MSS = strain specific antibody against A/Miami/63 virus.

‡ NSS = strain specific antibody against A/Newmarket/79 virus.

§ Post-challenge antibody status of two immune ponies: (1) CR only; (2) CR + MSS.

antibody levels of $> 70 \text{ mm}^2$ and $> 65 \text{ mm}^2$ and HI titres of 32 and < 8 to A/Miami/63 and A/Newmarket/79 respectively.

Antibody responses to challenge in the unvaccinated control ponies were very weak, when measured with the HI test. However, the number of vaccinated ponies responding was the same whether measured by SRH or HI tests.

(b) *Specificity*

In those ponies in which SRH antibody to either strain was detectable before challenge (six ponies in group 2, 16 ponies in group 3), there was significantly ($P < 0.05$) more SRH antibody to A/Miami/63 virus than to A/Newmarket/79 virus (Table 2). This was also the case in all the vaccinated ponies (groups 2 and 3) after challenge (significant at $P < 0.01$). However in the unvaccinated controls (group 1) more antibody to A/Newmarket/79 virus than antibody to A/Miami/63 was detectable after challenge (significant at $P < 0.05$). In order to examine the antigenic specificity of the antibody responses, virus adsorption experiments were carried out with 23 representative pre- and post-challenge sera from vaccinated ponies and four sera from the unvaccinated control ponies. As many of the sera from vaccinated ponies collected immediately before challenge contained little or no SRH-detectable antibody, the pre-challenge adsorption experiments were carried out on sera collected 17 weeks before challenge when antibody levels were high following vaccination. All pre-challenge sera from vaccinated ponies contained CR antibody which produced SRH zones with both A/Miami/63 and A/Newmarket/79 viruses and antibody which was SS for A/Miami/63 virus (Table 3). When these ponies were examined after challenge, all sera contained CR antibody and, for many of the ponies (14/23), this was the only type of antibody detectable. Nine ponies developed SS antibody after challenge yet this was mainly (eight out of nine ponies) directed against A/Miami/63 virus; only two out of nine ponies developed SS antibody to A/Newmarket/79 virus. One pony developed SS antibody to both viruses. Serum from each unvaccinated control pony after challenge, contained SS antibody to A/Newmarket/79 virus and CR antibody.

Clinical signs of respiratory diseases

Coughing, dyspnoea and nasal discharges were observed from 3 to 4 days after challenge in all the control ponies and in many of the vaccinated ponies but as individual ponies could not be monitored on a continuous basis no attempt was made to quantify these responses. However, it was evident that some ponies experienced secondary bacterial infection as demonstrated by the isolation of heavy growths of β -haemolytic streptococci and *Bordetella bronchiseptica* from profuse mucopurulent nasal secretions.

DISCUSSION

This study was carried out to examine the relationship between SRH-detectable antibody to A/equine/Newmarket/79 virus stimulated by A/equine/Miami/63 virus vaccines and the protection provided against infection with A/equine/Newmarket/79 virus.

Although earlier reports (Doll, 1961, Lief & Cohen, 1965; Blaskovic *et al.* 1966) have described failure to experimentally infect horses with influenza virus or to reproduce clinical signs of disease, in this study, all seronegative control ponies became infected with A/Newmarket/79 virus and developed typical clinical signs. This demonstrates that the challenge procedure used in this study was effective. In previous studies on protection provided by equine influenza vaccines against experimental or natural infection, the HI test has been used to measure antibody to the HA (Rouse & Ditchfield, 1970; Kumanimido & Akiyama, 1975; Bryans, 1973; Powell *et al.* 1977). Protective titres of HI antibody estimated from these studies range from 8 to 128. The disparity in the HI titres quoted is partly a reflection of the variability in the sensitivity of the HI test (Frerichs, Frerichs & Burrows, 1973) particularly when measuring HI antibody to H3N8 strains of equine influenza. This arises from the necessity to treat the antigen used in the test with a detergent (Tween 80) and ether to increase sensitivity (John & Fulginiti, 1966) but it has been shown that HI titres measured with different batches of Tween 80/ether treated antigen can vary as much as 10-fold (Burrows *et al.* 1981). The SRH test has been shown to be a sensitive and reproducible technique for measuring antibody to the HA's of both sub-types of equine influenza (Hamilton, 1978; Bockman, 1977; Fontaine *et al.* 1981; Yamagishi *et al.* 1982; Wood *et al.* 1983*b*), and therefore more suitable for determining protective levels of antibody.

From the results of this challenge study and those described in the preceding paper (Wood *et al.* 1983*b*) it is possible to estimate the level of SRH antibody associated with protection and to assess the likely duration of immunity provided by vaccines of known antigenic content. In this study only two vaccinated ponies were protected against experimental infection as judged by failure to develop antibody responses, pyrexia or to excrete virus. Both ponies had SRH antibody levels $> 65 \text{ mm}^2$ against the challenge strain A/Newmarket/79 in pre-challenge sera. One of these ponies had received two doses of aqueous whole virus vaccine containing $50 \mu\text{g}$ HA 22 weeks and 4 weeks before challenge; the other pony received one dose of adjuvanted whole virus vaccine containing $15 \mu\text{g}$ HA 22 weeks before challenge followed by a second dose of aqueous vaccine containing $50 \mu\text{g}$

HA 18 weeks later. However, protective levels ($> 65 \text{ mm}^2$) of antibody to A/Newmarket/79 virus could not be demonstrated at the time of challenge in any of the ponies receiving one vaccine dose, 22 weeks before challenge, or two vaccine doses, 18 and 22 weeks before challenge. By reference to the duration of SRH antibody stimulated by the graded doses of vaccines used in the accompanying study (Wood *et al.* 1983*b*) it is possible to speculate that a conventional non-adjuvanted equine influenza vaccine containing approximately $15 \mu\text{g}$ HA A/Miami/63 per dose (e.g. Vaccine B in the study of Wood *et al.* 1983*b*) would provide only shortlived protection against experimental infection with A/Newmarket/79. Antibody levels, 1 week after the second dose of vaccine were marginally lower when tested against A/Newmarket/79 than when tested against A/Miami/63 (mean SRH zone sizes 76 mm^2 and 80 mm^2 respectively). Thus, protective levels of antibody (SRH zone size $> 65 \text{ mm}^2$) would not be expected to persist longer than 4 weeks following a primary course of two doses of vaccine. On the other hand the adjuvanted vaccine maintained protective levels of antibody for approximately 12 weeks.

Although only two vaccinated ponies (SRH $> 65 \text{ mm}^2$) were protected against infection, virus excretion and febrile responses were modified in other vaccinated ponies. Lower levels of antibody provided some clinical protection in susceptible seropositive (SRH $4\text{--}65 \text{ mm}^2$) vaccinees and the degree of clinical immunity in terms of reduction in duration of pyrexia and virus excretion, was related to pre-challenge antibody levels. Previous experience of vaccine in the absence of detectable antibody, failed to prevent febrile responses but significantly reduced virus excretion. This applied to ponies immunized with one or two doses of vaccine (individual data not shown). In the seropositive pony group (SRH $\geq 4 \text{ mm}^2$), six ponies did not develop febrile responses, although four of these did excrete virus. Subclinical equine influenza infections in vaccinated horses have been reported previously (Burrows, 1979; Burrows *et al.* 1982) and are of particular importance in the epidemiology of equine influenza. In the seronegative (SRH $< 4 \text{ mm}^2$) vaccinated ponies, where evidence of immunological priming was provided by anamnestic antibody responses to challenge, the clinical responses to infection were also modified.

Although, in the present study, the relationship between protection and levels of humoral antibody has been investigated, other features of the immune system should not be overlooked. The role of secretory antibody in protective mechanisms to influenza virus remains unclear. In horses, a relationship between secretory antibody and resistance to challenge has been demonstrated (Rouse & Ditchfield, 1970). In man, inactivated influenza vaccines given parenterally are capable of stimulating low levels of secretory IgA (Kasel *et al.* 1969). However further studies have shown that IgG in nasal secretions is probably more important than secretory IgA in resistance to influenza virus (Couch *et al.* 1981). Cell-mediated immunity has been implicated in recovery from influenza virus infection in mice (Yap & Ada, 1978) and protection against infection in mice (Lin & Askonas, 1981), although little is known about cellular immunity to influenza virus in horses.

The poor protection provided by vaccine in this study could be partly due to low levels of humoral antibody at the time of challenge, although the antigenic differences that exist between the vaccine virus and the challenge virus (Hinshaw

et al. 1982; Douglas, personal communication) may also have important implications for protection. Burrows & Denyer (1982) have shown that post-vaccination pony sera in HI tests do not readily detect differences between A/Miami/63 virus and currently circulating H3N8 strains. However, the present studies with SRH and virus adsorption clearly demonstrated that a proportion of the circulating antibody in vaccinated ponies before challenge was directed against strain specific (SS) determinants of the vaccine virus which would not react with the recent H3N8 variant. It is expected that the limited protection provided by vaccination could be due to the presence of cross-reacting (CR) antibody. Previous studies in animals (Haaheim & Schild, 1980) and in man (Couch *et al.* 1979) have confirmed that high levels of CR antibody are protective. Moreover it has been established that SS antibody is more efficient in conferring passive immunity to influenza-infected animals, than is CR antibody (Virelizier, 1975; Haaheim & Schild, 1980).

Analysis of the specificity of SRH antibody induced by experimental infection has demonstrated responses similar to those observed in humans. The responses of the unvaccinated control ponies following experimental challenge resembled those reported for young, unprimed children following natural infection, who reacted by producing predominantly SS antibody to the infecting virus and smaller amounts of CR antibody (Oxford *et al.* 1981). However, responses of vaccinated ponies following challenge were different in that large amounts of CR antibody were produced and any SS antibody produced was largely directed against the vaccine virus and not the challenge virus. These are similar results to those obtained in adult humans following vaccination or natural infection (Schild *et al.* 1977; Oxford *et al.* 1979, 1981) and they may be explained in terms of 'original antigenic sin' described in man (Davenport, Francis & Hennessey, 1953).

Further challenge studies are in progress which employ homologous vaccine and challenge viruses.

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