Studies on the Antigenicity of an Inactivated, Aluminum Hydroxide Adjuvant Equine Influenza Vaccine

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ABSTRACT

An inactivated, aluminum hydroxide adjuvant equine influenza vaccine was tested in horses and guinea pigs to determine the levels of antigen that would elicit maximum serological responses. Vaccine containing serial twofold increments of A/Equi-1/Prague and A/Equi-2/Miami strains of equine influenza virus was administered to random groupings of both types of test animals. The hemagglutination inhibition antibody response for each group was then measured. Results in horses and guinea pigs were compared to determine if the equine serological values could be related to a potency test in laboratory animals.

The highest mean hemagglutination inhibition antibody response in horses occurred in groups vaccinated, respectively, with 128 or 256 hemagglutination units of A/Equi-1 and 512 or 1024 hemagglutination units of A/Equi-2 antigen. Groups vaccinated with further two- or fourfold increases in these antigens had mean hemagglutination inhibition titers that were somewhat lower than the maximum levels. When graded doses of vaccine were given to guinea pigs, their hemagglutination inhibition antibody titers reached a plateau of maximum values, similar to the serological response in vaccinated horses.

Test horses remained clinically free from signs of equine influenza during the year following vaccination and no untoward post-vaccination reactions were observed.

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RÉSUMÉ

Cette expérience visait à éprouver, chez des chevaux et des cobayes, un vaccin contre l'influenza équin, inactivé et contenant un adjuvant d'hydroxyde d'aluminium, afin de déterminer quelles concentrations d'antigène provoqueraient les meilleures réponses sérologiques. On administra donc à des groupes d'animaux des deux espèces, choisis au hasard, un vaccin qui contenait une série d'augmentations doubles du virus de l'influenza équin des souches A/Equi-1/Prague et A/Equi-2/Miami. On mesura ensuite, pour chacun des groupes, les taux d'anticorps décelables par l'épreuve de l'inhibition de l'hémagglutination. On compara aussi les résultats obtenus chez les chevaux et les cobayes, afin de déterminer si on pouvait relier à une épreuve effectuée avec des animaux de laboratoire, les données sérologiques obtenues chez les chevaux.

Chez le cheval, le taux moyen le plus élevé d'anticorps décelés par l'épreuve de l'inhibition de l'hémagglutination se produisit au sein des groupes vaccinés respectivement avec 128 ou 256 unités hémagglutinantes de la souche A/Equi-1 et 512 ou 1024 unités hémagglutinantes de la souche A/Equi-2. Les groupes vaccinés avec des doses deux ou quatre fois plus élevées de ces antigènes développèrent des taux d'anticorps décelables par l'épreuve de l'inhibition de l'hémagglutination, un peu plus bas que les taux les plus élevés obtenus auparavant. L'administration de doses déterminées de vaccin aux cobayes se traduisit par des concentrations maximales d'anticorps décelables par l'épreuve de l'inhibition de l'hémagglutination, comparables à celles qu'on enregistra chez les chevaux.

Les chevaux vaccinés ne manifestèrent aucun signe clinique d'influenza, au cours de l'année ultérieure à leur vaccination; ils ne développèrent aucune réaction défavorable, à la suite de la vaccination.

INTRODUCTION

The antigenicity of an equine influenza vaccine is influenced by 1) the levels of antigen it contains and 2) the vehicle in which the antigens are suspended. The effects of the latter characteristic have been well established. Based on studies with equine and human influenza vaccines, use of a suitable adjuvant will enable the vaccine to produce higher and/or longer lasting antibody levels than vaccines containing equal amounts of antigen but no adjuvant (1, 5, 8, 9, 12). The influence of antigenic mass on the potency of equine influenza vaccines, on the other hand, has not been fully delineated. Bryans and his group reported that vaccination with increasing amounts of inactivated A/Equi-1 and A/Equi-2 virus produced a corresponding increase in serum antibody response to both antigens (1). These findings were reported only in relative terms, however, and were inconclusive for the A/Equi-2 virus where four graded levels of antigen all produced hemagglutination inhibition (HI) titers $\leqslant$10. It remained to be determined if a maximum serological response could be elicited by certain levels of each antigen.

To make this determination, twofold increments of inactivated A/Equi-1 and A/Equi-2 antigen were administered to groups of seronegative horses and the serological response to each preparation was measured. A similar test was conducted in guinea pigs to determine if the serological values in horses could be related to a potency test in laboratory animals. Test animals were given two doses of inactivated vaccine containing graded levels of both antigens suspended in an aluminum hydroxide adjuvant. To clinically evaluate the safety and efficacy of the vaccine, test horses were observed for untoward reactions to vaccination and signs of disease under natural conditions.

MATERIALS AND METHODS

VACCINE

A/Equi-1/Prague and A/Equi-2/Miami virus strains[1] were inoculated by the allantoic route into ten-day old embryonated, specific pathogen-free eggs.[2] Following an incubation period, virus-laden allantoic fluids were aseptically removed, inactivated with formaldehyde and then purified and concentrated. To determine the amount of antigen present in the viral concentrate it was evaluated by a standard hemagglutination test using serial twofold dilutions. Based on this assay, the viral concentrate was diluted into various aliquots containing the desired number of hemagglutination (HA) units, (one HA unit being the amount of antigen in a 0.25 ml suspension that would agglutinate 0.5 ml of a 0.5% suspension of chicken erythrocytes).

Four graded, bivalent preparations of vaccine were used in horses. The preparations contained 1024 and 2048, 512 and 1024, 256 and 512, and 128 and 256 HA units of A/Equi-1 and A/Equi-2 virus, respectively, per dose. In each case, the antigens were combined with the aluminum hydroxide adjuvant so that the total volume per dose was 2.0 ml.

Eleven graded, bivalent preparations of vaccine were used in guinea pigs. The preparations contained serial twofold dilutions of the two antigens ranging from 1024 and 2048 HA units to 1 and 2 HA units per dose of A/Equi-1 and A/Equi-2 virus, respectively. In each preparation, antigens were suspended in the aluminum hydroxide adjuvant so that the total volume per dose was 1.0 ml.

TEST ANIMALS

Veterinary practitioners who participated in the study were asked to identify bands of horses without a recent history of equine influenza infection. Initial blood samples were taken from 411 clinically normal horses selected from these bands.

[1]Obtained from Dr. Marion T. Coleman, Center for Disease Control, Atlanta, Georgia.

[2]SPAFAS, Inc., Norwich, Connecticut.

A total of 331 of the horses had detectable HI titers to one or both of the equine influenza viruses, disqualifying them from the study. Of the 80 remaining horses, those that showed a marked antibody rise following administration of an initial dose of vaccine, suggesting an anamnestic response due to prior exposure, were also disqualified. Only those horses that were serologically negative to both antigens prior to and at the time of administration of a second immunizing dose were considered in the statistical evaluation of the serological response to vaccination.

The guinea pigs used for testing graded levels of antigen were normal adult animals weighing 350-400 grams each.

Serological Testing

Serum samples were heat inactivated and tested for HI antibodies by the receptor destroying enzyme (RDE) method recommended by the Center for Disease Control (10). RDE treated sera were evaluated in serial twofold dilutions using microtitration equipment. Equine serum samples were evaluated individually and geometric mean titers were then calculated for each test group. Equal aliquots of sera taken from individual guinea pigs were pooled by test group and HI titers then measured.

Antigenicity Studies in Horses

The four graded preparations of vaccine were tested respectively in random groupings of the test horses. Horses received two doses of vaccine administered intramuscularly ten weeks apart. HI antibody levels were measured by evaluating blood samples taken at the time of each vaccination and at various intervals following vaccination. Mean HI antibody levels for each test group were compared to determine the levels of A/Equi-1 and A/Equi-2 antigen that elicited a maximum serological response. Test horses were also observed for signs of disease and untoward reactions to vaccination.

Antigenicity Studies in Guinea Pigs

The 11 graded preparations of vaccine were tested respectively in random groupings of guinea pigs with each group consisting of ten to 12 animals. Two doses of vaccine were administered intramuscularly four weeks apart. Two doses of the aluminum hydroxide adjuvant without the viral antigens were administered to a control group. HI antibody levels for each group were measured before and after vaccination to determine the serological response to the various levels of the A/Equi-1 and A/Equi-2 antigens.

RESULTS

Antigenicity Studies in Horses

The serological response of horses vaccinated with graded levels of A/Equi-1 and A/Equi-2 antigen is summarized in Table I. Test groups vaccinated with either 128 or 256 HA units of A/Equi-1 virus showed mean HI antibody titers approximately one twofold dilution higher than horses vaccinated with greater amounts of this antigen. Of the groups evaluated for response to the A/Equi-2 antigen, those vaccinated with 512 or 1024 HA units showed mean HI titers slightly higher than the groups receiving greater or lesser amounts.

The response of individual horses within each test group suggests that more animals achieved the maximum postvaccination HI titer at an earlier date when 256 and 1024 HA units of the A/Equi-1 and A/Equi-2 antigens, respectively, were administered. Although individual horses receiving more or less antigen sometimes developed HI titers comparable to horses in those two

TABLE I. Mean Serological Response of Horses to Graded Levels of A/Equi-1/Prague and A/Equi-2/Miami Antigens

Vaccine HA Units Prague/Miami	HI Antibody Titers[a] Prague (No. Animals)	Miami (No. Animals)
1024/2048	78 (12)	26 (12)
512/1024	60 (15)	36 (13)
256/512	123 (11)	36 (16)
128/256	126 (3)	22 (4)

[a]Geometric mean titers

TABLE II. HI Antibody Response of Horses Vaccinated With 256 HA Units of A/Equi-1/Prague Antigen

Horse	HI Antibody Titers				
	1st Dose	7 Days Post 1st Dose	2nd Dose, 10 Weeks Post 1st Dose	7 Days Post 2nd Dose	14 Days Post 2nd Dose
50	<10	<10	<10	10	±320
51	<10	<10	<10	±10	160
52	<10	<10	<10	±10	± 80
56	<10	<10	<10	20	80
57	<10	<10	<10	10	40
58	<10	<10	<10	10	160
87	<10	<10	<10	80	160
225	<10	<10	<10	<10	40
227	<10	<10	<10	20	±160
228	<10	<10	<10	±20	640
428	<10	<10	<10	±80	80

groups their serological response tended to be less rapid. Tables II and III show the response of individual horses in the groups receiving 256 HA units of A/Equi-1 and 1024 HA units of A/Equi-2 antigen.

Antigenicity Studies in Guinea Pigs

The serological responses of guinea pigs to graded doses of vaccine are summarized in Tables IV and V. Up to a point, HI titers rose as antigen levels were increased. Following vaccination with 32 HA units of A/Equi-1 virus, however, HI titers reached a plateau above which they did not appreciably rise. When four times more A/Equi-1 virus, or 128 HA units, was administered, HI titers showed only a twofold increase. When 1024 HA units of the antigen were administered, the resulting HI titer was a twofold dilution below the level that developed following vaccination with 32 HA units. Similarly, HI titers for the A/Equi-2 antigen reached a plateau when 64 HA units were administered. Titers did not rise appreciably above this level following vaccination with as much as 32 times more A/Equi-2 antigen.

Clinical Observations

Test horses remained clinically free from signs of equine influenza during the year following vaccination. In addition, no untoward local or systemic reactions were observed following vaccination with any of the preparations of the aluminum hydroxide adjuvant vaccine.

DISCUSSION

Results of the tests in horses and guinea pigs indicate that the HI antibody response to graded doses of both types of equine influenza virus reaches a plateau beyond which it does not increase (and may even decrease) despite vaccination with increasing levels of these antigens. The antigenicity test in horses demonstrates that the highest HI titers of which the animal is capable can be predetermined and elicited by a vaccine containing suitable levels of antigen combined with a specified adjuvant. These findings were the basis for developing a commercial bivalent, aluminum hydroxide adjuvant equine influenza vaccine,[3] designed to elicit a maximum serological response in horses.

The tests in guinea pigs resulted in postvaccination HI titers (Tables IV and V) that exceeded those reported in guinea pig potency tests conducted for a variety of other equine influenza vaccines, including several commercial preparations (2, 6). The highest HI titers for the A/Equi-2 antigen, in particular, exceeded by several fold those reported for other commercial aqueous and oil adjuvant vaccines. These comparisons further suggest that the most suitable approach to vaccine development is to first determine the maximum serological capabilities in the species to be immunized and then produce a vaccine designed to elicit a corresponding response.

Test results also indicate there is a

[3]Flumune, Norden Laboratories, Lincoln, Nebraska.

TABLE III. HI Antibody Response of Horses Vaccinated With 1024 HA Units of A/Equi-2/Miami Antigen

Horse	HI Antibody Titers				
	1st Dose	7 Days Post 1st Dose	2nd Dose, 10 Weeks Post 1st Dose	7 Days Post 2nd Dose	14 Days Post 2nd Dose
36	<10	<10	<10	<10	< 10
37	<10	<10	<10	80	80
38	<10	<10	<10	40	80
42	<10	<10	<10	40	< 10
44	<10	<10	<10	80	±160
45	<10	<10	<10	<10	80
46	<10	<10	<10	20	40
218	<10	<10	<10	40	80
220	<10	<10	<10	40	80
221	<10	<10	<10	<10	<10
222	<10	<10	<10	20	± 20
223	<10	<10	<10	40	±320
251	<10	<10	<10	40	40

TABLE IV.HI Antibody Response of Guinea Pigs to Graded Levels of A/Equi-1/Prague Antigen

HA units	HI Antibody Titers[a]				
	1st Dose	7 Days Post 1st Dose	2nd Dose, 4 Weeks Post 1st Dose	7 Days Post 2nd Dose	14 Days Post 2nd Dose
1024	<10	ND[b]	1280	640	±640
512	<10	ND	1280	1280	1280
256	<10	ND	5120	1280	1280
128	<10	ND	±5120	±5120	2560
64	<10	<10	640	1280	1280
32	<10	<10	640	2560	1280
16	<10	<10	±320	640	320
8	<10	<10	160	320	±320
4	<10	<10	80	160	80
2	<10	<10	20	40	±40
1	<10	<10	<10	20	±20
control	<10	ND	<10	<10	<10

[a]Values for sera pooled by test group
[b]ND — not done

TABLE V. HI Antibody Response of Guinea Pigs to Graded Levels of A/Equi-2/Miami Antigen

HA Units	HI Antibody Titers[a]				
	1st Dose	7 Days Post 1st Dose	2nd Dose, 4 Weeks Post 1st Dose	7 Days Post 2nd Dose	14 Days Post 2nd Dose
2048	<10	ND[b]	±320	±160	160
1024	<10	ND	40	80	160
512	<10	ND	160	±160	160
256	<10	ND	160	±320	160
128	<10	<10	±160	±320	±320
64	<10	<10	80	160	160
32	<10	<10	80	80	160
16	<10	<10	40	80	160
8	<10	<10	40	80	80
4	<10	<10	± 20	40	20
2	<10	<10	<10	20	20
control	ND	<10	<10	< 10	< 10

[a]Values for sera pooled by test group
[b]ND — not done

broad correlation in the antibody response of horses and guinea pigs to varying levels of the two antigens. Because it is impractical to conduct potency tests in horses, development of a definitive guinea pig potency test for equine influenza vaccines would be a useful as well as a feasible objective for future studies.

The study did not include controlled challenge tests, so conclusions on vaccine efficacy cannot be made. It is noteworthy, however, that 80% of 411 original serum samples were positive for equine influenza virus, indicating the disease was endemic and that under these conditions the 80 susceptible test horses remained clinically normal during the year following vaccination.

Three of the test horses vaccinated with 1024 HA units of A/Equi-2 antigen showed little or no increase in HI titer after administration of two doses (Table III). Reasons for the lack of seroconversion are speculative but it has been shown that very young horses often are not fully immunocompetent. Doll and Bryans, for example, noted an absence of SN titers in yearlings vaccinated for equine rhinopneumonitis (3). Gerber *et al* (7) found that six- to eight-month old horses showed only slight increases in SN titers following equine rhinopneumonitis vaccination and a decrease in titer following revaccination one month later. Following a third vaccination given a year afterwards, however, an increase in SN titer resulted. It should also be noted that the A/Equi-2 virus is a relatively poor antigen, producing lower mean titers than the A/Equi-1 agent (Table I). Thus, low levels of maternal immunity, below the sensitivity of the HI tests, may have prevented seroconversion in the horses vaccinated with the A/Equi-2 antigen.

An earlier study has shown that an aqueous influenza vaccine has produced HI antibody titers that compare favorably with those elicited by certain vaccines containing an adjuvant (2). It has been correctly observed, however, that duration of immunity produced by an aqueous vaccine has not been demonstrated (11). Studies have, in fact, indicated that influenza vaccines with an adjuvant produce longer lasting antibody titers than aqueous vaccine (5, 8). Based on the available evidence, equine influenza vaccine of acceptable potency with an adjuvant would tend to be efficacious for a longer period than an aqueous vaccine. The aluminum hydroxide adjuvant was, therefore, retained in the commercial vaccine that was developed on the basis of this study.

Absence of postvaccination disease and untoward reactions indicate that the aluminum hydroxide adjuvant system is a safe vehicle for use in an equine influenza vaccine. This is an important consideration in view of the fact that adjuvants in equine and human vaccines have often been associated with localized tissue reactions (1, 2, 4, 9, 11).

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REFERENCES

1. **BRYANS, J. T., E. R. DOLL, J. C. WILSON and W. H. McCOLLUM.** Immunization for equine influenza. J. Am. vet. med. Ass. 148: 413-417. 1976.
2. **DEMEIO, J. L., D. E. GUTEKUNST, J. M. BEILER, I. M. PATON and A. N. DESANCTIS.** The evaluation of an experimental bivalent equine influenza vaccine. J. Am. vet. med. Ass. 155: 278-281. 1969.
3. **DOLL, E. R. and J. T. BRYANS.** Immunization of young horses against viral rhinopneumonitis. Cornell Vet. 53: 24-40. 1963.
4. **FORSYTH, J. R. L.** An assessment of oil adjuvant and aqueous influenza vaccines. I. reactions to vaccines. J. Hyg. 65: 485-495. 1967.
5. **FORSYTH, J. R. L. and E. E. RUSSELL.** An assessment of oil adjuvant and aqueous influenza vaccines. II. antibody responses to the vaccines. J. Hyg. 65: 497-504. 1967.
6. **FRERICHS, G. H. and C. C. FRERICHS.** Serological response of chickens, rabbits and guinea-pigs to equine influena vaccines. Res. vet. Sci. 14: 187-193. 1973.
7. **GERBER, J. D., A. E. MARRON, E. P. BASS and W. H. BECKENHAUER.** Effect of age and pregnancy on the antibody and cell-mediated immune responses of horses to equine herpesvirus 1. Can. J. comp. Med. 41: in press. 1977.
8. **HENLE W. and G. HENLE.** Effects of adjuvants on vaccination of human beings against influenza. Proc. Soc. exp. Biol. Med. 59: 179. 1945.
9. **KUCERA, C. J.** The case for an adjuvanted equine influenza vaccine. J. Am. vet. med. Ass. 155: 281-284. 1969.
10. **RECOMMENDED METHOD FOR THE USE OF ADENOVIRUS, ENTEROVIRUS, MYXOVIRUS, REOVIRUS, AND VACCINIA HEMAGGLUTINATION (HA) AND HEMAGGLUTINATION INHIBITION (HI) REAGENTS.** Center for Disease Control. 1971.
11. **REISINGER, R. C.** Comments on aqueous and adjuvanted equine influenza xaccines. J. Am. vet. med. Ass. 155: 287-293. 1969.
12. **SCHIECHMEISTER, I. L., T. AESCHLIMAN and W. G. KAMMLADE.** Use of sodium alginate adjuvant in immunization against influenza. Am. J. vet. Res. 28: 1373-1378. 1967.